INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Université d'Ottawa • University of Ottawa

IDENTIFICATION OF DECAY-ACCELERATING FACTOR (CD55) AS A HeLa CELL RECEPTOR FOR ENTEROVIRUS 70

A Thesis Submitted to the

School of Graduate Studies

University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

Faculty of Medicine

By

Timothy M. Karnauchow

ABSTRACT

To identify the cellular receptor for Enterovirus 70 (EV70), mice were immunized with HeLa cells or HeLa cell membranes. Hybridomas were generated and screened for the production of antibody that protected HeLa cells against infection by EV70, and one monoclonal antibody (mAb), EVR1, was isolated. EVR1 recognized a trypsin-resistant HeLa cell surface epitope, and interfered with EV70 binding to HeLa cell monolayers. The specificity of EVR1 for EV70 was demonstrated by the inability of this mAb to protect HeLa cells against infection by poliovirus (PV) or coxsackievirus B3 (CVB3). Furthermore, EVR1 did not bind to monkey kidney (LLC-MK_2) cells, nor did it protect these cells against EV70 infection.

In Western blots and immunoprecipitations, EVR1 recognized a HeLa cell glycoprotein of approximately 75 kDa. The properties of this protein suggested that it may be decay-accelerating factor (DAF/CD55). DAF is a 70-75 kDa glycosyl-phosphatidylinositol (GPI)-anchored membrane protein that is a regulator of complement activation, and that has also been shown to act as a receptor for a number of human enteroviruses. Several experiments were performed to demonstrate that EVR1 recognized DAF. HeLa cells treated with phosphatidylinositol-specific phospholipase C (PI-PLC) had a greatly reduced ability to bind EVR1, indicating that the ligand of EVR1 is attached to cells via a GPI anchor. Furthermore, EVR1 bound to Chinese hamster ovary (CHO) cells constitutively expressing human DAF, and DAF-specific mAbs inhibited EV70 binding to HeLa cells and protected them against EV70 infection. Using a vaccinia virus based system, human DAF was

transiently expressed in murine NIH/3T3 cells. Transient expression of human DAF conferred virus binding activity to these cells, and a stably transformed NIH/3T3 cell line expressing human DAF supported low levels of EV70 replication.

To map the EV70 binding site of DAF, chimeric DAF/membrane cofactor protein (MCP) molecules were transiently expressed in NIH/3T3 cells and tested for their ability to bind EV70. Expression of chimeric receptors, in which individual extracellular short consensus repeat (SCR) domains of DAF were exchanged with those of MCP indicated that DAF SCR1 contained sequences essential for EV70 binding. Sequences within DAF SCR2 may also play a minor role in EV70 binding, but SCRs 3 and 4 were not specifically required to retain binding activity of the molecule. Similarly, the juxtamembrane domain and the GPI anchor of DAF were not specifically required for EV70 binding. Chimeric receptor expression was also used to identify DAF SCR1 as the binding site for EVR1.

These results indicate that DAF acts as a HeLa cell receptor for EV70. However, whether or not DAF is necessary or sufficient to mediate EV70 infection of cells is not yet clear. The widespread *in vivo* distribution of DAF and the restricted *in vivo* tropism of EV70, as well as the discovery that other human enteroviruses also use DAF as a receptor indicate that DAF expression is not the sole determinant of EV70 tropism. In addition, it remains to be determined if EV70 uses DAF homologs or entirely different surface molecules as receptors on cells of non-human origin. The identification of co-receptors or accessory factors that govern the specificity of EV70 infection awaits further investigation.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Ken Dimock, for his guidance, patience, gentle prodding, and friendship throughout my stay in his laboratory. I am better for having known him. I would also like to thank the members of my advisory committee, Dr. S.A. Sattar, Dr. B. Tuana, and Dr. E. Brown, for their assistance. Thank you to Dr. K. Wright and the late Dr. E. Perry for their kind encouragement, advice, and friendship.

I also gratefully acknowledge Dr. E. Altman, Dr. D. Tolson, and Blair Harrison, of the National Research Council of Canada, who provided the expertise, materials, and facilities that led to the production of the thesis-saving antibody, EVR1. I would also like to thank Dr. D. Lublin of Washington University for providing most of the DAF-related reagents used in this thesis, and for many useful discussions relating to this project.

The friendships of André Bergeron, John Mbithi, Ajay Thaker, Simian Zou, Marie-Claude Tardif, Mireille Prud'homme, Gina Grazziani-Bowering, and Sylvie Faucher, have made my time here more enjoyable. My heartfelt thanks to Gina for her generous help with the FACS analyses that make up the latter part of this thesis. Thank you to Karen Meysick, who has been an extraordinary friend. I also owe a great debt of gratitude to Drs. K. Jarrell, A. Kropinski, and J. Goodman. Finally, thank you to my family for their lifetime of understanding, love, and support, and to my wife Kathy, for her love, companionship and encouragement throughout all this.

I dedicate this thesis to my parents.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A_{xxx}	absorbance at xxx nm
Å	Angstrom
ABTS	2,2'-azino-bis (3-ethylbenz-thiazoline sulphonic acid)
Ad	adenovirus
AHC	acute haemorrhagic conjunctivitis
ATP	adenosine triphosphate
BSA	bovine serum albumin
CA24v	coxsackievirus A24 variant
cDNA	complementary DNA
CHO	chinese hamster ovary
CHO-DAF	chinese hamster ovary cells constitutively expressing human DAF
CIAP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
CPE	cytopathic effect
CPM	counts per minute
CVA	group A coxsackievirus
CVB	group B coxsackievirus
DAF	decay-accelerating factor
Dmn	DAF molecule with SCRn replaced with corresponding SCR of MCP
DMEM	Dulbecco's minimal essential medium

DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
EV70	enterovirus 70
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMDV	foot-and-mouth disease virus
FS	forward scatter
xg	force of gravity
GPI	glycosyl phosphatidylinositol
HAV	hepatitis A virus
HCAR	human coxsackievirus and adenovirus receptor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HIV	human immunodeficiency virus
HRV	human rhinovirus
ICAM-1	intracellular adhesion molecule 1
LCMV	lymphocytic choriomeningitis virus
LLC-MK_2	Lilly Laboratories Culture-monkey kidney cell line
mAb	monoclonal antibody
MCAR	mouse coxsackievirus and adenovirus receptor
MCP	membrane cofactor protein (CD46)

MCP-PI	MCP with GPI anchor of DAF
MEM	minimal essential medium
MHC-I	major histocompatibility complex class 1 molecule
MOI	multiplicity of infection
MTT	3, [4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide
NP-40	nonidet P40
nt	nucleotide
OPD	o-phenylenediamine dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-BSA	PBS containing 2% (w/v) BSA and 0.1% (w/v) sodium azide
PBS-Tween	PBS containing 0.02% (v/v) Tween-20
PEG	polyethylene glycol
PFU	plaque forming units
PI-PLC	phosphatidylinositol-specific phospholipase C
PMSF	phenylmethylsulfonylfluoride
PV	poliovirus
PVR	poliovirus receptor
R buffer	buffer containing 20 mM NaCl, 50 mM $MgCl_2$, 10 mM Tris HCl, pH7.5
RGD	arginine-glycine-aspartic acid
RIPA	radioimmunoprecipitation assay

rpm	revolutions per minute
SCR	short consensus repeat
SDS	sodium dodecyl sulphate
SOC	medium containing Bacto-tryptone, yeast extract, NaCl, and $MgSO_4$
SS	side scatter
SV40	simian virus 40
TAE	tris-acetate buffer
TBS	tris-buffered saline
3T3	NIH/3T3 cell line
3T3-DAF	NIH/3T3 cells constitutively expressing human DAF
3T3-RDAF	NIH/3T3 cells transfected with the DAF gene in reverse orientation with respect to its promoter
TM-DAF	DAF with transmembrane domain of MCP
Tris	tris (hydroxymethyl) aminomethane

INTRODUCTION

I. Acute haemorrhagic conjunctivitis

In 1969, the sudden appearance of an explosively contagious form of haemorrhagic conjunctivitis was reported in Ghana, West Africa (Chatterjee *et al.*, 1970). This new clinical entity, known as acute haemorrhagic conjunctivitis (AHC), was characterized by severe conjunctivitis of sudden onset and short duration, with severe subconjunctival haemorrhage and conjunctival swelling. The initial outbreak of AHC spread rapidly, eventually reaching pandemic proportions, with tens of millions of individuals affected between 1969 and 1971. From its focus in West Africa, AHC swept through the coastal regions of East and North Africa. The Indian subcontinent, South East Asia, Hong Kong and Japan were all affected in 1970-1971 by outbreaks of epidemic conjunctivitis displaying the same clinical and epidemiological characteristics of the conjunctivitis reported in Africa the previous year (Kono,1975; Kono *et al.*, 1972). During 1971-1972, a small number of nosocomial cases of AHC were reported in various European cities (Yin-Murphy, 1984), and in the same time period, epidemics were reported in the Middle East. Between 1972 and 1979, AHC was reported only sporadically throughout the Eastern Hemisphere, but there was a widespread outbreak (1974-1975) in Thailand, Singapore and India (Reeves *et al.*, 1986).

Until 1980, all cases of AHC had been confined to the Eastern Hemisphere, primarily in coastal, tropical and subtropical areas with high humidity and high population densities (Hierholzer and Hatch, 1985; Reeves *et al.*, 1986; Yin-Murphy, 1984). By early 1980, an increase in the number of reported cases heralded the beginning of a second pandemic, which

swept through regions of the world affected in the first pandemic, and reached the Western Hemisphere for the first time. From a focus in Southern India, AHC spread rapidly throughout India, Pakistan, Nigeria, and in early 1981, arrived in northern Brazil (Linhares *et al.*, 1989). From Brazil, epidemic conjunctivitis swept through the Caribbean, Central America, and southern Florida (Asbell *et al.*, 1985; Malison *et al.*, 1984; Morgan *et al.*, 1981; Partriarca, 1989; Reeves *et al.*, 1986; Waterman *et al.*, 1984). A small number of cases were reported in North Carolina, Minnesota, California, and as far north as New York, and in Toronto, Ontario (Spence and Vellend, 1982; Wright *et al.*, 1992). Early 1982 saw the arrival of AHC in the islands of the West Pacific and in New Zealand (Ghendon, 1989).

Since the second pandemic, sporadic outbreaks of AHC continue to be reported. These have occurred in Singapore (1983; Goh *et al.*, 1990), Beijing, P.R. of China (1984; Ku, 1989) Sao Paulo, Brazil (1984; Linhares *et al.*, 1989), Nigeria (1985; Babalola *et al.*, 1990), Saudi Arabia (1985; Moustafa *et al.*, 1989), Delhi, India (1986; Kishore *et al.*, 1989), American Samoa (1991; Bern *et al.*, 1992), and Pune, India (1991; Bhide *et al.*, 1994). Most recently, AHC caused by EV70 has been reported in Osaka, Japan (1993; Yamazaki *et al.*, 1995), Delhi, India (1994; Satpathy *et al.*, 1996), and in Okinawa, Japan (1994-95; Uchio *et al.*, 1996).

A. Discovery of the etiologic agent of AHC

Following the first pandemic, efforts to identify the etiologic agent of AHC led to a surprising discovery. Conjunctival scrapings recovered from patients of the 1970 outbreak of epidemic conjunctivitis in Singapore yielded an antigenic variant of coxsackievirus A24 (CA24v), which was referred to as Singapore Epidemic Conjunctivitis (SEC) virus (Yin-

Murphy and Lim, 1972). Subsequently, a virus which was antigenically distinct from CA24v was isolated from the conjunctivae of patients of the 1971 epidemic in Japan. This virus was identified as a previously unknown enterovirus, and was initially referred to as Acute Haemorrhagic Conjunctivitis (AHC) virus (Kono *et al.*, 1972). Through a collaborative study of representative AHC virus strains isolated from three regions affected in the outbreaks of 1971 (Singapore, Japan, Morocco), AHC virus was classified and recognized by the World Health Organization as a new enterovirus, type 70 (EV70; Mirkovic *et al.*, 1973).

Despite its relatively short history as a human pathogen, EV70 has been responsible for two pandemics and numerous epidemics and localised outbreaks of AHC, affecting well in excess of 80 million individuals in virtually all regions of the globe. Although the impact of CA24v-related epidemics cannot be overlooked, this virus is quantitatively less significant than EV70 as a cause of AHC (Uchio *et al.*, 1996). Epidemics of CA24v have been restricted to parts of Southeast Asia and the Indian Subcontinent (Lin *et al.*, 1993).

B. Clinical presentation of AHC due to EV70

Typically, AHC due to EV70 follows a rapid, benign course. Following a short incubation period of 10 to 24 hours, infected individuals usually experience sudden onset of unilateral conjunctivitis, which spreads, in 80% of cases, to the other eye within 24 hours (Uchida, 1989b). Symptoms include ocular redness, excessive lacrimation, pain, foreign-body sensation, preauricular lymph node enlargement, and intense subconjunctival haemorrhage (Higgins, 1982; Uchida, 1989b; Wright *et al.*, 1992). Unlike most enteroviral infections, which are characterized by a high proportion of subclinical manifestations (Melnick 1996), the number of asymptomatic cases of AHC is low, estimated to be less than 10% (Wright *et*

al., 1992). Systemic involvement is rare, and fever is infrequently reported. Symptoms normally peak within 24 hours of onset, persist for 3 to 5 days, and resolve without complication within 2 weeks.

On rare occasions, ocular infection is followed by neurological disorders of short or long duration. These neurological sequelae, which may result in significant morbidity, are estimated to occur in 1:10,000 to 1:17,000 cases of AHC. These figures are thought to be an underestimate, due to failure of physicians to associate EV70 with these symptoms, and because consistent isolation of the agent has proven difficult (Uchio *et al.*, 1996). Neurologic manifestations of EV70 infection vary in severity, ranging from mild cranial nerve palsy to radicular pains in muscles of the limbs, to asymmetrical flaccid paralysis reminiscent of acute paralytic poliomyelitis (Higgins, 1982; Vejjajiva, 1989; Wadia *et al.*, 1983; Wright *et al.*, 1992). These complications normally appear 10 to 20 days after the onset of conjunctivitis, but may develop during conjunctivitis, or as late as 3 months after infection. Approximately 25 to 30% of affected individuals sustain permanent debilitating neurologic impairment. Although EV70 has never been isolated from either the conjunctivae or cerebrospinal fluid (CSF) of patients with neurological complications, neutralizing antibody against EV70 has been found in serum and CSF of these individuals. This observation was interpreted to indicate that EV70 replicates within the central nervous system (Higgins, 1982; John *et al.*, 1981). The association of EV70 infection with central nervous system effects was substantiated experimentally (Kono *et al.*, 1973). Intraspinal or intrathalamic inoculation of cynomolgous monkeys with high titre EV70 produced polio-like paralysis, and histologically, lesions in the anterior horn of the spinal cord similar to those observed in acute poliomyelitis.

C. Transmission of EV70

AHC due to EV70 is highly infectious, and spreads rapidly in densely-populated areas with hot, humid climates and poor levels of hygiene (Hierholzer and Hatch, 1985). Outbreaks show no seasonal variation (Yin-Murphy, 1984). All age groups are susceptible to infection, although infection of children appears to be less frequent and less severe (Uchida, 1989b; Wright *et al.*, 1992).

Epidemic spread of AHC is due to the rapid replication rate of EV70, and the unusual mode of transmission of the virus (Johnson, 1994). The role of faecal-oral transmission, so important in infections with other enteroviruses, appears to be of little significance (Hung and Kono, 1979; Higgins, 1982), since there is no evidence that EV70 replicates in the enteric tract. Rather, the prime replication site of EV70 is the conjunctival epithelium, ocular discharge from infected individuals is infectious (Higgins, 1982), and transmission of AHC involves bringing the virus in contact with the eye. This may result from direct person-to - person contact via contaminated hands, as well as by indirect contact through contaminated fomites (Malison *et al.*, 1984; Reeves *et al.*, 1986). Intrafamilial spread is an important contributor to the epidemic potential of EV70. The infection rate of household members is known to be as high as 70 to 100% (Malison *et al.*, 1984; Patriarca, 1989; Reeves *et al.*, 1986). Furthermore, it has been shown that high humidity, moisture on fomites, and perspiration on hands, all aid virus survival in the environment (Sattar *et al.*, 1988).

D. Origin and evolution of EV70

The exceptional simultaneous emergence of two viruses, CA24v and EV70, associated with new and clinically indistinguishable ocular infections, prompted speculation that these

agents may have a common origin (Kono 1978). Subsequent analyses have shown that this is not the case. CA24v and EV70 are serologically distinct (Miyamura, 1989) and display little sequence similarity (Natori *et al.*, 1984). CA24v was, with the exception of its ocular tropism, found to be a typical enterovirus (Ghendon, 1989; Mirkovic *et al.*, 1974), and is not associated with paralytic illness (Chen *et al.*, 1989). As will be discussed in a subsequent section, EV70 displays a number of characteristics that are not commonly found among human enteroviruses.

The origin of EV70 remains obscure. Clinical, epidemiological and phylogenetic evidence are consistent with the hypothesis that EV70 emerged from a single source in West Africa, sometime around 1967 (Kew *et al.*, 1983; Kono 1985; Miyamura *et al.*, 1986; Takeda *et al.*, 1984). Initially it was suggested that the progenitor of EV70 was (i) an animal virus that acquired pathogenicity for humans; (ii) a human virus, which through the accumulation of mutations gained new pathogenic properties; or (iii) an insect picornavirus (Kono *et al.*, 1981).

The hypothesis that EV70 was originally an animal virus was supported by seroepidemiological studies of animal sera recovered in Africa and Japan. These demonstrated the presence of neutralizing antibody in cows, horses, sheep and swine, prior to the first pandemic in 1969 (Kono *et al.*, 1981; Sasagawa *et al.*, 1982). However, neutralizing activity was associated with IgM, not IgG, indicating that this activity was in all probability non-specific, the result of cross-reactive antibodies directed against another animal virus (Sasagawa *et al.*, 1982).

Although the molecular relationships among the enteroviruses are complex and at times confusing, genetic sequence data suggest a close relationship between EV70 and other human enteroviruses. The most recent phylogenetic analyses (Pöyry *et al.*, 1996; Rodrigo and Dopazo, 1995; Stanway, 1990) seem to indicate that EV70 is most closely related to polioviruses, coxsackieviruses A21 and A24, some of the B group coxsackieviruses (B3, B5), but also to Bovine Enteroviruses (BEV). EV70 displays the least sequence similarity to insect picornaviruses.. The EV70 progenitor was therefore probably a human enterovirus, however, the events that resulted in the acquisition of new pathogenic properties remain unresolved. Regardless of its origins, EV70 is evolving at a constant, rapid rate (Miyamura *et al.*, 1989; Yamazaki *et al.*, 1995), suggesting that the emergence of EV70 as a human pathogen was the result of an accumulation of nucleotide substitutions. The rapid rate of evolution raises concern that more virulent, oculotropic and neurotropic strains may emerge, and cause more widespread and severe infection (Johnson, 1994; Wright *et al.*, 1992).

II. The family *Picornaviridae*

EV70 is a member of the family *Picornaviridae*, which is one of the largest and most important families of human and animal pathogens known . These small, non-enveloped RNA viruses are subdivided into genera according to physicochemical and biological characteristics such as acid lability, buoyant density, sedimentation coefficients, antigenic similarities, and major disease syndromes and histopathology in laboratory animals and in humans (Melnick, 1996; Palmenberg, 1990; Rueckert, 1996). Currently, five genera are described (Table 1): *Rhinovirus*, *Enterovirus*, *Aphthovirus*, *Cardiovirus*, and *Hepatovirus*, although more recent

Table 1. The Family *Picornaviridae*

Genus *Rhinovirus*	
Human Rhinoviruses	102 types
Bovine Rhinoviruses	3 types
Genus *Enterovirus*	
Polioviruses	types 1-3
Coxsackieviruses, group A	types A1-22, 24
Coxsackieviruses, group B	types B1-6
Echoviruses	types 1-7, 9, 11-27, 29-34
Human Enteroviruses	types 68-71
Enteroviruses, other species	29 types
Genus *Aphthovirus*	
Foot-and- mouth-disease virus (FMDV)	7 types
Genus *Cardiovirus*	
Encephalomyocarditis virus (EMCV)	1 type
Theiler's murine encephalomyelitis virus (TMEV)	1 type
Genus *Hepatovirus*	
Human hepatitis virus A	1 type

Adapted from Rueckert (1996)

comparisons of genetic organization, cleavage patterns and sequence relationships suggest that this classification scheme requires revision (Hyypiä *et al.*, 1997; Palmemberg 1990; Rodrigo and Dopazo,1995; Stanway *et al.*, 1994).

Despite the considerable diversity among picornaviruses with respect to pathogenesis, host range, serology and physicochemical properties, these viruses are similar in morphology, genome organization, and replication strategy (Hyypiä *et al.*, 1997; Rueckert, 1996). Picornaviruses are small, unenveloped particles approximately 30 nm in diameter. The capsid, which displays icosahedral symmetry, is composed of 60 copies of each of four structural proteins, designated VP1, VP2, VP3, and VP4. This protein shell encloses a single strand of infectious positive sense RNA of 2.5 X 10^6 to 2.9 X 10^6 Da, or 7,200 to 8,500 nucleotides (nt) (Koch and Koch, 1985).

The extremely compact picornavirus genome has a small virally-encoded peptide (VPg) covalently linked to its 5' end, and a 3' poly(A) tract. The viral RNA (vRNA) also has untranslated regions (UTRs) at the 5' end (624 to 1200 nt) and at the 3' end (47 to 126 nt). These UTRs flank a single open reading frame of approximately 6600 nt (Ansardi *et al.*, 1996; Palmenberg, 1990; Rueckert, 1996).

A. Picornavirus replication

The replication of picornaviruses is a complex process, and has recently been reviewed elsewhere (Ansardi *et al.*, 1996; Wimmer *et al.*, 1993). Following virus binding to a specific cell surface receptor, vRNA is introduced into the cytoplasm of the cell. In the cytoplasm, picornavirus genomic RNA is directly translated into a large polyprotein, which is co-translationally processed via a cascade of proteolytic cleavages mediated by virally-encoded

proteases (Palmenberg, 1990). The products of these cleavages include the viral coat proteins, several viral proteases, the viral RNA-dependent RNA polymerase, and VPg (Ansardi *et al.*, 1996; Palmenberg, 1990; Rueckert, 1996). The general strategy of replication involves the synthesis of full length complementary (negative sense) strands which serve as templates for the synthesis of progeny (positive sense) genomes. This takes place in association with smooth membranes in the cytoplasm of infected cells, on which replication complexes are localized (Baltimore, 1968; Bienz *et al.*, 1992; Takeda *et al.*,1986). These complexes contain RNA replication intermediates and all of the viral and host cell proteins required for the replication and encapsidation of vRNA. All nascent and negative sense template RNAs have VPg attached to their 5' termini. Although its role in replication is unclear, VPg has been shown to be an absolute requirement for genome replication (Reuer *et al.*, 1990). Vpg is proposed to have a role in the packaging of vRNA genomes into capsids (Nomoto *et al.*, 1977; Rueckert, 1996), and uridylated VPg (5'VPg-pU) is believed to function as a primer for the initiation of RNA synthesis (Takegami *et al.*, 1983).

B. Picornavirus capsid structure

The capsids of picornaviruses are composed of 60 copies of each of four structural proteins, VP1, VP2, VP3, and VP4 (Hogle *et al.*, 1985). The outer surface of the capsid is formed by aspects of the three major, and largest of these proteins, VP1, VP2, and VP3. The fourth, and smallest structural protein, VP4, lines the inner surface of the capsid.

Among the picornaviruses, there is size variation and sequence dissimilarity between the different capsid proteins (Rueckert, 1996) Despite these differences, the folding patterns and assembly of VP1, VP2, and VP3 are widely conserved (Hogle *et al.*, 1985; Rossman *et*

al., 1985; Rueckert, 1996). Each capsid protein is folded into an eight-stranded antiparallel β-barrel configuration, which forms a wedge. Extending from the corners of these wedges, are peptide loops with hypervariable sequences, and which have been shown to contain binding sites for neutralizing antibodies (Rossman *et al.*, 1985). The association of one copy of each protein (VP1-VP4) forms the fundamental structural unit of the capsid, the protomer. Five protomers associate to form a pentamer, and finally, twelve pentamers associate to produce a capsid displaying the icosahedral symmetry characteristic of picornaviruses.

The capsid serves to protect viral RNA from nucleases in the environment, and directs the packaging of nascent genomes. Furthermore, discrete regions of the capsid interact with specific molecules expressed on the surfaces of target cells (i.e., virus receptors), and following binding, actively participate in the delivery of viral RNA into the cell cytoplasm (Ansardi *et al.*, 1996; Johnson, 1996; Rueckert, 1996).

Crystallographic studies of human rhinovirus 14 (HRV-14) revealed the presence of twelve symmetrically arranged deep surface depressions or "canyons" encircling each of the twelve five-fold vertices of the capsid (Rossman *et al.*, 1985). These canyons are formed by the packing of five copies of VP1 around the five-fold axis of symmetry of the capsid. Each VP1 contains prominent loops which create a concave surface, and the close packing of five of these proteins forms a circular groove, or canyon, the walls of which are formed by the amino acid residues of VP1 and VP3 (Rossman *et al.*, 1985). The dimensions of these canyons (estimated to be 25 Angstroms (Å) deep, 30 Å at the entrance, and 12 Å at the bottom, or floor) were considered to be too narrow to accomodate the broad antigen binding region (Fab) of neutralizing antibodies. Rossman and colleagues (1985) speculated that the

residues comprising the canyon floor were involved in host-cell receptor recognition, and that these residues were shielded against mutation and the selective pressure of the host's immune system and as a result, preserve receptor-specificity of the virus.

The involvement of canyon structures in receptor recognition was confirmed by site-directed mutagenesis, which showed that mutation of certain residues on the canyon floor abrogated virus binding (Colonno *et al.*, 1988), and by the demonstration that small organic molecules which could enter and bind under the canyon floor induced conformational alterations of the floor, inhibiting virus binding or uncoating (Badger *et al.*, 1988; Pevear *et al.*, 1989). Crystallographic and cryoelectromicroscopic analyses have also directly shown the association of HRV complexed with its receptor (Olson *et al.*, 1993; Rossman *et al.*, 1994).

Similar canyon structures have been observed in poliovirus type 1 (Mahoney strain) (Hogle *et al.*, 1985), and most likely exist in the group B coxsackieviruses (CVB) (Jenkins *et al.*, 1987), and coxsackievirus A21 (CVA21) as well (Chapman and Rossman, 1993). Although the three-dimensional structures of only a small number of picornaviruses have been elucidated, it already appears that canyons are not a uniform or universal feature of these viruses. For example, the surface of the Aphthovirus Foot-and -mouth-disease virus (FMDV) does not show any deep crevices. The surface of this virus is marked with ridges, created by short, disordered loops of amino acids from VP1 (Acharya *et al.*, 1989). Similarly, no significant surface depression appears on the capsid of the Cardiovirus mengo virus (Krishnaswamy and Rossman, 1990).

C. Enterovirus pathogenesis

The genus *Enterovirus* consists of nearly 70 serotypes of human pathogens (Table 1), including polioviruses, coxsackieviruses, echoviruses (enteric cytopathogenic human orphan), and the higher-numbered enteroviruses. As the name of this genus suggests, most of its members replicate in the enteric tract (Rueckert, 1996).The spectrum of disease caused by enteroviruses ranges from non-specific febrile illness and common colds, to encephalitis, aseptic meningitis, myocarditis, pericarditis, and poliomyelitis (Cherry, 1987; Melnick 1996).

Enterovirus transmission is normally from person to person, directly via the faecal-oral route, respiratory route, or indirectly, via fomites and contaminated water or food. Typically, infection is initiated by the ingestion or inhalation of enteric or respiratory excretions of infected hosts. After an incubation period of 7 to 14 days (ranging from 2 to 35 days), viral replication takes place in the lymphatic tissue of the oropharynx and intestinal tract, which results in a mild viraemia. As a result of this mild viraemia, virus spreads to and multiplies in secondary sites of infection. Viral replication in these secondary sites coincides with the onset of clinical symptoms. At this time, major viraemia may be established. Haematogenous spread of the virus exposes many tissues to the virus, and secondary replication takes place in select target organs, the identity of which varies with the infecting virus. Usually virus is detectable in the faeces for several weeks, and can be detected in the pharynx for 1 to 2 weeks post-infection (Cherry, 1987; Melnick, 1996). The diversity of enterovirus target tissues is reflected in the diversity of sites from which virus has been isolated. These sites include the faeces, pharynx, cerebrospinal fluid, spinal cord, brain, cardiac and skeletal muscle, and the conjunctivae (Melnick, 1996).

III. Unusual characteristics of EV70

The morphologic, physicochemical, and serological data obtained in the initial characterization of EV70 (Table 2; Kono *et al.*, 1972; Kono *et al.*, 1975; Mirkovic *et al.*, 1973; Yamazaki *et al.*, 1974), and subsequent genome sequence and organization analyses (Ryan *et al.*, 1990; Takeda *et al.*, 1984; Yamazaki *et al.*, 1974), supported its classification as a human enterovirus. Although EV70 shares many characteristics with other members of its genus, it is, in many respects, an atypical human enterovirus.

A. Ocular tropism

The primary replication site of EV70 is the conjunctival epithelium. While conjunctivitis may sometimes appear as an ancillary symptom in certain coxsackievirus and echovirus infections (Melnick,1996), epidemics of a distinctive form of conjunctivitis due to an enterovirus were unknown prior to the discovery of EV70 and CA24v (Mirkovic *et al.*, 1973). EV70 (and CA24v) produces disease not previously recognized as characteristic of enteroviruses.

EV70 was first isolated from conjunctival specimens (Kono *et al.*, 1972), and almost all strains of EV70 were isolated from conjunctival scrapings or swabs collected during the acute phase of AHC (Nakazono and Kondo, 1989). In contrast to other enteroviruses (including CA24v), EV70 is rarely found in the faeces of infected patients (Mirkovic *et al.*, 1973; Wadia *et al.*, 1983). The detection of EV70 in faeces may be incidental, the result of an acid-stable virus replicating in the conjunctivae and being secreted into the alimentary tract via the nasopharynx (Hung and Kono, 1979, Yamazaki and Miyamura, 1989). Neurological complications following ocular infection with EV70 suggested that EV70 gains entry to and

Table 2. Characteristics of Enterovirus 70

Morphology		
	Shape	spherical
	Diameter	30 ±1 nm
Physicochemical properties		
	Buoyant density	1.34 g/cm^3 in cesium chloride
	Sedimentation coefficient	1.24 g/cm^3 in sucrose
	RNA molecular weight	2.5 X 10^6 daltons
	Structural proteins	VP1, VP2, VP3, VP4
	Stability of infectious virus	pH 3.0-8.6
	Reactions to chemicals	
	sodium deoxycholate	Resistant
	chloroform and ether	Resistant
Virus replication		
	Site of replication	Cytoplasm

Adapted from Yamazaki and Miyamura (1989)

replicates in the central nervous system. Neurovirulence studies in which cynomolgous monkeys received intracerebral or intrathalamic inoculations with virus provided direct evidence associating EV70 infection and neurological symptoms (Uchida, 1989b). The mechanism by which EV70 reaches the central nervous system is not known. Studies of neurovirulence in monkeys suggest that EV70 may reach the CNS via a blood route, thanks to the heavy vascularization of the eye, or may gain access to the CNS directly via nervous tissue (Uchida, 1989b).

B. Temperature-sensitive growth

The optimal temperature for growth of most human enteroviruses is 37°C, the temperature of the gastrointestinal tract (Melnick, 1996; Cherry, 1987). In contrast, replication of EV70 is optimal at 32-34°C (Miyamura *et al.*, 1974), the temperature encountered in the conjunctiva (Hung and Kono, 1979; Stanton *et al.*, 1977). At 37°C, the replication rate of EV70 is markedly decreased, and virus yield is significantly lower than at 33°C (Miyamura *et al.*, 1974), while at 39 °C, no replication is observed (Miyamura *et al.*, 1974, 1976). EV70 is the only enterovirus with a naturally occurring temperature-sensitive phenotype, and is, in this respect, more similar to the rhinoviruses than to other enteroviruses (Melnick, 1996; Miyamura *et al.*, 1976; Yamazaki and Miyamura,1989). The inability of EV70 to replicate at 39°C appeared to be due to a block in initiation of viral RNA synthesis (Miyamura *et al.*, 1974), and Takeda *et al.* (1984) attributed this to a failure to form 5'-Vpg-pU at the non-permissive temperature.

C. Host range

While the replication of human enteroviruses is normally restricted to primary or continuous cell lines of human or lower primate origin (Cherry, 1987; Melnick, 1996), a small number of exceptions has been noted. Coxsackievirus B5 has been shown to replicate in some porcine cell lines (Graves, 1973), and coxsackieviruses A7, B3, B4, as well as echovirus 4 have been shown to replicate in primary cultures of kidney cells from pig, hamster, calf, lamb and mouse (Lenahan and Wenner, 1960).

Although EV70 *in vivo* tropism is limited to the conjunctiva, this virus has a wider *in vitro* host range than typical human enteroviruses. EV70 was first isolated in cultures of human (HeLa) cells, and was subsequently adapted to monkey kidney cells (Kono *et al.*, 1972, Mirkovic *et al.*, 1973). Yoshii *et al.* (1977) demonstrated that EV70 is able to replicate in continuous cell lines derived from rabbits (RK13, RK17), mice (L), hamsters (BHK21), and pigs (ESK and IB-RS-2), and in primary cultures of bovine kidney (BK1) cells. Growth of EV70 in cells is generally associated with the cytopathic effects of picornavirus infection (Mirkovic *et al.*, 1973). Infected cells round up, shrink, exhibit marked nuclear pyknosis, eventually degenerate and detach from their growth surface (Ressig *et al.*, 1956). The continuous cell cultures of rabbit origin and the primary bovine culture yielded virus titres as high as primate culture controls, and EV70 infection of these cells produced typical CPE. Replication in murine, hamster, and porcine cell lines yielded lower EV70 titres than did primate cell lines, and failed to produce CPE. Two of the cell lines examined (porcine kidney, PK15; bovine kidney, MDBK) were non-permissive for EV70. Since similar results were obtained using three separate strains of EV70, the ability to replicate in non-primate derived

cell lines is considered a general property of this virus (Yamazaki and Miyamura, 1989). It should be noted that replication of CA24v, the second etiologic agent of AHC, is restricted to primate cells (Langford and Stanton, 1980).

The basis of the restricted *in vivo* tropism and extended *in vitro* host range of EV70 is a poorly studied aspect of the biology of this virus.

IV. Virus host range and tropism

The host range of a virus describes the species origin of the cells that are susceptible to virus infection. Tropism may be defined as the capacity of a virus to grow within particular cells of a host, or as the predilection of a virus for specific cells and tissues (Fields, 1993). The first step in the infectious cycle of many viruses is attachment to specific receptors on the surface of host cells (Bass and Greenberg, 1992; Lentz, 1990; Norkin, 1995). This attachment is mediated by the binding of sites present on the polypeptides forming the envelope of enveloped viruses or the capsid of non-enveloped viruses (viral attachment proteins, VAPs), to a complementary molecule(s) present on the host cell surface which act(s) as a virus receptor (Crowell and Hsu, 1986; Lentz, 1990; Norkin, 1995; Rossman *et al.*, 1985; Wimmer, 1994).

Infection requires that viruses gain entrance into host cells, and binding of virus to a receptor is followed by the delivery of viral genomes to the interior of the cell. For enveloped viruses, entry may be effected by surface fusion between the viral envelope and the cell plasma membrane, or by receptor-mediated endocytosis and fusion within the cell, at endosomal membranes (Knipe, 1996; Marsh and Helenius, 1989). Entry of non-enveloped

viruses such as picornaviruses is not well understood. After binding to the cellular receptor,viral RNA is released from the capsid (uncoating) and delivered into the cytoplasm. It has been proposed that for certain picornaviruses, receptor binding induces conformational changes of the viral particle, which increases capsid hydrophobicity. This promotes the association of the capsid with the plasma membrane and penetration of the lipid bilayer by viral RNA (Fricks and Hogle, 1990; Rueckert, 1996). However, whether picornaviral RNA is delivered to the cytoplasm at the cell surface, or from within endocytic vesicles following receptor-mediated endocytosis, is the subject of some controversy (Gromeier and Wetz, 1990; Hoover-Litty and Greve, 1993; Kaplan *et al.*, 1990; Mason *et al.*, 1993; Rueckert, 1996). Following uncoating and delivery, the viral genome is translated and replicated, viral proteins are processed, and progeny virions assembled.

While many factors affecting steps subsequent to attachment may influence viral infectivity and replication, the expression of appropriate host cell receptors is believed to be a major determinant of viral host range, as well as cell and tissue tropism (Crowell and Landau, 1983; Haywood, 1994; Mims, 1986; Norkin, 1995).

The virus-receptor relationship has been the subject of intense study, not only as a starting point to understanding the interaction of viruses with host cells, but also as a potential strategy for controlling virus infection and its spread. The literature concerning virus receptors is vast, and a comprehensive treatise on this subject would be impossible in these pages. Therefore, discussion will largely be restricted to picornaviruses and their receptors.

V. Cellular receptors as determinants of picornavirus host range and tropism

The most compelling evidence of a direct relationship between susceptibility to virus infection and the expression of specific host cell receptors arose from studies of polioviruses (PV). Alexander and colleagues (1958) showed that purified PV RNA was infectious, a discovery that led to the first demonstration of the importance of cell-specific receptors in the initiation of picornavirus infection. McLaren *et al.* (1959) observed that only cells of simian (monkey kidney) and human (HeLa) origin adsorbed PV and were susceptible to infection. Subsequently, Holland and colleagues (Holland *et al.*, 1959a,b) showed that introduction of purified poliovirion RNA into non-permissive cells resulted in a single round of virus replication. From this it was concluded that the block in poliovirus replication in non-primate cells was due to the absence of a virus-specific receptor. Thus it was proposed that PV host range, at least in cultured cells, was controlled either by the expression of a PV receptor, or at the stages of virus entry or uncoating. Debris from PV susceptible primate cells were found to neutralize PV infectivity (Holland and McLaren, 1959), and virus binding activity was subsequently localized to the microsome fraction of susceptible cells (Holland and McLaren, 1961).

Demonstration of the existence of cellular receptors for PV prompted others to study the relationship between the receptors for different picornaviruses, and provided the impetus to study the nature of the receptors for other enteroviruses.

The specificity of picornavirus receptors was first investigated by competition binding experiments (Abraham and Colonno, 1984; Burness and Pardoe, 1983; Crowell, 1966; Lonberg-Holm and Korant, 1972; Lonberg-Holm *et al.*, 1976; Quirsin-Thiry and Nihoul,

1961; Sekiguchi *et al.*, 1982). In these studies, cell receptors were saturated with one virus, and the ability of a second virus to bind to these cells was assessed. If binding of the first virus prevented attachment of the second, the two viruses were assumed to utilize a common receptor.

Six distinct picornavirus receptor families have been described using this technique. The human rhinoviruses (HRV) are divided into a major group (91serotypes) and a minor group (10 serotypes) (Abraham and Colonno, 1984; Rueckert, 1996), the group B coxsackieviruses (CVB) form the third group, the PVs form a fourth group, and the foot-and mouth disease viruses (FMDV; Sekiguchi *et al.*, 1982) and encephalomyocarditis viruses (EMCV, Burness and Pardoe, 1983) form the remaining two families.

Overall, the grouping of picornaviruses according to receptor specificities parallels the traditional taxonomic groupings that were based on disease and histopathology. This observation forms the basis of the hypothesis that cellular receptors are important determinants of picornavirus tropism. Some inconsistencies were observed, however, and it appeared that certain unrelated viruses share receptor molecules. The group A coxsackieviruses (A13, 18, 21) share a receptor with the major group rhinoviruses (Lonberg-Holm *et al.*, 1976), and adenovirus type 2 and coxsackievirus B3 also use a common receptor (Lonberg-Holm *et al.*, 1976). Human rhinovirus 87 attachment is not inhibited by other human rhinoviruses (Uncapher *et al.*,1991). Furthermore, it is known that virus attachment to cells does not necessarily lead to infection of cells (Angel and Burness, 1977; Colonno *et al.*, 1990; Hsiung and Melnick, 1958; Mims, 1986; Yoshii *et al.*, 1977; Zajac *et al.*, 1991).

VI. Early approaches to the identification of picornavirus receptors

A. Biochemical approaches

The earliest investigations of picornavirus receptors were limited by the availability of suitable reagents. Information regarding the biochemical nature of picornavirus receptors was obtained by treating virus- susceptible cells or membranes prepared from these cells with various enzymes and chemicals, and determining the effect of these agents on virus binding activity (Levitt and Crowell, 1967; Lonberg-Holm, 1975; Zajac and Crowell, 1965a,b). These experiments indicated that picornavirus receptor molecules are predominantly integral membrane glycoproteins.

The thorough characterization of virus receptors requires isolation and identification of these molecules. Historically, this has proven to be difficult, because receptors may be present on the cell surface in small numbers, and because standard protein isolation procedures may result in the loss of virus binding activity (Crowell and Hsu, 1986; Tardieu *et al.*, 1982). Several researchers have used an affinity based approach to receptor identification, termed the virus overlay protein blotting assay (VOPBA). This technique exploits the affinity of virus particles for cellular components: cellular proteins separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose are incubated with virus, and virus binding is used to identify proteins found uniquely in susceptible cells. This method has been used in the study of several viruses, including Sendai virus (Gershoni *et al.*, 1986), reovirus (Verdin *et al.*, 1989), and lymphocytic choriomeningitis virus (LCMV; Borrow and Oldstone, 1992), as well as for the minor group HRV (Mischak

et al., 1988a), and Theiler's murine encephalomyelitis virus (TMEV; Kilpatrick and Lipton, 1991).

B. Immunological approaches

The development of monoclonal antibody (mAb) technology has had a tremendous impact on virus receptor research. MAbs capable of selectively inhibiting the attachment of different viruses have been produced by immunizing mice with whole cells or with cell membranes, and are identified by their ability to block virus attachment and infection (Bass and Greenberg, 1992). Campbell and Cords (1983) were the first to produce monoclonal antibody that specifically inhibited the infection of HeLa cells by CVB1, B5, and B6. Subsequently, Nobis *et al.* (1985), Minor *et al.* (1984), and Shepley *et al.* (1988) obtained antireceptor mAbs for PV, Colonno *et al.* (1986) produced an antireceptor mAb for the major group HRVs, and Crowell and colleagues successfully produced mAbs specific for receptors for CVB (Crowell *et al.*, 1986; Hsu *et al.*, 1988; Mapoles *et al.*, 1985).

Antireceptor mAbs have proven to be useful tools for the biochemical and functional characterization of virus receptors. The ability of these mAbs to selectively block virus binding was exploited to confirm the receptor specificities of picornaviruses, and the results of these antibody blockade experiments showed a strong correlation with the results of earlier virus binding competition assays (Bass and Greenberg, 1992; Hsu *et al.*, 1990).

Receptor-specific antibodies have also provided a means by which receptor molecules can be isolated from membranes of susceptible cells. An example of the application of antireceptor mAbs in the basic characterization of virus receptors is the work of Crowell and colleagues with CVB.

Briefly, Krah and Crowell (1985) demonstrated that the receptor for CVBs could retain its binding activity following detergent solubilization of HeLa cell membranes. The high affinity of the receptor protein for virus permitted the isolation of a virus-receptor complex (Mapoles *et al.*, 1985), which consisted of viral capsid proteins and a 46 to 49 kDa HeLa cell membrane glycoprotein protein. This protein was isolated and used to produce antireceptor mAb RmcB, which protected HeLa cells against infection by all six serotypes of CVB (Hsu *et al.*, 1988). During the course of their studies, Crowell and colleagues isolated a CVB3 host range variant that acquired the ability to grow in the human rhabdomyosarcoma (RD) cell line, which is normally resistant to CVB infection, and also acquired the ability to agglutinate erythrocytes (Reagan *et al.*, 1984). Interestingly, CVB3-RD retained the ability to grow in HeLa cells, and it was eventually shown that this virus recognized two distinct receptors on HeLa cells . This conclusion was reached after it was shown that CVB3-RD could saturate HeLa cell receptors and prevent the binding of prototype CVB3, but that saturation of HeLa cells with prototype virus did not prevent CVB3-RD from binding to cells (Reagan *et al.*, 1984; Crowell *et al.*, 1986). To identify this additional receptor, another antireceptor mAb (generated against HeLa cells) was used. This mAb, RmcA, identified a 60 to 70kDa glycoprotein from both RD and HeLa cells, and protected RD cells from infection by CVB3-RD and HeLa cells from infection by prototype strains of CVB1, B3, B5, as well as Echovirus 6, and CVA21 (Crowell *et al.*, 1986). Although the receptors for CVB were only partly characterized, this work was important, providing evidence that adaptation of a picornavirus to a new host cell corresponded with the acquisition of the ability to utilize multiple receptors.

At the time that this project was initiated, the application of basic biochemical techniques, including enzyme sensitivity of receptors, binding competition experiments, affinity purification and the production of antireceptor antibodies had resulted in the identification of relatively few virus receptors (Lentz, 1990; Bass and Greenberg, 1992). From the characterizations that were performed, it was apparent that a large number of different plasma membrane components could serve as virus receptors, including phospholipids and glycolipids, as well as hormone and neurotransmitter receptors and other integral membrane glycoproteins (Co *et al.*, 1986; Lentz, 1990). For the most part, these receptor identifications were not definitive.

VII. Contemporary approaches to the identification of virus receptors

In order to definitively identify a receptor molecule, receptor function (virus binding followed by infection of cells) must be demonstrated. When this project was initiated, the most thoroughly characterized receptors were those for human immunodeficiency virus (HIV-1), the major group human rhinoviruses (HRV) and poliovirus (PV). A brief description of the strategies used to identify these cellular receptors follows.

A. The receptor for HIV

The identification of the cellular receptor for HIV was in itself significant, but the successful application of immunologic and molecular biology techniques in its identification also heralded the beginning of a new era in virus receptor research.

The observations that $CD4^+$ lymphocytes are depleted in HIV-infected individuals and that HIV appeared to have a specific tropism for these cells initially led researchers to suspect

that CD4 was the receptor for HIV. Klatzman *et al.*, (1984) demonstrated that mAbs directed against CD4 prevented infection of $CD4^+$ lymphocytes, and Dalgleish and colleagues (Dalgleish *et al.*, 1984) demonstrated that vesicular stomatitis virus-HIV pseudotype viruses displayed tropism that was restricted to cells expressing CD4, and also that CD4-specific mAbs prevented infection of CD4-expressing cells. The groundbreaking work of Maddon (Maddon *et al.*, 1986) definitively demonstrated that the interaction between HIV and CD4 is of biologic significance. This group converted HIV-resistant $CD4^-$ human epithelial cells into HIV- susceptible cells by introducing the gene encoding CD4. This evidence strongly suggested that CD4 acted as a receptor for HIV. However, CD4 expression was not necessarily sufficient to ensure HIV infection of cells. While a number of human $CD4^-$ HIV-resistant cell lines were rendered susceptible following expression of CD4, several other cell lines of mice, rabbits, and lower primates could not be infected by HIV. These observations indicated that additional human cell-specific factors are required for HIV entry into cells (Maddon *et al.*, 1986). The role of co-receptors / accessory proteins in virus infection will be addressed in more detail in the discussion section

CD4 is an integral membrane glycoprotein, containing four contiguous immunoglobulin-like extracellular domains, and is a member of the immunoglobulin superfamily of proteins (White and Littman, 1989). The HIV binding site of CD4 has been localized to the most distal, amino terminal domain of the molecule (Landau *et al.*, 1988; Arthos *et al.*, 1989).

Of additional interest was the observation that some human cells could be infected in the absence of CD4 expression. HIV therefore appeared to be able to use more than one

receptor, and galactosyl ceramide has been proposed as an alternative receptor for HIV-1 on human neural cell lines (Bhat *et al.*, 1991; Harouse *et al.*, 1991).

Thus, the model of HIV provided an example of: (1) receptor-influenced tropism, (2) the insufficiency of receptor expression to guarantee infection, and (3) the ability of viruses to utilize different receptors in different cell types. The genetic transformation experiments of Maddon and colleagues represented the first direct demonstration of receptor function for a candidate receptor molecule, and established a new criterion for receptor identification studies: genetic transfer of receptor activity to receptor-negative cells.

B. The receptor for the major group HRV

The first picornavirus receptor identified through the application of immunological and molecular techniques was the receptor for the major group HRV (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989a). Human rhinoviruses initiate infection by binding to one of two different receptors expressed on susceptible cells (Abraham and Colonno, 1984; Colonno *et al.*, 1986). Colonno and colleagues produced a mAb that blocked binding of major group HRV, and protected HeLa cells against infection (Colonno *et al.*, 1986). Using this mAb, a 90 kDa HeLa cell protein was identified by immunoaffinity chromatography, and the candidate receptor protein was characterized (Tomassini *et al.*, 1989b). Protein sequence analysis permitted molecular probes to be designed, and these were used to screen HeLa cell cDNA libraries for receptor-specific sequences (Tomassini *et al.*, 1989a). DNA sequence analysis revealed similarity to intracellular adhesion molecule 1 (ICAM-1). A similar approach was used by Greve *et al.*, (1989). The similarities between the candidate HRV receptor described by Tomassini (Tomassini *et al.*, 1989b) and ICAM-1

led Staunton *et al.*, (1989) to directly demonstrate that these were the same molecule. Collectively, these studies showed that ICAM-1 specific mAbs prevented HRV binding to HeLa cells and prevented infection, and that COS (Staunton *et al.*, 1989) or murine L cells (Greve *et al.*, 1989) engineered to express ICAM-1 bound virus.

ICAM-1 is the natural cell surface ligand of the integrins lymphocyte function-associated antigen (LFA-1) and Mac-1 (Diamond *et al.*, 1991), and is involved in a variety of cellular interactions (White and Littman, 1989). Like CD4, ICAM-1 is a member of the immunoglobulin superfamily of proteins: primary sequence analysis predicts a molecule with five contiguous extracellular immunoglobulin-like domains (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989a). Using ICAM-1 deletion mutants and chimeric ICAM-1 molecules (containing murine and human ICAM-1 sequences) the HRV binding site has been localized to the first and second amino terminal domains of the molecule (McClelland *et al.*, 1991; Staunton *et al.*, 1990). In spite of the demonstration that ICAM-1 expression mediates HRV binding and infection of cells, the widespread distribution of ICAM-1 *in vivo* (Dustin *et al.*, 1986) and the restricted replication of HRV suggested that ICAM-1 is not the sole determinant of HRV tissue tropism (Greve *et al.*, 1989).

C. <u>The receptor for PV</u>

By the mid-1980s, several antireceptor mAbs were available for the study of the poliovirus receptor (PVR; Minor *et al.*, 1984; Nobis *et al.*, 1985; Shepley *et al.*, 1988) however, none of these reacted with solubilized membrane material, and thus could not be used for the biochemical purification of receptor protein (Mendelsohn *et al.*, 1986). In order to circumvent this problem, Mendelsohn *et al.* (1989) used a genetic approach involving

DNA-mediated gene transfer, to isolate a clone of the PVR gene. This group first demonstrated that susceptibility to PV infection could be transferred to the normally non-susceptible murine L cell line via genomic DNA transformation. Genomic DNA from HeLa cells was introduced into L cells, and primary and secondary transformants were detected on the basis of their susceptibility to infection and their reactivity with receptor-specific mAb. A genomic library constructed from the DNA of one positive L-cell transformant was then screened for the presence of human sequences. Positive recombinants were analyzed by restriction digestion, and a human DNA fragment common to all receptor-positive transformants was identified. This fragment was then used to isolate HeLa cell cDNA clones encoding the PVR. When cDNA clones were introduced into L cells, functional PV receptors were expressed (Mendelsohn *et al.*, 1989). The predicted amino acid sequence revealed that PVR was a 43 to 45 kDa transmembrane glycoprotein with three extracellular, contiguous immunoglobulin-like domains, typical of members of the immunoglobulin superfamily (White and Littman, 1989). The predominant mature PVR species in HeLa cells is 80 kDa (Bernhardt *et al.*, 1994). Deletion mutants of PVR (Freistadt and Racaniello, 1991; Koike *et al.*, 1991a), and chimeric ICAM-1/PVR molecules (Selinka *et al.*, 1991) were used to map the PV binding site to the most distal amino terminus domain of PVR.

The normal cellular function of PVR protein is unknown. Northern blot (Mendelsohn *et al.*, 1989) and Western blot analyses (Friestadt *et al.*, 1990) showed that PVR mRNA is found in virtually all human tissues, whether or not these bind or can be infected by PV, and transgenic mice also expressed PVR RNA in both susceptible and non-susceptible tissues (Ren *et al.*, 1990). The widespread distribution of PVR mRNA and protein suggested that

PVR alone is not the determinant of PV tropism. However, tissue-specific variations in PVR molecular weight and immunoreactivity were observed (Friestadt *et al.*, 1990), giving rise to speculation that PV tissue tropism may be regulated by tissue-specific post-translational control. The existence of a cofactor required for PV infection was suggested by the work of Shepley *et al.* (1988). This group produced a mAb that blocked PV binding to HeLa cells and protected cells against infection. When this mAb was used to probe cell membrane preparations derived from a variety of cell lines and tissues, a 100 kDa protein was detected only in cell lines and tissues permissive for PV.

D. Integrins as picornavirus receptors

The discovery that the cellular receptors for PV and HRV were members of the immunoglobulin superfamily was consistent with the canyon hypothesis for picornavirus binding (Rossman, 1989), and led to speculation that perhaps members of this family of proteins may be receptors for all the picornaviruses (Eisner, 1992).

Previous observations that FMDV lacks a canyon (Acharya *et al.*, 1989) suggested that this generalization would not hold true. The surface of FMDV features a prominent VP1 surface loop containing the tripeptide sequence arginine- glycine- aspartic acid (RGD), which is found in a number of extracellular matrix and adhesion proteins that bind to members of a family of cell surface receptors called integrins (Hynes, 1992). Fox *et al.* (1989) and Baxt and Becker (1990) demonstrated that oligopeptides containing the RGD sequence could compete with FMDV for binding sites on the cell, and thus proposed that the cellular receptor for FMDV may be an integrin. Subsequently, the RGD motif was also identified on the distinct C-terminal extension of capsid protein VP1 of the enterovirus CVA9. Roivainen *et al.* (1991)

showed that RGD-containing peptides effectively blocked virus attachment, suggesting that integrins are also involved in CVA9 binding. Interestingly, trypsin cleavage of the VP1 extension yielded virus that was still infectious, though to a reduced level, indicating that CVA9 was able to bypass RGD-dependent entry, and thus may use an alternative receptor (Roivainen *et al.*, 1991).

By 1991, relatively little was known about the identities of many of the host cell receptors for picornaviruses (Table 3, and above). These molecules appeared to be integral membrane proteins, but other than for HRV and PV, comprehensive descriptions and direct evidence of their role in binding and infection were lacking. The hypothesis that receptor specificity is an important determinant of picornavirus tropism which then influences resulting disease was generally supported by the data available at the time this project was begun, and was a concept that could be tested only through the identification and thorough characterization of additional virus receptors.

In this context, it was felt that the *in vivo* and *in vitro* replication characteristics of EV70 may be determined by the nature and distribution of its cellular receptor. Therefore, in an attempt to understand the molecular basis of the tropism and host range of this virus, identification of the EV70 receptor was undertaken.

VIII. Precedent studies of the host cell receptor for EV70

Prior to the work described in this thesis, little information had been published regarding the nature of host cell receptors for EV70. The important early experiments of Yoshii *et al.* (1977), which originally established that EV70 replication was supported by a

Table 3. Picornavirus receptors identified by 1991

Virus	Receptor	Reference
Poliovirus	PVR (Immunoglobulin superfamily member)	Mendelsohn, *et al.*, 1989
Human Rhinovirus (major group)	ICAM-1	Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989
Human Rhinovirus (minor group)	120 kDa	Mischak *et al.*, 1988a,b
Encephalomyocarditis virus	glycophorin A	Allaway and Burness, 1986
Foot-and-mouth disease virus	Integrin	Fox *et al.*, 1989; Baxt and Becker, 1990
Coxsackievirus A9	Integrin	Roivainen *et al.*, 1991
Coxsackievirus A13, 15,18,21	ICAM-1	Colonno *et al.*, 1986; Lonberg-Holm *et al.*, 1976
Coxsackievirus B1-6	49 kDa	Mapoles *et al.*, 1985
Coxsackievirus B3-RD	60-70 kDa	Hsu *et al.*, 1988,1990
Theiler's murine encephalomyelitis virus	34 kDa	Kilpatrick and Lipton, 1991

number of non-primate cell lines *in vitro*, also examined the interaction of EV70 with these various cell types. Virus binding was assessed using a virus adsorption test. In this assay, monolayers of cells were incubated with a known number of PFU of EV70, then unadsorbed virus was removed from monolayers and titrated for infectivity by plaque assay. High levels of EV70 adsorption were observed with all the cell lines tested. Some cells (rabbit RK13 and RK17; bovine BK1) supported high levels of virus replication and exhibited CPE, while other cells supported somewhat lower levels of virus replication and exhibited no CPE (murine L cells; hamster BHK21 cells; porcine IB-RS-2 and ESK cells). Finally, although they adsorbed high levels of EV70, porcine PK15 and bovine MDBK cells were not permissive for EV70. These results suggested that receptors for EV70 were widely distributed, and that, in view of the observation that non-permissive cells also bound virus, in some cells, expression of a receptor was not sufficient to ensure productive infection.

The observation that high titres of EV70 could agglutinate human "O", guinea pig, and chicken erythrocytes (Kono *et al.*, 1978) led Utagawa *et al.* (1982) to investigate the biochemical nature of the erythrocyte receptor for EV70. The neuraminidase sensitivity of EV70 binding to erythrocytes was compared with the neuraminidase sensitivity of binding by two other human enteroviruses, Echoviruses 7 and 11, and of the cardioviruses mengovirus and EMCV. Results indicated that the erythrocyte receptor for EV70 and for cardioviruses was sensitive to enzyme treatment, thus implicating terminal sialic acid residues in virus binding to the erythrocyte surface. The interaction of Echoviruses 7 and 11 with erythrocytes was unaffected by neuraminidase treatment. Neuraminidase sensitivity of erythrocyte receptors for EMCV (Angel and Burness, 1977) and for rhinovirus 5 (Scott and Killington,

1972) had previously been reported, but had never before been shown for a human enterovirus. While these data provided additional evidence that EV70 was an unusual enterovirus, no further information regarding the nature of the erythrocyte receptor for EV70 has been published

Finally, Yamazaki and Miyamura (1989) briefly commented on attempts to prepare a mAb directed against the EV70 receptor on HeLa cells. A mAb which blocked adsorption of EV70 to HeLa and rabbit RK13 cells, and which also exerted a partial inhibitory effect on adsorption of CVB5 to HeLa cells, was apparently isolated. However, no detailed description of these data has ever been published, and requests for an aliquot of this antibody went unanswered.

IX. Rationale and objectives

When this work was initiated, very few virus receptors were unequivocally identified. However, considerable evidence suggested that for many viruses, the specificity of the virus-host cell receptor interaction was an important, though not exclusive determinant of host range and tropism.

EV70 possesses biological and pathogenic properties that are unusual for a human enterovirus. Among the atypical features of this virus are its restricted *in vivo* tropism and broad *in vitro* host range. A long term objective of this laboratory is to elucidate the molecular basis of these characteristics.

In view of the proposed importance of receptors in governing the host range and tropism of picornaviruses, it was hypothesized that the *in vivo* and *in vitro* replication

characteristics of EV70 were determined by the nature and distribution of its receptor, and that the receptor for EV70 would be different from the picornavirus receptors identified to date. Therefore, the objectives of this thesis were : (1) to identify the receptor for EV70, and (2) to clone the gene encoding the receptor for EV70.

A candidate receptor molecule was identified and partially characterized, and appeared to be similar to decay-accelerating factor (DAF/CD55). As a result of this observation, the objectives of this thesis were changed to: (1) demonstrating that the EV70 receptor was DAF, and (2) initiating characterization of the virus-receptor interaction by mapping the EV70-binding site of DAF.

MATERIALS AND METHODS

I. Cells and cell culture

A. Sources of cell lines

Human HeLa cells were obtained from the NIAID AIDS Research and Reference Reagent Program, Rockville, MD. Rhesus monkey (*Macaca mulatta*) kidney cells, LLC-MK_2, were purchased from Flow Laboratories, Rockville, Maryland. African green monkey (*Cercopithecus aethiops*) kidney cells, CV-1, were obtained from ATCC (Rockville, MD), and murine NIH/3T3 (3T3) cells were provided by Dr. E.G. Brown, of the Department of Microbiology and Immunology, University of Ottawa. Chinese Hamster Ovary (CHO) cells and CHO cells stably expressing human decay-accelerating factor (CHO-DAF; Lublin and Coyne, 1991) were provided by Dr. D. Lublin (Washington University School of Medicine, St. Louis, MO). 3T3 cells transfected with the gene for human DAF under control of the SV40 early promoter and 3T3 cells transfected with the DAF gene in reverse orientation with respect to the promoter (3T3-DAF and 3T3-RDAF; White *et al.*, 1992), were gifts of Dr. J. Atkinson (Washington University School of Medicine, St. Louis, MO).

B. Cell culture

For HeLa, LLC-MK_2 , CV-1, and 3T3 cells, growth medium consisted of Eagle's Minimal Essential Medium containing Earle's salts (MEM) supplemented with 0.2% (w/v) sodium bicarbonate, 2mM L-glutamine, 50 μg/mL gentamycin sulphate (Roussel Canada, Montreal, PQ) and either 5% (v/v) (LLC-MK_2; 3T3) or 10% (v/v) (HeLa; CV-1) heat-

inactivated fetal bovine serum (FBS). CHO cells and CHO-DAF cells were grown in Ham's F12 medium supplemented with 5% (v/v) FBS, 2 mM L-glutamine, and 50 μg/mL gentamycin sulphate. CHO-DAF cells were maintained in medium containing 0.5 mg/mL Genetecin (G418, Boehringer Mannheim Canada, Laval, PQ) (Lublin and Coyne, 1991). 3T3-DAF and 3T3-RDAF cells were maintained in MEM supplemented as above, but containing 10% (v/v) FBS and 0.5 mg/mL Genetecin (White *et al.*, 1992).

Cells were routinely grown as monolayer cultures in 100 mm diameter polystyrene tissue culture dishes (Corning Science Products, Richmond Hill, ON), at 37°C in a humidified 5% CO_2 incubator (Sheldon Manufacturing Inc., Portland, OR). When large numbers of cells were required, monolayers were grown in sterilized Pyrex dishes (33 x 23 x 5 cm; Corning, UK). Each confluent Pyrex dish contained approximately 1 x 10^8 cells, the equivalent of approximately twenty-two 100 mm diameter tissue culture dishes. All media and supplements were from Gibco/BRL Life Technologies Canada (Burlington, ON), unless stated otherwise.

As cells approached confluency, they were passaged as follows. Culture medium was aspirated, monolayers were washed with warmed Tris-buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose, and 25 mM Tris-HCl pH 7.2), and then trypsinized in 0.05% trypsin-0.02% EDTA (Gibco/BRL) for 3 to 5 min at 37°C. Cells were removed by pipetting and were added directly to fresh growth medium in new tissue culture dishes at an appropriate dilution. For routine propagation, cells were split at either a 1:5 or 1:10 ratio.

II. Viruses and virological methods

A. Sources of virus strains

Enterovirus 70 (EV70) prototype strain J670/71 was obtained from Dr. M. Hatch and Dr. M. Pallansch of the Centers for Disease Control, Atlanta, GA. Poliovirus (PV) strain Sabin 1 was obtained from Dr. D. Denicourt, Health Canada, Ottawa, ON, and coxsackievirus B3 (CVB3) was provided by Dr. S.A. Sattar of the Department of Microbiology and Immunology, University of Ottawa. Recombinant vaccinia virus vTF7-3, expressing bacteriophage T7 RNA polymerase (Fuerst *et al.*, 1986) was obtained from ATCC (Rockville, MD), and was propagated and titrated in CV-1 cells.

B. Growth and purification of EV70

Regular virus stocks were prepared by infecting confluent monolayers of LLC-MK_2 cells in 100 mm tissue culture dishes at a multiplicity of infection (MOI) of 0.01 - 0.1 plaque forming units (PFU) per cell in serum and antibiotic-free MEM. After allowing the virus to adsorb for 1 h at 33°C (in an atmosphere of 5% CO_2), the inoculum was removed, 4 mL of fresh complete MEM were applied, and cells were incubated at 33°C until the cytopathic effects (CPE) of infection were near-maximal (24 to 36 h). Cells and growth medium were harvested and placed at -80°C. Following three cycles of freezing and thawing, cellular debris were removed by centrifugation (4,000xg for 10 min at 4°C), and aliquots of virus-containing supernatants were stored at -80°C.

C. Preparation of concentrated virus stocks

(i) **EV70:** LLC-MK_2 cell monolayers were grown in 8 to 10 sterile Pyrex dishes. Monolayers were infected (1 h, 33°C) at a MOI of 0.1 PFU/cell in serum and antibiotic-free

growth medium, and then incubated in complete medium at 33°C for approximately 30 h. With the aid of a rubber policeman, virus was collected by harvesting medium and cells (125 mL per dish), followed by three cycles of freezing and thawing, and clarification at 4,000xg for 10 min at 4°C. Pooled supernatants were concentrated by tangential flow ultrafiltration using a Minitan ultrafiltration apparatus with PTHK 100K filters (Millipore Ltd., Mississauga, ON). Virus was pelleted from concentrated supernatant at 110,000xg for 4 h at 4°C, resuspended in R buffer (0.2 M NaCl, 50 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5) (Abraham and Colonno, 1984), and placed on a linear density gradient of 10 to 40% (w/v) sucrose in R buffer. After centrifugation at 154,000xg for 3.5 h at 4°C, 500 µL gradient fractions were collected by bottom puncture, and portions of each fraction were tested by plaque assay. Peak virus-containing fractions were pooled, centrifuged at 200,000xg for 2.5 h at 4°C, resuspended in either R buffer or in phosphate-buffered saline, pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), divided into aliquots, and stored at -80°C.

(ii) **Radiolabelled EV70:** As described above, monolayers of LLC-MK_2 cells grown in Pyrex dishes were infected with EV70 at a MOI of 0.1 for 1 h at 33°C. The inoculum was removed, and serum-free methionine-free growth medium (ICN Biomedicals Canada Ltd., Mississauga, ON) was added to the monolayers. After 3 h, 5 to 8 µCi of Tran^{35}S-label (ICN) were added per mL of medium. Incubation was continued for a further 30 h, and virus was harvested as described above. Sucrose gradient fractions were recovered and analyzed for the presence of radiolabelled virus using a RackBeta 'Excel' liquid scintillation counter (LKB Wallac, Turku, Finland). Peak fractions were pooled, centrifuged

at 200,000xg for 2.5 h at 4°C, resuspended in R buffer or PBS, divided into aliquots, and stored at -80°C.

(iii) **CVB3:** Freshly confluent HeLa cells in 100 mm tissue culture dishes were infected at a MOI of 0.1 as described above, but at 37°C. Incubation was continued at 37°C until monolayers were approximately 80% destroyed. Cells and supernatant were recovered and subjected to three cycles of freezing and thawing. Cellular debris was removed by centrifugation at 32,000xg for 15 min at 4°C. Supernatants were transferred to new centrifuge tubes and Nonidet P-40 (NP-40; Sigma Chemical Company, St. Louis, MO) was added to a final concentration of 1% (v/v). After gentle, thorough mixing, virus was pelleted by centrifugation at 110,000xg for 4 h at 4°C. The resulting pellet was resuspended in R buffer, and applied to a 10 to 40% (w/v) sucrose linear density gradient. Fractions were recovered, and tested by plaque assay. Peak virus fractions were pooled, centrifuged at 200,000xg for 2.5 h at 4°C, and the final virus pellet was resuspended in a small volume of PBS. Aliquots were stored at -80°C.

D. Plaque assay

All virus stock titers were determined by plaque assay. Monolayers of LLC-MK_2 cells were grown to near confluency in 60 mm polystyrene tissue culture dishes (Corning). Growth medium was removed, and cells were infected with 0.5 mL of virus serially diluted in serum-free MEM. After 1 h incubation with occasional rocking at 33°C, the inoculum was removed, and monolayers were overlaid with 3 mL of a solution of growth medium containing 0.8% (w/v) agarose (Bio-Rad Laboratories Ltd., Mississauga, ON). After incubation for 4 days at 33°C, the monolayers were fixed with a solution of 10% formol-saline (10% [v/v] formalin,

0.8% [w/v] NaCl) for 1 h at 37°C, the overlays were removed, and cells were stained with a solution of 0.1% (w/v) crystal violet. Plaques were counted, and virus titers were expressed as PFU per mL.

III. Cell membrane preparation

Eight to ten Pyrex dishes of HeLa cells were used to prepare membranes for immunization of mice (see below). After removal of growth medium, freshly confluent monolayers were washed with PBS, then incubated for 15 min at 37°C with PBS containing 50 mM EDTA. Cells were removed with the aid of a rubber policeman, pooled, and then pelleted at 1,000xg for 5 min at 4°C. Cells were washed three times with cold PBS, and the final pellet was resuspended in 13 mL of 10 mM sodium phosphate buffer, pH 7.4. Resuspended cells were incubated on ice for 15 min, then disrupted in a Dounce homogenizer in the presence of protease inhibitors (1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride [PMSF] (both from Sigma), and 5 mM EDTA). Following centrifugation at 1,400xg for 5 min at 4°C, the supernatant was transferred to fresh tubes and centrifuged at 200,000xg for 2 h at 4°C. The final membrane pellet was resuspended in PBS, protein concentration was determined by Bradford assay (Bio-Rad), and sample concentration was adjusted to 10 mg/mL. Aliquots of the membrane preparation were stored at -80°C.

IV. Immunological methods and reagents

A. Production of anti-receptor antibodies

Six to eight week-old inbred female BALB/c mice (Charles River Laboratories, St. Constant, PQ) were maintained in the Animal Care Unit at the National Research Council, Ottawa, ON. Two groups of mice were immunized by intraperitoneal injection, at three-week intervals. One group was injected with HeLa cell membranes, the other with whole HeLa cells. Primary immunizations consisted of 50 μg of membrane protein or 4 X 10^6 cells emulsified in Complete Freund's Adjuvant, and were followed by injections with 80 μg of membrane protein or 5 X 10^6 cells in incomplete Freund's Adjuvant, and finally, with 120 μg of membrane protein or 1 X 10^7 cells in PBS. In order to confirm antibody production against the antigen, 200 μL of blood were recovered from each mouse by tail bleed, and dilutions of sera were examined by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA microtiter plates (Corning) were coated by drying 100 μL of a 10 μg/mL membrane preparation diluted in distilled water, in each well. ELISAs were performed using standard methods (Engvall and Perlman, 1971). Dilutions of mouse sera were used as primary antibody, and a 1:1000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG/M (Jackson Immunoresearch Laboratories Inc., West Grove, PA) was used as secondary antibody. The substrate, 2,2'-azino-bis (3-ethylbenz-thiazoline sulphonic acid) (ABTS), was from Sigma. ELISA plates were read at A_{490} using a Bio-Rad Model 3550 microplate reader.

Three days prior to cell fusion, mice were primed by tail vein injection of 175 μg of membrane protein or 1.5 X 10^7 cells in PBS. Splenocytes were prepared essentially as

described by Kennet *et al.* (1978). The spleens of two mice from each group were removed and placed in a 60 mm petri dish containing Dulbecco's Minimal Essential Medium (DMEM) with 20 % (v/v) FBS (DMEM-20% FBS). Cells were perfused from the spleens using a 1mL syringe fitted with a 26 gauge needle (Becton Dickinson and Co., Rutherford, NJ). Spleen cells were removed, centrifuged at 200xg for 10 min, resuspended in cold 0.17 M NH_4Cl, and diluted with an equal volume of DMEM-20% FBS. After recentrifugation, the cells were resuspended in cold DMEM-20% FBS, counted and tested for viability using trypan blue exclusion.

Polyethylene glycol (PEG) - mediated fusion of spleen cells and the non-secreting SP2/0 parental myeloma cell line (Schulman *et al.* , 1978) followed the protocol described by Kennet (1979). Spleen cells and SP2/0 cells were mixed at a ratio of 10:1, and washed with DMEM-20% FBS. Following removal of the supernatant, the spleen-SP2/0 cell pellet was gently resuspended in 200 µL of 30% (w/v) PEG 1450 (J.T. Baker Chemical Co., Phillipsburg, NJ) prepared in serum- free DMEM. After 8 min at 37°C, the cells were pelleted at 400xg for 5 min, the PEG solution was slowly diluted by gentle mixing with 5 mL of serum-free DMEM, and finally, an equal volume of DMEM-20% FBS was added. The cells in diluted PEG were pelleted at 200xg for 10 min, and resuspended in 30 mL of HT medium (DMEM with 4.5 g/L glucose, 10% (v/v) NCTC 109 medium, 20% (v/v) FBS, 50µg/mL gentamycin sulphate, and 1 mM oxaloacetic acid, 0.56 mM sodium pyruvate, 0.200 units/mL bovine insulin, 16 µM thymine, 0.1 mM hypoxanthine [all from Sigma]). After 30 min at room temperature, the cells were gently distributed into microtiter plates (approximately 1.7 X 10^4 cells per well). The following day, an equal volume of HT medium containing 16 µM

aminopterin (Sigma) was added to each well, to begin selection of hybrids. After 7 days, additional HT medium was added to each well. On day 8, medium was carefully aspirated and replaced with fresh HT medium. Clones appeared within eight days.

On day 10, the supernatants of all viable hybridomas were screened for the secretion of anti-EV70 receptor antibodies by the cell protection assay described below. Any hybridoma supernatants that were protective or showed signs of delaying the onset of EV70-induced CPE were subjected to further rounds of screening. Hybridomas of interest were transferred and grown in 24-well tissue culture dishes, and finally, 25 cm^2 tissue culture flasks (Nunclon, Gibco/BRL).

Hybridomas producing protective antibodies were cloned twice by limiting dilution and their supernatants were retested. Positive clones were grown, and were used to produce ascites fluid (Hoogenraad *et al.*, 1983). Retired BALB/c breeders received intraperitoneal injections of 0.5 mL of pristane (2,6,10,14, tetramethylpentadecane; Sigma) ten days prior to being inoculated with 1 X 10^7 hybridoma cells. Ten days later, ascites fluid was collected.

B. Antibody isotyping

Isotyping was performed by indirect ELISA using standard methods (Engvall and Perlman, 1971). HeLa cell membrane protein (3μg per well) was adsorbed to ELISA microtiter plate wells and undiluted tissue culture supernatants from cloned cells were used as primary antibody. Horseradish peroxidase-conjugated goat anti-mouse isotype-specific antibodies (Caltag, South San Francisco, CA) diluted 1:2000 were used as secondary antibodies, ABTS (Sigma) was used as the substrate, and A_{490} values were recorded.

C. Antibody quantification

Antibodies in culture supernatants and ascites fluids were quantified by antibody-sandwich ELISA (Hornbeck, 1991). Briefly, ELISA microtiter plates coated with 1 μg/well rat anti-mouse IgG (H+L) (Jackson) capture antibody, were washed with PBS-0.05% (v/v) Tween 20 (Sigma), blocked with PBS-2% (w/v) bovine serum albumin (BSA, Sigma), washed, and then incubated with dilutions of antibody samples, either as tissue culture supernatant or ascites fluid. After 1 h at room temperature, plates were washed, and 100μL (1.25 mg/mL) of a biotinylated mouse-specific IgG_1 monoclonal antibody were added to each well. After 1 h at room temperature, wells were washed, and incubated with 100 μL per well (30 ng/mL) peroxidase-conjugated streptavidin (Jackson) for 15 min at room temperature. After a final wash, o-phenylenediamine dihydrochloride substrate (OPD, Sigma) was added to each well. Thirty minutes later, the reaction was stopped by addition of 0.5 M H_2SO_4 (50 μL/well), and A_{490} values were recorded. Antibody concentrations were determined from a standard curve constructed with known amounts of mouse IgG_1 (Coulter Electronics, Burlington, ON) as primary antibody. EVR1 hybridoma supernatant contained 17.5 μg Ab/mL, and EVR1 ascites contained 1.1 mg Ab/mL.

D. Sources of other antibodies

DAF-specific mAbs 1H4 (35 μg/mL), 8D11 (36 μg/mL), 11D7 (40 μg/mL) (Coyne *et al.*, 1992), and 1F7 (1.2 mg/mL) (Bergelson *et al.*, 1994) were used. 1H4 hybridoma culture supernatant was provided by Dr. D. Lublin (Washington University School of Medicine, St. Louis, MO). 8D11 and 11D7 hybridoma culture supernatants were gifts from Dr. W. Rosse (Department of Medicine, Duke University Medical Center, Durham, NC), and

1F7 ascites fluid was from Dr. R.W. Finberg (Dana-Farber Cancer Institute, Boston, MA). The DAF domain specificities of these antibodies are presented in Table 4. Anti-lymphocytic choriomeningitis virus (LCMV) ascites fluid (IgG_1) (4 mg/mL) was provided by Dr. K. Wright (Department of Microbiology and Immunology, University of Ottawa). Fluorescein isothiocyanate - conjugated (FITC) and non-conjugated mouse antibodies specific for human HLA ABC class I IgG_1 (anti-MHC I), and mouse anti-human CD46 IgG_{2a} (clone E4.3) were from Serotec Canada (Toronto, ON). FITC-conjugated sheep anti-mouse Ig was from Amersham Canada (Oakville, ON). Anti CVB3 receptor mAb RmcB was provided by Dr. R. Crowell (Hahnemann University School of Medicine, Philadelphia, PA).

V. Cell protection assays

A. Hybridoma culture supernatants and mAb EVR1

Flat-bottomed 96-well tissue culture plates (Corning) were seeded with either LLC-MK_2 or HeLa cells. Once monolayers approached confluency (approximately 2 x 10^4cells per well), growth medium was removed and the monolayers were incubated with 100 µL of undiluted hybridoma culture supernatant for 1 h at 37°C. Cells were washed with PBS and EV70 was added at a MOI of 1 in serum and antibiotic-free MEM. After 45 min at 33°C, the inoculum was removed, fresh complete MEM containing FBS was added and the cells were placed at 33°C. Monolayers were monitored at regular intervals by light microscopy, for signs of virus-induced CPE. Under the conditions of this assay, control wells (receiving no antibody pretreatment) showed complete monolayer destruction by 48 h post-infection. Results of antibody screening were confirmed on duplicate plates using a colorimetric assay

Table 4. Domain specificities of anti-DAF antibodies

Antibody	DAF domain specificity	Source
11D7	SCR1[a]	W. Rosse
1F7	SCR2[b]	R. Finberg
1H4	SCR3[c]	D. Lublin
8D11	SCR4[d]	W.Rosse

[a] Coyne *et al.*, 1992
[b] Bergelson *et al.*, 1994
[c] Coyne *et al.*, 1992
[d] *ibid*

based on the ability of viable cells to metabolize the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT; Sigma) (Hansen *et al.*, 1989). Cell protection assays using PV and CVB3 were performed in an identical manner, except that all incubations were performed at 37°C.

B. DAF-specific mAbs

DAF-specific mAbs 1H4, 8D11, 11D7, and 1F7 were tested for their ability to protect monolayers against virus infection as described above. Antibodies were diluted appropriately in MEM, except for MAb 1F7, which was diluted in RPMI 1640 medium containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (Bergelson *et al.*, 1994).

VI. Virus binding assays

A. Virus binding assays

Cells (2.5×10^5 per well) were grown to confluency in 24-well tissue culture plates (Corning). Growth medium was aspirated, monolayers were washed with 200 µL of PBS, and ^{35}S-labelled EV70 (^{35}S-EV70; 2,000 to 4,000 cpm per well) was added in serum-free MEM, in a total volume of 150 µL. After a 45 to 60 min incubation at 33°C, the inoculum was removed and the monolayers were washed with 200 µL of PBS. The inoculum and PBS wash were pooled, and represent the unbound fraction of input virus. The monolayers were then disrupted with 150 µL of PBS containing 1% (w/v) SDS, and the wells were washed with 200 µL of PBS. These samples, representing the bound fraction of input virus, were also pooled. Samples were analyzed by scintillation counting. The percent of input virus bound to cells was determined by dividing the counts per minute (cpm) of labelled EV70 in the

bound fraction by the total number of cpm recovered per well. Binding of virus to positive control wells (typically 25 to 35% of input ^{35}S-EV70) was set as 100 percent virus binding, and binding of virus to test wells was expressed as the percent of virus bound relative to these positive control wells.

B. Virus binding competition assays

LLC-MK_2 cells were grown to confluency in 24-well tissue culture plates (Corning). Increasing amounts of unlabelled competitor virus (EV70, PV, or CVB3) were added to a fixed amount of ^{35}S-EV70 (approximately 2 X 10^6 PFU per well; 2,000 cpm per well) in a final volume of 150 µL. After removal of growth medium and a PBS wash, the mixtures of ^{35}S-EV70 plus competitor were applied to monolayers, and the assay proceeded as described above for the virus binding assay.

C. Virus binding inhibition assays

After removal of growth medium and washing with PBS, monolayers were treated with 100 µL of test sample (mAb EVR1, 1H4, 8D11, 11D7, anti-MHC 1, or MEM) for 1 h at 37°C. The test sample was then removed, monolayers were washed with PBS, and virus binding assays were performed as described above.

VII. Indirect immunofluorescence

Cells were removed from 100 mm plates by scraping or by treatment with 0.05% trypsin-0.02% EDTA (Gibco/BRL) in PBS, and washed in PBS containing 2% (w/v) BSA and 0.1% (w/v) sodium azide (Sigma) (PBS-BSA). Cells (approximately 5 X 10^5 per sample) were dispensed into 5 mL polypropylene tubes (Sarstedt of Canada, St. Laurent, PQ), pelleted

at 1500 rpm for 5 min in a Sorvall GLC-2B centrifuge (Dupont Inc., Mississauga, ON), and resuspended in 25 µL of undiluted hybridoma cell culture supernatant, or ascites fluid diluted 1:100 in PBS-BSA. After incubation on ice for 1 h, cells were washed in PBS-BSA, and the cell pellets were resuspended in 20 µL of secondary antibody (FITC-conjugated sheep anti-mouse IgG_1) diluted 1:10 in PBS-BSA. After 1 h on ice, the cells were washed, resuspended in 90% glycerol/PBS, pH 9.6, deposited on glass slides under coverslips (Fisher Scientific, Nepean, ON) and examined using a Zeiss Aristoplan epifluorescent microscope (Zeiss Canada Ltd., Don Mills, ON).

For 3T3 cells transiently expressing human DAF (see below), growth medium was removed from monolayers cultured in 24-well plates (Corning), and after gentle washing with PBS, cells were trypsinized and removed by pipetting. Two to three wells of transfected cells (approximately 2 X 10^5 cells per well) were combined and processed for immunofluorescence as described above.

All photography was performed at 40X power (eyepiece magnification 10X), using Kodak Ektachrome 400 ASA film (Kodak Canada Inc., Toronto, ON).

VIII. Phospholipase sensitivity of the ligand of EVR1

The susceptibility of the ligand of EVR1 to phosphatidylinositol-specific phospholipase C (PI-PLC) was examined essentially as described by Bergelson *et al.* (1994). HeLa cell monolayers were dispersed by trypsin treatment, dispensed (1 X 10^6 cells per sample) into 5 mL tubes (Sarstedt), and washed three times with PBS. Cells were resuspended in 25 µL of PI-PLC buffer (RPMI 1640 medium; 0.2% BSA; 50 µM 2-

mercaptoethanol; 10 mM HEPES; 0.1% (w/v) sodium azide), or in PI-PLC buffer containing 0.5 units of *Bacillus thuringiensis* PI-PLC (Oxford Glycosystems, Rosedale, NY). Following incubation at 37°C for 1 h, samples were washed with PBS, then processed for indirect immunofluorescence as described above (EVR1 and 1H4 culture supernatant, undiluted; anti-MHC I, 1:10).

IX. Protein analysis

A. Radioimmunoprecipitations

Nearly confluent monolayers of cells grown in 100 mm tissue culture dishes were washed with PBS and then incubated in 9 mL of glucose-free medium (Gibco/BRL) supplemented with 5% (v/v) FBS, for 2 h at 37°C. Following this starvation period, 15 µCi of D-[6-^{3}H]-glucosamine-hydrochloride (Amersham) were added per mL of medium. After 16 h at 37°C, cells and medium were recovered and centrifuged at 1000 rpm for 5 min (Sorvall GLC-2B). After three washes with cold PBS, the cell pellet was resuspended in detergent-free RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl) , with 1 mM PMSF and 1 mM benzamidine-HCl. An equal volume of RIPA buffer containing 2X detergent (2% (w/v) sodium deoxycholate (Difco Laboratories, Detroit, MI), 2% (v/v) NP-40) was added, and after mixing by inversion, samples were given a short pulse in a bench-top microcentrifuge to pellet cellular debris. Aliquots of cell lysates were stored at -80°C.

Protein G Sepharose 4 Fast Flow beads (Pharmacia Canada, Baie d'Urfe, PQ) were washed in RIPA buffer containing 2% ovalbumin, and resuspended in 100 µL of RIPA buffer. To this, 200 µL of cell lysate and 10 µL of undiluted ascites fluid were added. Samples were

allowed to mix overnight at 4°C, after which time the beads were washed five times in RIPA buffer containing 1X detergent. Pelleted beads were resuspended in 25 μL of sample buffer (60.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue) and heated at 95°C for 5 min to dissociate antibody-protein complexes from beads. Beads were removed by brief centrifugation and samples were analyzed by electrophoresis in 8% polyacrylamide gels containing SDS, as described below.

B. SDS-Polyacrylamide gel electrophoresis

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), performed essentially as described by Laemmli (1970). All chemicals were obtained from BioRad Laboratories Ltd., unless stated otherwise. Samples to be analyzed were mixed with an equal volume of 2X electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2%(w/v) SDS, 20% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue), and heated at 95°C for 5 min before being applied to gels. Resolving gels consisted of 8% acrylamide, 0.21% bis-acrylamide, 0.1% SDS and 750 mM Tris-HCl pH 8.8. Stacking gels consisted of 5% acrylamide, 0.13% bis-acrylamide, 0.1% SDS and 73 mM Tris-HCl pH 6.8.

Electrophoresis was carried out at 100 V in electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), using a BioRad Mini-Protean II electrophoresis cell. Following electrophoresis, gels were fixed in a solution of 30% (v/v) methanol, 10% (v/v) glacial acetic acid for 20 to 30 min, and incubated in Amplify (Amersham) for 1 h. Gels were then dried under vacuum and exposed to X-ray film (Amersham). Prestained high range molecular mass markers (Amersham) were: myosin (200 kDa); phosphorylase b (97.4 kDa); bovine serum

albumin (69 kDa); ovalbumin (46 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (21.5 kDa); and lysozyme (14.3 kDa).

C. Western blots

Lysates of unlabelled cells were prepared as described above for lysates of radiolabelled cells. Proteins (25μg/lane) were separated by electrophoresis at 4°C, in 8% polyacrylamide gels, as described above, with the following modifications. Gels and running buffers contained 0.05% SDS, and samples were prepared without heating in electrophoresis sample buffer containing 0.05% SDS and no 2-mercaptoethanol. Proteins were then transferred from gels to Schleicher & Schuell NC nitrocellulose membranes (Mandel Scientific Co. Ltd., Guelph, Ontario). Prior to transfer, membranes and gels were equilibrated in transfer buffer (2 mM Tris base, 192 mM glycine, 20% (v/v) methanol) (Towbin *et al.*, 1979). Transfer was performed at 4°C using a BioRad Mini Trans-Blot apparatus (2 h, 100 V). Following transfer, membranes were blocked in PBS containing 3% (w/v) BSA (PBS-3%BSA), for 30 min at 37°C. After three 10 min 37°C washes in PBS-0.02% (v/v) Tween 20 (PBS-Tween), membranes were incubated for 1 h at 37°C with monoclonal antibody as either hybridoma supernatant (1H4, 1:3000) or ascites fluid (EVR1, 1:1000; anti-LCMV IgG_1, 1:300) diluted in PBS- 3% BSA. After three washes in PBS-Tween, membranes were incubated with peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson) diluted 1:20,000 in PBS-3%BSA, for 1 h at 37°C. Membranes were washed with PBS-Tween, and colour was developed in a solution consisting of 10 mM $(NH_4)_2SO_4$, 10 mM KH_2PO_4 pH 6.0, 0.03% (v/v) H_2O_2 and 0.01% (v/v) of a solution of 0.5% (w/v) o-dianisidine (Sigma) in methanol. The reaction was stopped with repeated washes in distilled water.

X. EV70 Replication studies

EV70 replication in 3T3-DAF and 3T3-RDAF cells was examined as follows. Cells were grown to confluency in 24-well tissue culture dishes (Corning), and infected with EV70 at a MOI of 5. After 1 h at 33 °C, monolayers were washed three times with 1 mL of PBS in order to remove non-adsorbed virus. One mL of fresh complete MEM was added to each well, and incubation was continued at 33°C. At regular intervals, cells and growth medium were recovered, and stored at -80° C. After two cycles of freezing and thawing, virus titer at each time point was determined by plaque assay.

XI. DNA Manipulations

A. Isolation of plasmid DNA

(i) **Small scale plasmid isolation:** Isolation of plasmid DNA was performed by a procedure modified from that described by Birnboim and Doly (1979). Individual bacterial colonies were grown overnight at 37°C with shaking, in 3 mL of YT medium (0.8% (w/v) tryptone, 0.8% (w/v) yeast extract, 0.5% (w/v) NaCl) containing 50 μg/mL of ampicillin. Cells were pelleted in a bench top microcentrifuge, and were resuspended in 100 μL of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) containing 50 μg/mL DNAse-free RNAse A. One hundred μL of freshly prepared Solution II (1% (w/v) SDS, 0.2 N NaOH) were added, and tubes were gently mixed by inversion. Lysates were neutralized by addition of 100 μL of ice cold Solution III (60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL H_2O). After mixing, samples were centrifuged for 5 min at 4°C at 13,000 rpm (microfuge). Supernatants were extracted with an equal volume of chloroform,

centrifuged for 1 min (4°C, 13,000 rpm), and DNA was precipitated from the aqueous phase by addition of 750 µL of ethanol and incubation for 30 min at -80°C. Following a final centrifugation (13,000 rpm, 10 min, 4°C), pellets were resuspended in 50 µL of sterile water.

(ii) **Large scale plasmid DNA isolation:** Isolation of larger amounts of purified plasmid DNA was performed using Nucleobond AX kits (AX-100 cartridges; Vector Biosystems, Toronto, ON), according to instructions provided by the manufacturer. Purified plasmid DNA was quantified spectrophotometrically (A_{260}).

(iii) **Transformations:** Stratagene *E. coli* Epicurian Sure cells for electro-transformation were purchased from PDI Bioscience Inc. (Aurora, ON). Electroporation of plasmid DNA into cells was accomplished using the BRL Cell-Porator Electroporation System (Gibco/BRL) with a field strength of 16.6 kV/cm. Ten ng of DNA were gently mixed with 20 µL of freshly-thawed competent cells, and the cell-DNA mixture was placed into microelectroporation chambers (Gibco/BRL). Electroporation was performed using the following settings: 400 V, 330 µF, with low Ω and fast charge settings on the cell porator, and 4 kΩ resistance on the voltage booster. Electroporated cells were recovered and immediately added to 1 mL of prewarmed SOC broth (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated with shaking, at 37°C for 1 h. Aliquots of the transformation mix were plated on YT-ampicillin plates and the plates were incubated overnight at 37°C.

B. <u>DNA analysis</u>

(i) **DNA agarose gel electrophoresis:** Electrophoresis of DNA samples was performed in 1% (w/v) agarose gels (Bio-Rad) in Tris-acetate buffer (TAE; 40 mM Tris-

acetate, 2 mM EDTA). DNA was mixed with loading buffer (5% (v/v) glycerol, 0.1% (w/v) SDS, 10 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol FF), and was applied to gels in a Horizon 58 submarine electrophoresis unit (Gibco/BRL). Following electrophoresis at 50 V for 30 min to 1 h, gels were stained in ethidium bromide (0.5μg/mL) and fragments were visualized using short-wave ultraviolet light. Low-melting temperature agarose gels (see below) were poured and allowed to set at 4°C, and electrophoresis of samples in these gels was performed at 4°C.

(ii) **Isolation and purification of DNA fragments from agarose gels:** DNA fragments were resolved in low-melting point agarose (FMC Bioproducts, Rockland, ME) gels (1% w/v in TAE buffer). After staining in ethidium bromide, fragments of interest were excised from gels and placed into microfuge tubes. DNA was purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI) according to the instructions provided by the manufacturer.

(iii) **Restriction and modification of DNA:** Unless stated otherwise, all restriction endonucleases and DNA modifying enzymes were from Pharmacia, New England Biolabs (Mississauga, ON), or Boehringer Mannheim.

(a) *Restriction Digestions*: Routinely, DNA (1μg/20 μL) was digested with 2 to 5 units of enzyme per sample. Digestions were performed for 1 h in buffers and at temperatures recommended by the supplier.

(b) *Ligations*: Restriction fragments were mixed with linearized plasmid at a molar ratio of 5:1 (vector:insert). Ligations were performed in 30 mM Tris-HCl, pH8.0, 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM ATP, with approximately 1 Weiss unit of T4 DNA ligase

(Gibco/BRL) per 20 µL reaction. Ligation mixtures were incubated overnight at 16°C, and following phenol-chloroform extraction, ligation products were precipitated with ethanol (Sambrook *et al.*, 1989).

To reduce self-ligation frequency, linearized vector was dephosphorylated prior to subcloning. Five µg of DNA was dephosphorylated using 0.5 to 1.0 unit of calf intestinal alkaline phosphatase (CIAP) in 50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine. After 30 min at 37°C, CIAP was inactivated by phenol:chloroform extraction.

XII. DAF expression studies

A. Assessment of DAF as a receptor for EV70

(i) **Source and description of constructs:** DAF and TM-DAF constructs were gifts of D. Lublin (Washington University School of Medicine, St. Louis, MO). Human DAF cDNA was provided in expression vector pcDNA3 (Invitrogen Corp., San Diego, CA). This vector contains the early gene product promoter of cytomegalovirus (CMV) as well as the promoter for bacteriophage T7 RNA polymerase, the latter required for the transient expression experiments described below.

A version of DAF with a transmembrane domain (TM-DAF) in place of the normal glycosylphosphatidylinositol (GPI) anchor was provided in expression vector pSFFV.neo. The construction of TM-DAF was described by Lublin and Coyne (1991). TM-DAF was produced by ligating a fragment of cDNA encoding the five extracellular domains of DAF (amino acids 1 to 304) to a fragment of cDNA encoding the transmembrane and cytoplasmic

domains of membrane cofactor protein (MCP/CD46; amino acids 270 to 350). Amino acid numbering begins with the first amino acid of the mature protein. The gene encoding TM-DAF was excised from plasmid pSFFV.neo by *Eco*RI digestion, the fragment was isolated, and ligated into the *Eco*RI site of linearized pcDNA3.

In transient expression experiments designed to assess the ability of DAF and of TM-DAF to bind EV70, cells transfected with pcDNA3 served as a negative control. For some experiments, HeLa cells served as a positive control for DAF expression.

B. Mapping the EV70 binding sites of DAF

(i) **Source and description of constructs:** DAF/MCP chimeras, (DM1, DM2, DM3, DM4, and MCP-PI), were gifts of Dr. D. Lublin (Washington University School of Medicine). The construction of these hybrid molecules (where DMn refers to a DAF molecule in which SCRn has been replaced with the corresponding SCR domain of MCP) was described by Manchester *et al.* (1995). Chimeras replaced DAF SCR1 (amino acids 1 to 62) with MCP SCR1 (amino acids 1 to 62), DAF SCR2 (amino acids 63 to 126) with MCP SCR2 (amino acids 63 to 125), DAF SCR3 (amino acids 127 to 188) with MCP SCR3 (amino acids 126 to 191), and DAF SCR4 (amino acids 189 to 251) with MCP SCR4 (amino acids 192 to 251). A GPI-anchored version of MCP (MCP-PI; Lublin and Coyne, 1991) consisted of amino acids 1 to 269 of MCP and amino acids 307 to 347 of DAF.

DM1, DM3, and DM4 were provided in expression vector pCRα. This modified form of vector pcDNA3 contains the R-U5' segment from human T-cell leukemia virus I, in addition to the early promoter of CMV and the promoter for bacteriophage T7 RNA polymerase. These elements are located upstream of the multiple cloning site into which the

chimeric cDNAs were inserted. DM2 and MCP-PI were provided in vector pSRαEN. This vector contains the promoter for bacteriophage T7 RNA polymerase, the Simian Virus 40 (SV40) early gene product promoter, and the R-U5' segment from human T-cell leukemia virus I, all located upstream of the multiple cloning site. In 3T3 cells, the vectors pSRαEN and pCrα give comparable levels of DAF/MCP chimera expression.

In transient expression experiments designed to map the EV70 binding site(s) of DAF, pCRαDAF was used as a positive control for DAF expression. This construct was generated by subcloning the *Eco*RI fragment containing DAF cDNA from pcDNA3/DAF into *Eco*RI linearized pCRα. For these transient expression experiments, two negative controls were included: (1) cells transfected with vector pCRα; (2) cells transfected with MCP-PI.

C. Transient expression

Twenty-four- well tissue culture plates (Corning) were seeded with 8 X 10^4 3T3 cells per well. The next day, growth medium was removed, and monolayers were carefully washed with warmed OptiMEM (Gibco/BRL). Cells were then transfected using LipofectAMINE reagent (Gibco/BRL), following instructions provided by the manufacturer. Briefly, plasmid DNA and LipofectAMINE (1 μg of DNA and 3 μL of LipofectAMINE per well, diluted in OptiMEM) were combined, and 500 μL of the mixture were applied per well. Transfection was allowed to proceed at 37°C for 9 h, at which time an equal volume of MEM with 10% (v/v) FBS was added to each sample. After an additional 15h at 37°C, lipid-DNA complexes were removed, monolayers were washed, and were infected with vaccinia virus vTF7-3 at a MOI of 15. After 1 h at 37°C, the inoculum was removed and the cells were incubated in 1 mL of MEM with 5% (v/v) FBS. Eighteen hours post-infection, expression of DAF or

DAF/MCP proteins was analyzed by indirect immunofluorescence and/or by flow cytometry. The ability of transfectants to bind virus was evaluated using the virus binding assay described above.

XII. Flow Cytometry

A. Sample preparation

(i) **HeLa, 3T3-DAF, and 3T3-RDAF cell lines:** In experiments examining DAF expression in HeLa cells and in the stable cell lines 3T3-DAF and 3T3-RDAF, cells were grown on 100 mm tissue culture dishes, monolayers were washed with PBS, cells were dispersed by trypsin treatment, counted, and 5 X 10^5 cells were dispensed into 5 mL polypropylene tubes (Sarstedt). These samples were then processed for flow cytometric analysis as described below.

(ii) **3T3 cells transiently expressing DAF and TM-DAF:** In the first experiments performed to examine the ability of DAF expression to confer EV70 binding activity to 3T3 cells, plasmids pcDNA3/DAF and pcDNA3/TM-DAF were transfected into cells as described above. Monolayers of transfected cells were washed once with OptiMEM, and then were dispersed by trypsin treatment. Common transfectant samples were pooled, dispensed (2 X 10^5 cells per tube), and processed for flow cytometric analysis.

(iii) **3T3 cells transiently expressing DAF/MCP chimeras:** Because MCP is sensitive to trypsin (Naniche *et al.*, 1992), cells used in experiments designed to identify the EV70 binding site(s) of DAF (transfected cells expressing DAF/MCP chimeras and DAF controls) were dispersed as follows. After washing with OptiMEM, monolayers (in 24-well

tissue culture plates) were incubated with 300 μL per well of PBS containing 50 mM EDTA. After 10 to 15 min on ice, cells were recovered by scraping, common transfectant samples were pooled and cells were disaggregated by three passes through a 1 mL syringe fitted with a 25 gauge needle (Becton Dickinson). Cells were then dispensed (2 X 10^5 per tube), and processed for flow cytometric analysis.

B. Cell staining

Cells dispensed into 5 mL tubes were washed with PBS-2%BSA (PBS containing 2% (w/v) BSA and 0.1% (w/v) sodium azide), or PBS-2%BSA containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$. After centrifugation at 1500 rpm in a Sorvall GLC-2B centrifuge (Dupont), cells were resuspended in 40 μL of PBS-2%BSA containing primary antibody (Table 5). Cells were incubated for 20 min at room temperature, washed, centrifuged, then incubated with FITC-conjugated sheep anti-mouse IgG_1 diluted 1:20 in PBS-2%BSA or in PBS-2%BSA with $MgCl_2$ and $CaCl_2$. After incubation for 20 min at room temperature, cells were pelleted, resuspended in approximately 500 μL of PBS-2%BSA or PBS-2%BSA with $MgCl_2$ and $CaCl_2$, and examined by flow cytometry.

Negative controls, for autofluorescence and for non-specific secondary antibody binding, were included for each sample analyzed. These samples, processed in the same manner as described above, consisted of cells incubated in the absence of both primary and secondary antibody (autofluorescence control), or incubated with secondary antibody only (secondary antibody control).

Table 5. Antibody dilutions used for Flow Cytometry

Antibody	Dilution[a]	(µg/mL)
EVR1	1:100	(1.1)
11D7	1:200	(0.2)
1F7	1:500	(2.4)
1H4	1:5	(7.0)
8D11	1:200	(0.2)
α-MCP	1:1000	(1.0)

[a] All antibodies diluted in PBS-BSA (PBS with 2% (w/v) BSA and 0.1% (w/v) sodium azide), except 1F7, diluted in PBS-BSA with 2 mM $MgCl_2$ and 2 mM $CaCl_2$.

C. Sample analysis

Stained cell suspensions were analyzed using an EPICS XL-MCL flow cytometer (Coulter Electronics of Canada, Burlington, ON), equipped with an argon ion laser tuned at 15 mW of 488 nm excitation light. Forward scatter (FS), log side scatter (SS) and fluorescence emission signal (log scale; LFL1) data were collected as listmode files. A FS versus log SS histogram was established to visualize viable cells, and for each sample analysed, a minimum of 5,000 events were counted in the established gate. Single parameter histograms (relative cell number versus fluorescence intensity) were generated using EPICS XL version 1.5 software (Coulter).

RESULTS

I. Analysis of EV70 binding to cells

The ability to reliably and rapidly measure virus binding to cells is central to the success of any study of virus receptors. Most contemporary investigations rely on assays that quantify the attachment of radiolabelled virus to cells. This method provides a more convenient and direct measurement of virus binding than the adsorption test used by Yoshii *et al.* (1977) in their early examination of EV70-target cell interaction. It was therefore desirable to develop a radiolabelled virus binding assay for EV70. Once the conditions for this assay were established, analyses of EV70 binding and EV70 receptor specificity were performed.

A. Time course of EV70 binding

In order to determine the optimal incubation period for EV70 binding, time course experiments were performed. The ability of EV70 to bind to and replicate in primate-derived cell lines is well established (Miyamura *et al.*, 1974; Yoshii *et al.*, 1977), and in our laboratory, rhesus monkey kidney (LLC-MK_2) cells have traditionally been used for the routine propagation and study of EV70. Consequently, virus binding analyses were carried out with LLC-MK_2 cells. In these experiments, cell monolayers were incubated with 3,000 cpm (approximately 2 X 10^6 PFU) of ^{35}S-EV70, at 33 °C. At intervals, unadsorbed virus particles were removed, monolayers were washed, and residual radioactivity bound to cells was measured by liquid scintillation counting. The amount of virus bound was expressed as a percentage of the total radioactivity applied to each monolayer culture.

Results presented in Figure 1 show that EV70 binding to LLC-MK_2 cells was time dependent, and reached a maximum (38 % of input virus bound) after 45 min of incubation. Therefore, an incubation time of 45 to 60 min was adopted for all subsequent binding assays.

B. Virus binding competition assays

Competition binding studies have shown that picornaviruses can be grouped into distinct receptor families (Abraham and Colonno, 1984; Lonberg-Holm *et al.*, 1976; Lonberg and Korant, 1972; Quirsin-Thiry and Nihoul, 1961). To investigate the specificity of the EV70 receptor, competition experiments were performed with EV70 and two other picornaviruses, PV (Sabin 1) and CVB3. These viruses (PV and CVB3) are known to belong to separate receptor families (Crowell, 1966) and phylogenetically, are closely related to EV70 (Pöyry *et al.*, 1996; Roderigo and Dopazo, 1995; Stanway, 1990).

In these experiments, LLC-MK_2 cell monolayers were incubated with a fixed amount of ^{35}S-EV70 mixed with increasing amounts of homologous or heterologous unlabelled virus. As shown in Figure 2A, unlabelled EV70 competitively inhibited attachment of ^{35}S-EV70 to cells. In the absence of competitor, 33% of input virus bound to cells. Competition for binding sites became apparent in the presence of a 50-fold excess of competitor (approximately 2.5 X 10^7 PFU; 100 PFU per cell), when virus binding was reduced to 11% of input. At or above a 100-fold excess of unlabelled EV70 (approximately 5 X 10^7 PFU; 200 PFU per cell), ^{35}S-EV70 binding decreased to 3% of input.

Figure 2B shows that CVB3 exerted a partial inhibitory effect on EV70 binding. In the absence of competitor, monolayers bound 32% of input ^{35}S-EV70. In the presence of a 50-fold excess of CVB3 (approximately 100 PFU per cell), binding decreased to 18% of

Figure 1. Time course of EV70 binding to HeLa and LLC-MK_2 cells. Confluent cell monolayers grown in 24-well tissue culture plates were incubated at 33°C, with 3,000 cpm of ^{35}S-labelled EV70 per well, for the length of time indicated on the x-axis. The percent of input virus bound to cells was then determined by scintillation counting. Results of duplicate determinations for each time point are represented.

- ● Binding to HeLa cell monolayers
- □ Binding to LLC-MK_2 cell monolayers

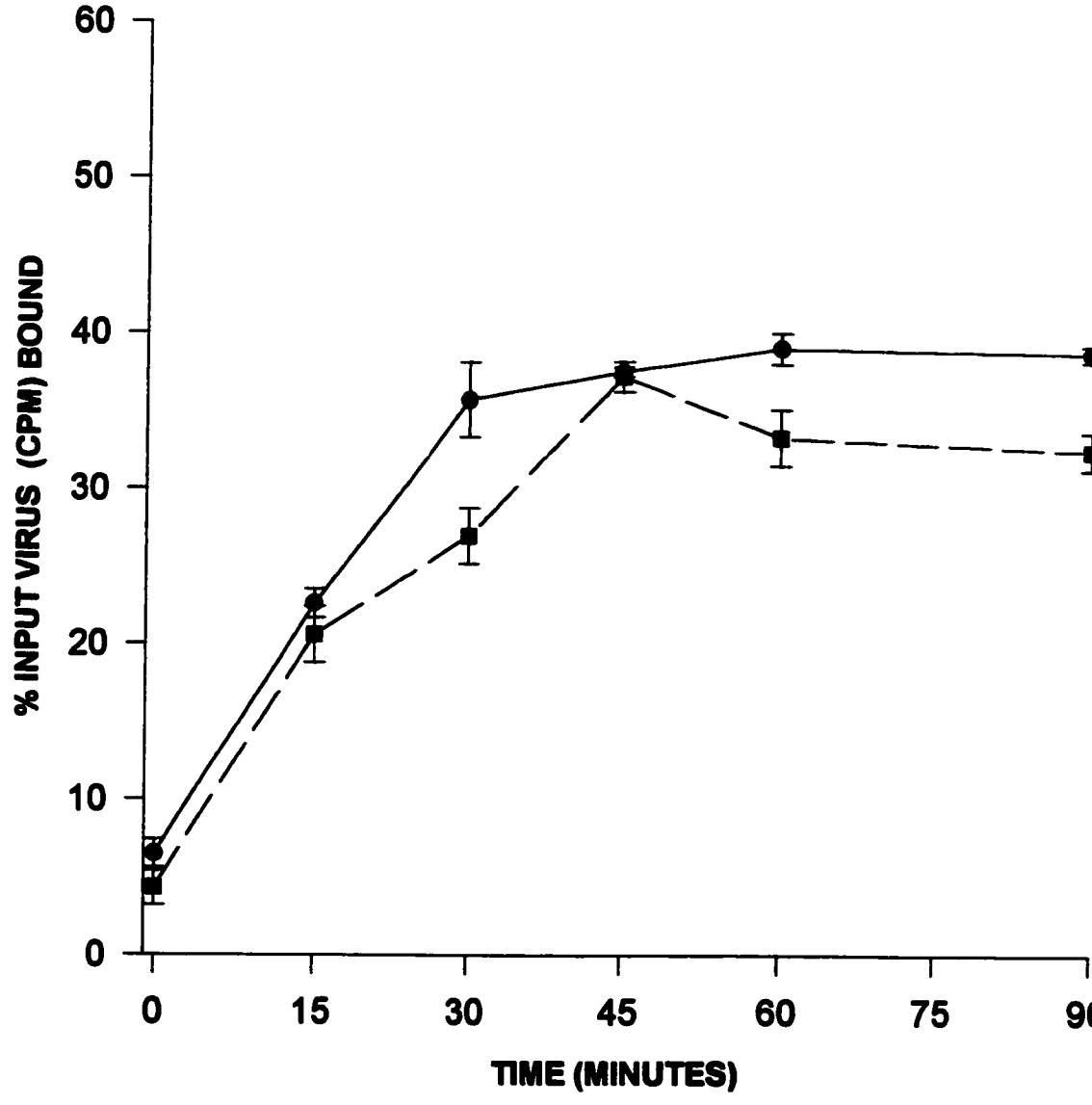
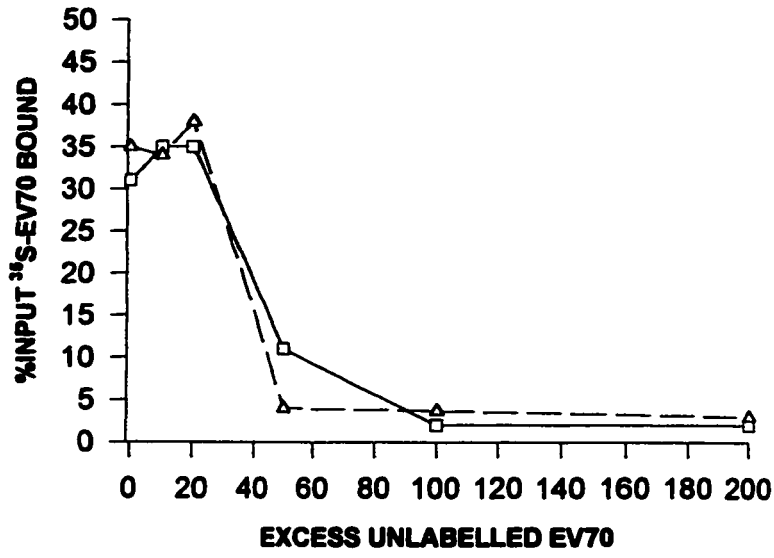
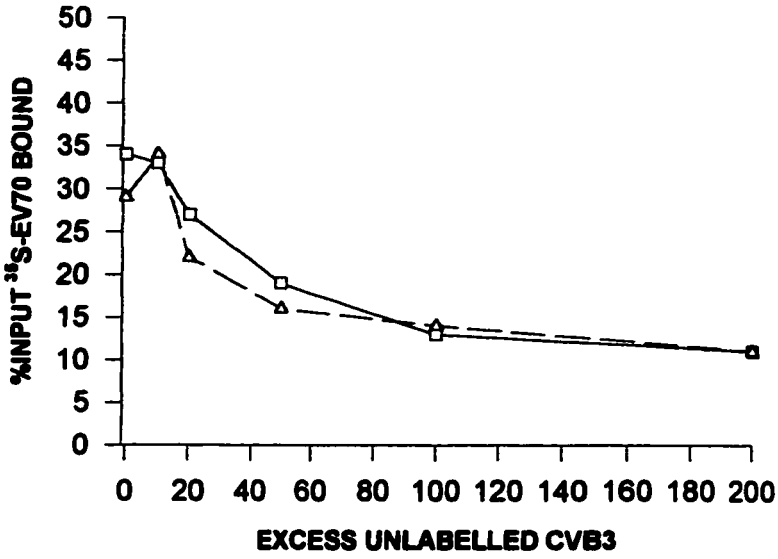

60
50
40
30
20
10
0
% INPUT VIRUS (CPM) BOUND
0
15
30
45
60
75
90
TIME (MINUTES)

Figure 2. Specificity of the EV70 receptor on LLC-MK_2 cells. Confluent LLC-MK_2 cell monolayers grown in 24-well tissue culture plates were incubated with a fixed amount of ^{35}S-labelled EV70 (2 X 10 ^{6} PFU per well; approximately 3,000 cpm) in the presence of increasing amounts of unlabelled competitor virus, at the ratios indicated. After a 1 h incubation at 33°C, the percent of input ^{35}S-EV70 bound to cells was determined by scintillation counting. Results of duplicate determinations at each fold-excess of unlabelled competitor virus are represented.

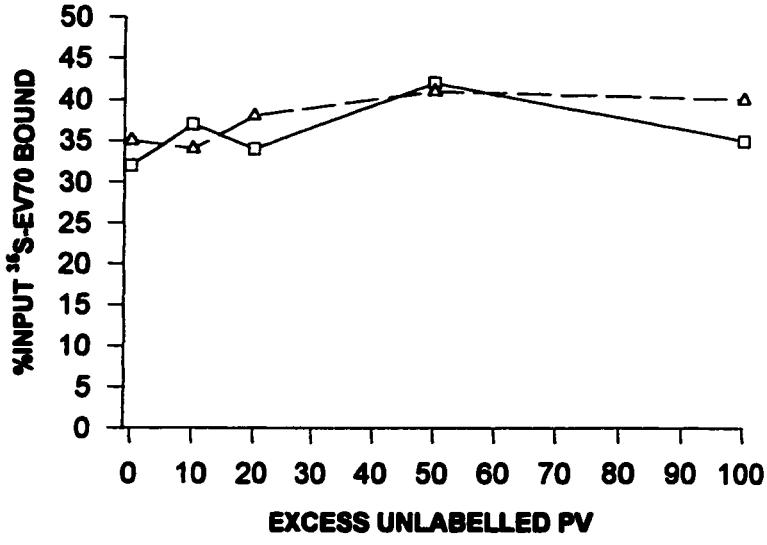
A) Unlabelled EV70 competitor
B) Unlabelled CVB3 competitor
C) Unlabelled PV competitor


%INPUT 35S-EV70 BOUND
50
45
40
35
30
25
20
15
10
5
0
0 20 40 60 80 100 120 140 160 180 200
EXCESS UNLABELLED EV70

A


%INPUT 35S-EV70 BOUND
50
45
40
35
30
25
20
15
10
5
0
0 20 40 60 80 100 120 140 160 180 200
EXCESS UNLABELLED CVB3

B


%INPUT 35S-EV70 BOUND
50
45
40
35
30
25
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
EXCESS UNLABELLED PV

C

input, while a 100-fold excess of CVB3 (approximately 200 PFU per cell) reduced binding to 14% of input. In contrast, PV had no effect on the ability of EV70 to bind to LLC-MK_2 cells (Figure 2C). Even in the presence of a 100-fold excess of PV (approximately 250 PFU per cell), binding of ^{35}S-EV70 remained at a level comparable to that seen in the absence of competitor (38% and 33% of input, respectively).

Competition experiments using homologous virus (Figure 2A) suggested that the LLC-MK_2 receptor for EV70 becomes saturated when incubated with a multiplicity of approximately 100 PFU or more per cell. In all subsequent assays described in this thesis, the amount of EV70 added to monolayers never exceeded a multiplicity of 30 PFU per cell, ensuring that conditions for binding were always below saturation. The proportion of ^{35}S-EV70 binding to LLC-MK_2 monolayers varied between experiments and between labelled virus preparations. Over the course of this project, the range of ^{35}S-EV70 binding to cells was 20 to 40% of input virus.

These experiments demonstrated that binding of EV70 to its cellular receptor was time-dependent and saturable. While the receptor for EV70 appeared to be distinct from that for PV, the relationship between the receptors for EV70 and CVB3 was not clear. Results suggested that either EV70 and CVB3: (1) compete for the same receptor; (2) share a component of a receptor complex; or that (3) CVB3 binds to the cell near enough to the receptor for EV70 to interfere with EV70 access to the cell surface.

II. Identification of a EV70-binding protein from HeLa cells

Initial attempts to identify receptor protein(s) involved in EV70 binding were made using a method based on the affinity of the virus for molecules present in the membranes of susceptible cells. As a first step, virus binding to membrane preparations of LLC-MK_2 cells was examined. In a dot-blot assay (Kilpatrick and Lipton, 1991), whole cells and membrane preparations were adsorbed to a nitrocellulose membrane, which was then incubated with ^{35}S-EV70. After washing, and drying, the nitrocellulose membrane was exposed to X-ray film. Membrane preparations and whole cells retained virus-binding activity (data not shown). A virus overlay protein binding assay (VOPBA) which has been used to characterize the binding proteins for a number of picornaviruses (Kilpatrick and Lipton, 1991; Mischak *et al.*, 1988a; Jin *et al.*, 1994), was then used in attempts to identify specific cell membrane proteins involved in EV70 binding. Cell membrane proteins were separated by electrophoresis, transferred to nitrocellulose membranes, and the membranes were incubated with ^{35}S -EV70. After thorough washing, membranes were dried and exposed to X-ray film. This method failed to detect any specific virus binding proteins in LLC-MK_2 cells (data not shown). Numerous attempts were made to overcome problems of high background and non-specific binding encountered with VOPBAs, but these proved unsuccessful, and this technique was abandoned in favour of an immunological approach.

Many groups have successfully used anti-receptor antibodies for the identification of picornavirus receptors (Campbell and Cords, 1983; Crowell *et al.*, 1986; Greve *et al.*, 1989; Hsu *et al.*, 1988; Minor *et al.*, 1984; Nobis *et al.*, 1985; Tomassini *et al.*, 1989a,b). This method relies on the production of antibodies directed against cell surface molecules that are

involved in virus binding. It was hoped that the immunization of mice with either whole cells or with membranes prepared from cells susceptible to EV70 infection would lead to the isolation of a mAb directed against the cellular receptor for EV70, and that this antibody could subsequently be used to identify the EV70 receptor. Since the majority of anti-receptor antibodies specific for picornaviruses have been generated against HeLa cell epitopes, HeLa cells, rather than LLC-MK_2 cells were chosen for these studies.

Earlier work had demonstrated that HeLa cells are permissive for EV70 (Mirkovic *et al.* 1973, Yoshii *et al.* 1977), but no information was available concerning EV70 interaction with these cells. In time course experiments, ^{35}S-EV70 binding to HeLa cells was similar to that seen with LLC-MK_2 cells (Figure 1), with virus binding reaching a maximum (39 % of input virus) after 45 min of incubation. As with LLC-MK_2 cells, the proportion of ^{35}S-EV70 binding to HeLa cell monolayers varied between experiments and between labelled virus preparations. Over the course of this project, the range of ^{35}S-EV70 binding to HeLa cells was between 20 and 40%.

A. Identification of a mAb that blocks EV70 infection of HeLa cells

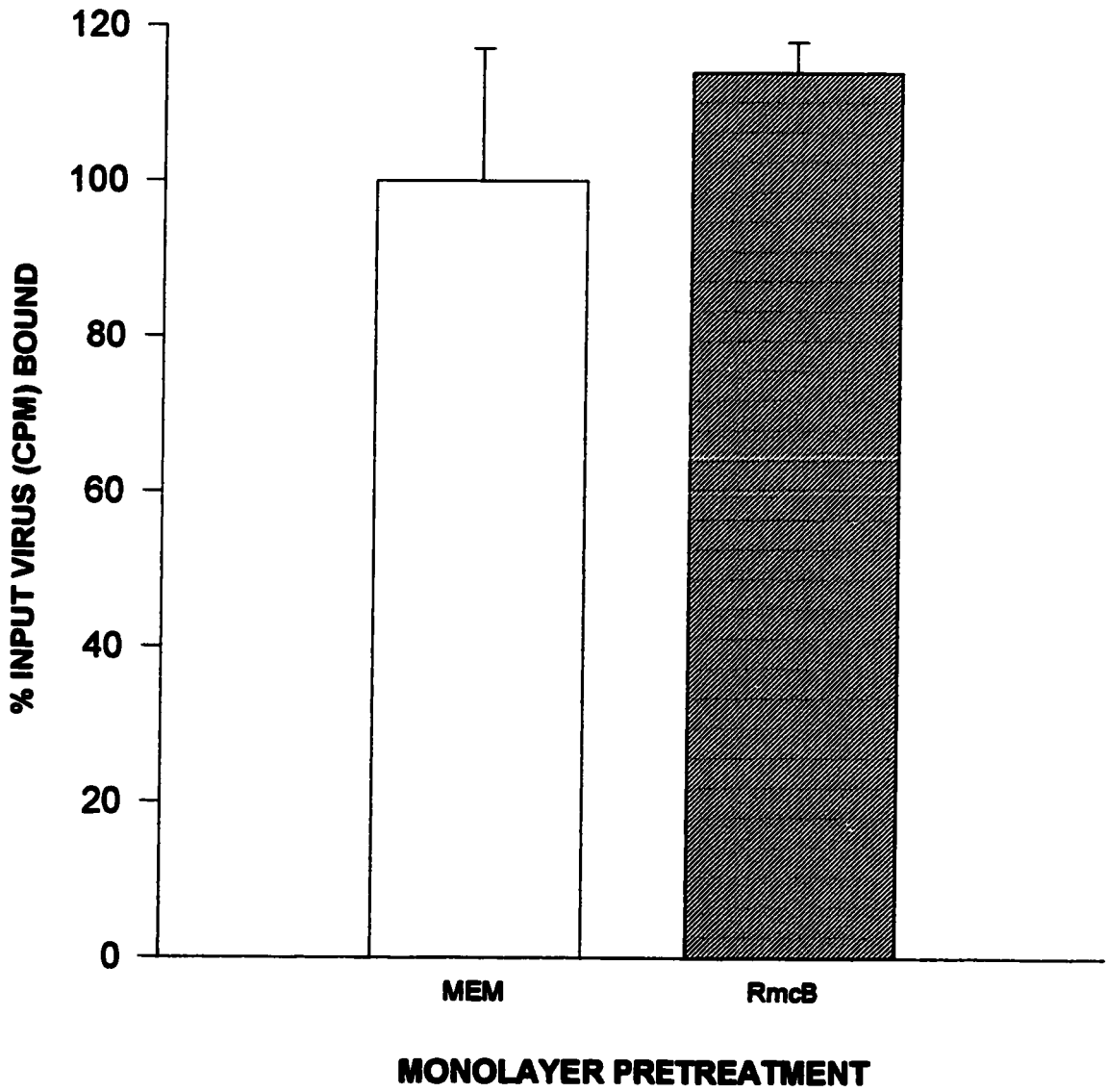
(i) **CVB3 receptor-specific mAb RmcB:** The earlier observation that CVB3 partially inhibited EV70 binding to LLC-MK_2 cells (Figure 2B) suggested that these viruses may share a receptor or a component of a receptor complex, or that CVB3 binding occurs near to the EV70 binding site, and thus sterically hinders EV70 access to its receptor. In order to investigate a possible relationship between the HeLa cell receptors for EV70 and CVB3, a mAb directed against the putative HeLa cell receptor for CVB3 was obtained. Antibody RmcB was previously shown to protect HeLa cells from infection by all six CVB

serotypes (Hsu *et al.*, 1988). To test EV70 receptor blocking activity of RmcB, monolayers of HeLa cells were treated with RmcB diluted 1:10 prior to infection with EV70 at a MOI of 0.1. Although this dilution of RmcB was protective against CVB3 infection of HeLa cells (R. Crowell, personal communication), it did not protect cells against infection by EV70. Signs of EV70-induced CPE became apparent by 24 h post-infection, and monolayers were completely destroyed within 48 h. To determine if RmcB could interfere with EV70 binding, monolayers of HeLa cells were treated with RmcB, and virus binding assays were performed. As shown in Figure 3, RmcB pretreatment had no effect on ^{35}S-EV70 binding to HeLa cells. These observations suggested that either EV70 and CVB3 utilize distinct receptors, or that RmcB binds to a shared receptor molecule, but at a site which does not interfere with EV70 attachment.

(ii) **Production of anti-EV70 receptor mAb EVR1:** At this stage, it was decided that attempts should be made to generate an antibody specific for the EV70 receptor. An anti-receptor antibody would be expected to bind to the surface of HeLa cells and selectively inhibit their infection by EV70. To produce this antibody, BALB/c mice were immunized with either whole HeLa cells, or with HeLa cell membrane preparations. Hybridomas were generated by fusion of spleen cells with SP2/0 cells, and in a cell protection assay, hybridoma culture supernatants were tested for their ability to protect HeLa cell monolayers against EV70 infection. Infected monolayers were monitored by light microscopy at regular intervals, and hybridoma culture supernatants were assessed for their ability to delay or prevent the appearance of EV70-induced CPE. During initial rounds of screening, visual evaluation of

Figure 3. Effect of mAb RmcB on EV70 binding to HeLa cells. Confluent HeLa cell monolayers grown in 24-well tissue culture plates were treated with mAb RmcB (1:10) for 1 h at 37°C. After washing, monolayers were incubated with 4,000 cpm of ^{35}S-labelled EV70 for 1 h at 33°C, and the amount of virus bound to cells was determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to the MEM control, ± standard deviation for three samples.

MEM: culture medium pretreatment
RmcB: RmcB (1:10) in culture medium


120
100
80
60
40
20
0
% INPUT VIRUS (CPM) BOUND
MEM
RmcB
MONOLAYER PRETREATMENT

protection was corroborated using a colorimetric assay based on the ability of viable cells to metabolize the tetrazolium salt MTT (Hansen *et al.*, 1989).

Under the conditions of the cell protection assay, infection with EV70 at a MOI of 1 resulted in complete destruction of HeLa monolayers in control cultures (receiving no antibody pretreatment) within 48 h of infection. More than 900 hybridomas were tested, and scored based on both the degree of protection provided to monolayers, and the duration of this protection. Seventeen hybridomas that either completely protected monolayers for 72 h post-infection, or that delayed the appearance of significant CPE during the screening period, were identified. From these 17 hybridomas, a total of 408 clones were isolated and screened. Nine clones (from one hybridoma, derived from mice immunized with HeLa cell membranes) provided complete protection to monolayers, preventing the appearance of CPE for the duration of the screening period (72 h). All nine clones secreted immunoglobulin of the IgG_1 isotype, and four of these clones were used for the production of ascites fluids. Monoclonal antibody, in ascites fluid or in the culture supernatant of cloned hybridoma cells, was designated EVR1. Cell protection assays also confirmed that EVR1 protection of HeLa cell monolayers was concentration-dependent (Table 9, p. 102).

To rule out the possibility that EVR1 protects HeLa cells by non-specific masking of the cell surface, the ability of the antibody to protect HeLa and LLC-MK_2 monolayers against infection by PV (strain Sabin 1) and by CVB3 was tested (Table 6). Undiluted EVR1 hybridoma supernatant completely protected HeLa cell monolayers against EV70 infection, with monolayers remaining free of CPE for the duration of the monitoring period (96 h). EVR1 did not protect either HeLa or LLC-MK_2 cells against PV or CVB3. In all cases,

Table 6. Specificity of EVR1 protection

	Cell Protection[a,b]	
Virus[c]	HeLa	LLC-MK_2
Enterovirus 70	+	-
Poliovirus Sabin 1	-	-
Coxsackievirus B3	-	-

[a] + no observable cytopathic effects for a minimum of 96 h post-infection;
- cytopathic effects observed within 45 h post-infection.
[b] EVR1 used at 17.5 μg/mL.
[c] EV70, PV, CVB3, MOI 1.0

monolayers infected with PV or CVB3 were completely destroyed within 18 h of infection. EVR1 also failed to protect LLC-MK_2 monolayers against EV70, as evidenced by the appearance of extensive CPE within 45 h of infection.

To show that EVR1 interfered with attachment of EV70, virus binding inhibition assays were performed, in which HeLa cell monolayers were incubated with various dilutions of hybridoma culture fluid prior to incubation with ^{35}S-EV70. Undiluted EVR1 reduced binding of EV70 by 84% when compared to control monolayers preincubated with either growth medium (MEM) or with a mAb directed against MHC I, a ubiquitous cell surface protein of nucleated cells (Figure 4). EVR1 inhibition of EV70 binding was concentration dependent (Table 10, p. 104).

Finally, the specificity of EVR1 for HeLa cells was confirmed by indirect immunofluorescence. Dispersal of HeLa cells by trypsin treatment prior to incubation with antibody did not affect EVR1 binding (Figure 5A), whereas LLC-MK_2 cells dispersed either manually (Figure 5B) or by trypsin treatment (not shown), did not bind antibody. Therefore, EVR1 recognized a trypsin-resistant epitope expressed on the surface of human (HeLa) cells, but not on the surface of monkey (LLC-MK_2) cells.

B. Identification of a HeLa cell protein recognized by EVR1

The results of cell protection, virus binding inhibition and indirect immunofluorescence studies provided compelling evidence that EVR1 was directed against a HeLa cell receptor for EV70. EVR1 was therefore used to begin characterization of the putative EV70 receptor.

(i) **Western blot analysis:** When HeLa and LLC-MK_2 cell membrane proteins separated by SDS-PAGE under denaturing conditions were analyzed by Western blot, no

Figure 4. Inhibition of EV70 binding to HeLa cells by EVR1. Confluent HeLa cell monolayers grown in 24-well tissue culture plates were treated with culture medium, antibody directed against an irrelevant antigen (MHC I-specific IgG_1) , or undiluted EVR1 hybridoma culture supernatant, for 1 h at 37°C. After washing, monolayers were incubated with 4,000 cpm of ^{35}S-labelled EV70 for 1 h at 33 °C, and the amount of virus bound to cells was determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to the MEM control, ± standard deviation for four samples.

MEM: culture medium pretreatment
anti-MHC 1: culture medium containing MHC I-specific IgG_1 (10 μg/mL)
EVR1: EVR1 hybridoma culture supernatant (17.5 μg/mL)

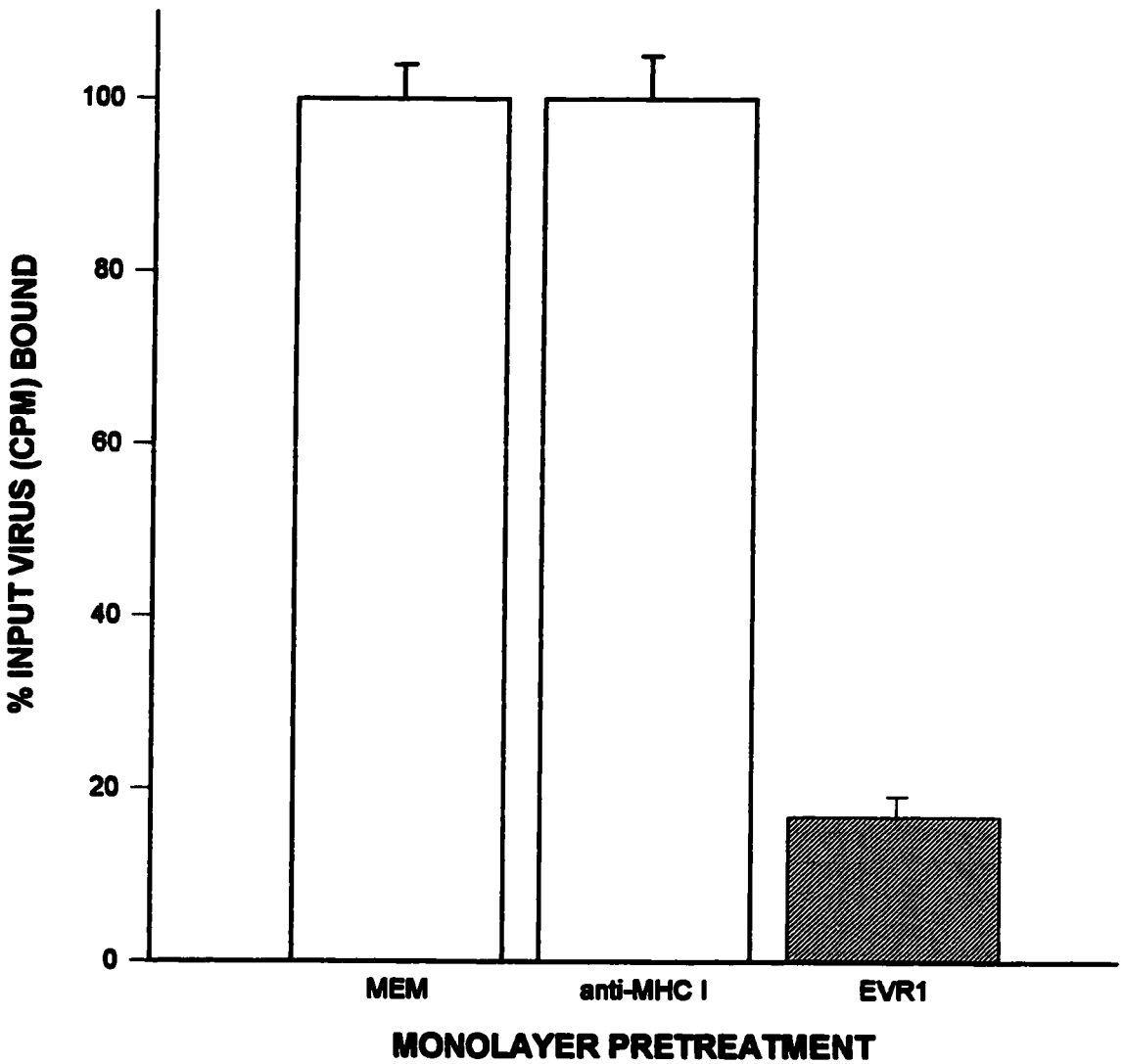

% INPUT VIRUS (CPM) BOUND
100
80
60
40
20
0
MEM
anti-MHC I
EVR1
MONOLAYER PRETREATMENT

Figure 5. HeLa cell specificity of EVR1 binding. Trypsin-dispersed HeLa and manually dispersed LLC-MK_2 cells were processed for indirect immunofluorescence analysis. After incubation with EVR1 ascites fluid (1:100) for 30 min at room temperature, cells were washed, and EVR1 binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:10). Cells were photographed through an FITC filter.

Panels:
A) HeLa cells
B) LLC-MK_2 cells

A

immune-reactive proteins were detected by EVR1 (data not shown). However, in Western blots of cell lysate proteins separated by SDS-PAGE under non-reducing conditions in the presence of reduced SDS (0.01%), EVR1 reacted specifically with a HeLa cell protein of approximately 75 kDa (Figure 6, lane 1). Again, no immune-reactive proteins were detected in LLC-MK_2 cell lysates (Figure 6, lane 2). These observations indicated that the HeLa cell epitope recognized by EVR1 was heat labile and sensitive to the reducing agent (2-mercaptoethanol) present in electrophoresis sample buffer. Furthermore, these results supported the earlier observation that EVR1 was specific for an epitope expressed on human (HeLa) cells, even though monkey (LLC-MK_2) cells also express a receptor for EV70.

(ii) **Immunoprecipitation of a HeLa cell glycoprotein by EVR1:** To further characterize the protein recognized by EVR1, and to confirm the results obtained by Western blot analysis, lysates of [^{3}H]-glucosamine-labelled HeLa and LLC-MK_2 cells were incubated with antibody, and immunoprecipitated proteins were analyzed by SDS-PAGE (Figure 7). EVR1 reacted specifically with a HeLa cell protein of approximately 70-75 kDa that appeared to migrate as a doublet (lane 6). Successful labelling after a 4 h (data not shown) or a 16 h incubation with ^{3}H-glucosamine indicated that this protein was glycosylated. No protein was immunoprecipitated from lysates of LLC-MK_2 cells incubated with EVR1 (lane 3), nor from HeLa or LLC-MK_2 lysates incubated with mouse immunoglobulin directed against an irrelevant antigen (LCMV-specific IgG_1), or with protein G beads alone (lanes 1, 2, 4, 5).

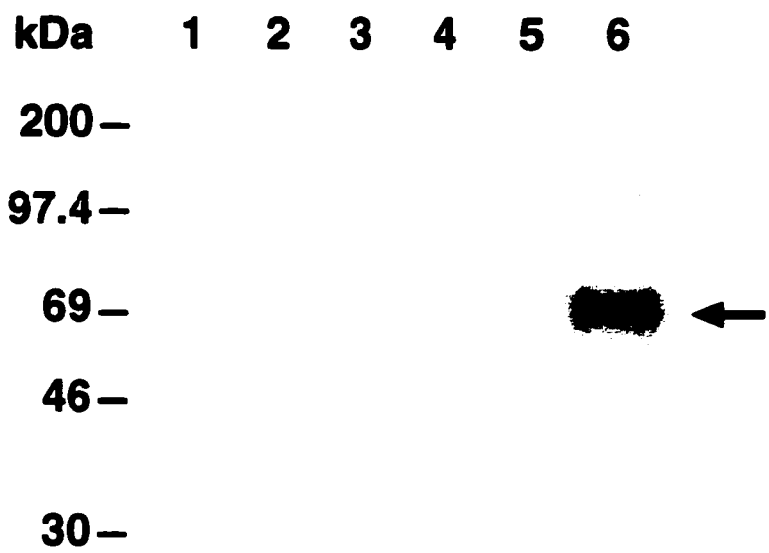
Figure 6. Western blot analysis of cell lysate proteins with EVR1. Proteins from cell lysates (25μg per lane) were separated under non-denaturing conditions on 8% polyacrylamide gels containing 0.01% SDS. Proteins were transferred to Immobilon-P, and after blocking, the membrane was incubated with undiluted EVR1 hybridoma culture supernatant for 1 h at 37°C. Primary antibody binding was detected with peroxidase conjugated goat anti-mouse IgG (H+L) (1:2000) and o-dianisidine solution, as described in Materials and Methods. Positions of molecular mass standards are indicated to the right of the figure.

Lanes:
1) HeLa cell lysate proteins
2) LLC-MK_2 cell lysate proteins


kDa
1
2
200
97.4
69
46
30
21.5

Figure 7. Immunoprecipitation of a HeLa cell glycoprotein by EVR1. HeLa and LLC-MK_2 cells were labelled with [^{3}H]-glucosamine for 16 h at 37°C, and lysed with RIPA buffer as outlined in Materials and Methods. Lysates were incubated with a 1:20 dilution of either EVR1 ascites fluid, antibody directed against an irrelevant antigen (LCMV-specific IgG_1), or culture medium. Following immunoprecipitation, samples were separated on 8% polyacrylamide gels containing 0.1% SDS, fluorography was performed, and the dried gel was exposed to X-ray film. Positions of molecular mass standards are indicated to the left of the figure.

Lanes:
1) LLC-MK_2 lysate; no antibody
2) LLC-MK_2 lysate; LCMV-specific IgG_1
3) LLC-MK_2 lysate; EVR1
4) HeLa lysate; no antibody
5) HeLa lysate; LCMV-specific IgG_1
6) HeLa lysate; EVR1


kDa
1
2
3
4
5
6
200
97.4
69
46
30

III. EVR1 recognizes decay-accelerating factor (DAF)

Anti-receptor antibodies may be used in several ways to identify virus receptor molecules and ultimately clone their coding sequences. One approach, similar to that used for the identification and cloning of the poliovirus receptor (Mendelsohn *et al.*, 1989), would involve the genetic transformation of receptor-negative cells with a cDNA expression library prepared from HeLa cells. Resulting transformants could then be screened for expression of the candidate receptor of EV70 using EVR1. Receptor-expressing transformants could subsequently be analyzed and the HeLa cell DNA fragment encoding the putative receptor isolated. An alternative approach, used in the identification of the receptor for the major group Rhinoviruses (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*,1989a) would involve the use of EVR1 to affinity purify receptor protein from HeLa cells. Isolated protein could subsequently be subjected to sequence analysis, and degenerate oligonucleotide probes designed in order to screen a HeLa cell cDNA library for receptor-specific sequences (Tomassini *et al.*, 1989a).

Before either approach could be initiated, reports appeared in the literature showing that the complement regulatory protein, decay-accelerating factor (DAF/CD55) served as a receptor for a number of Echoviruses. These viruses, like EV70, are human pathogens belonging to the family *Picornaviridae* (Bergelson *et al.*, 1994; Ward *et al.*, 1994). A comparison of the characteristics of DAF and of the candidate receptor for EV70 (molecular weight, glycosylation, trypsin resistance, sensitivity to reducing agents) suggested that these molecules were the same. The abundant expression of DAF on human erythrocytes (Nicholson-Weller *et al.*, 1982), and the observation that EV70 is a hemagglutinating

enterovirus (Kono *et al.*, 1978), further strengthened the possible link between DAF and the ligand of EVR1. DAF has been the subject of intense study, and as a result, a number of useful reagents were available. Several human DAF-specific mAbs (Table 4) had been generated, and the cDNA for DAF was cloned (Medof *et al.*, 1987a). In addition, some DAF-expressing cell lines had been established (Lublin and Coyne, 1991; White *et al.*, 1992).

DAF (Figure 8) belongs to a class of integral membrane proteins which attaches to cell membranes via a glycosylphosphatidyl inositol (GPI) anchor. Attachment of GPI-anchored proteins to the outer leaflet of cell membranes is through a glycophospholipid - containing phosphatidylinositol, which is covalently linked to the carboxy termini of the proteins (Davitz *et al.*, 1983; Medof *et al.*, 1986). Release of membrane proteins from the surface of cells by the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) is considered to be diagnostic for the presence of a GPI anchor (Low *et al.*, 1988). The sensitivity of the ligand of EVR1 to PI-PLC would therefore provide additional evidence that the protein recognized by EVR1 was DAF.

As shown in Figure 9, treatment of HeLa cells with PI-PLC prior to incubation with EVR1 greatly reduced antibody binding (panels A and B). Binding of the DAF-specific mAb 1H4 was similarly affected by PI-PLC pretreatment of cells (panels C and D), while binding of a mAb specific for human MHC I (an integral membrane glycoprotein not attached to membranes via a GPI anchor) was not affected by the enzyme (panels E and F).

Subsequently, the specificity of EVR1 for DAF was confirmed by indirect immunofluorescence and Western blot analyses. Binding of EVR1 to a CHO cell line constitutively expressing human DAF (CHO-DAF; Lublin and Coyne, 1991) and to the

Figure 8. Decay-accelerating factor (DAF). Schematic representation of the human DAF molecule. SCR, short consensus repeat; GPI, glycosyl-phosphatidylinositol; N-CHO, N-glycosylation site; O-CHO, O-glycosylation site. SCR domains are numbered sequentially from the amino-terminus of the protein. Adapted from Lublin and Atkinson, 1989. The arrow indicates the site of PI-PLC cleavage.

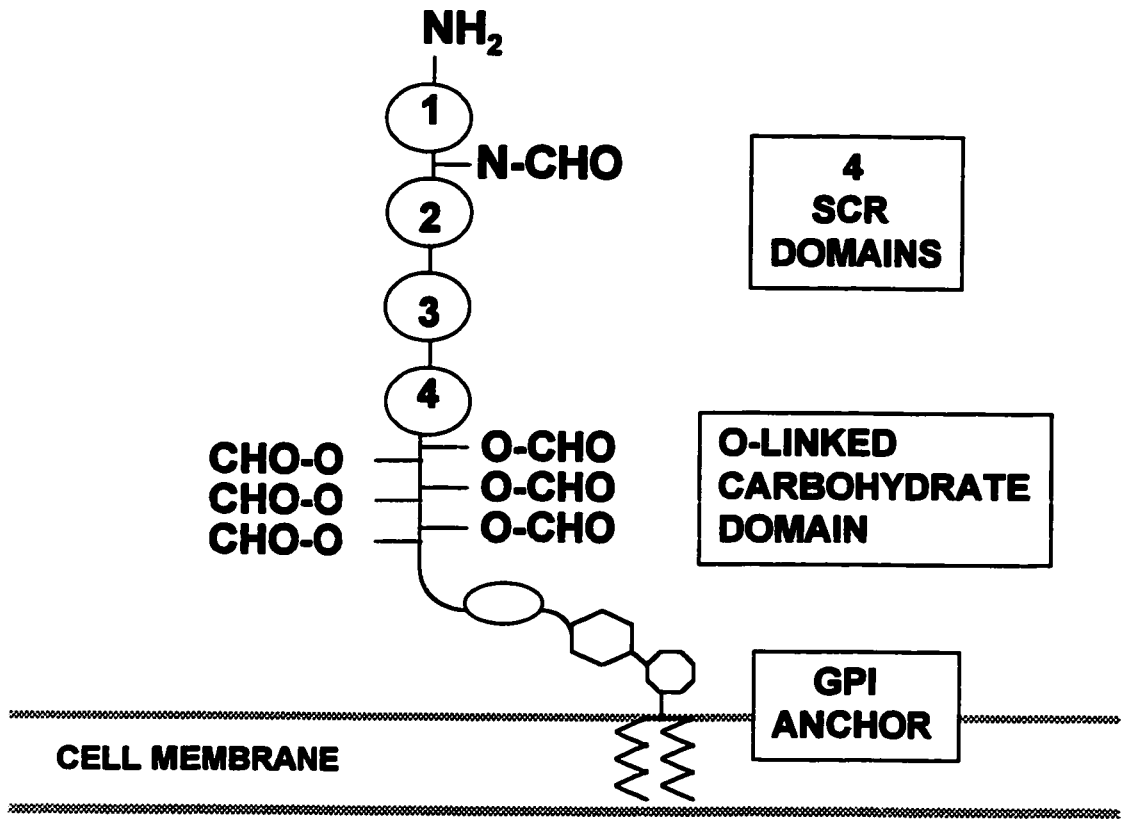
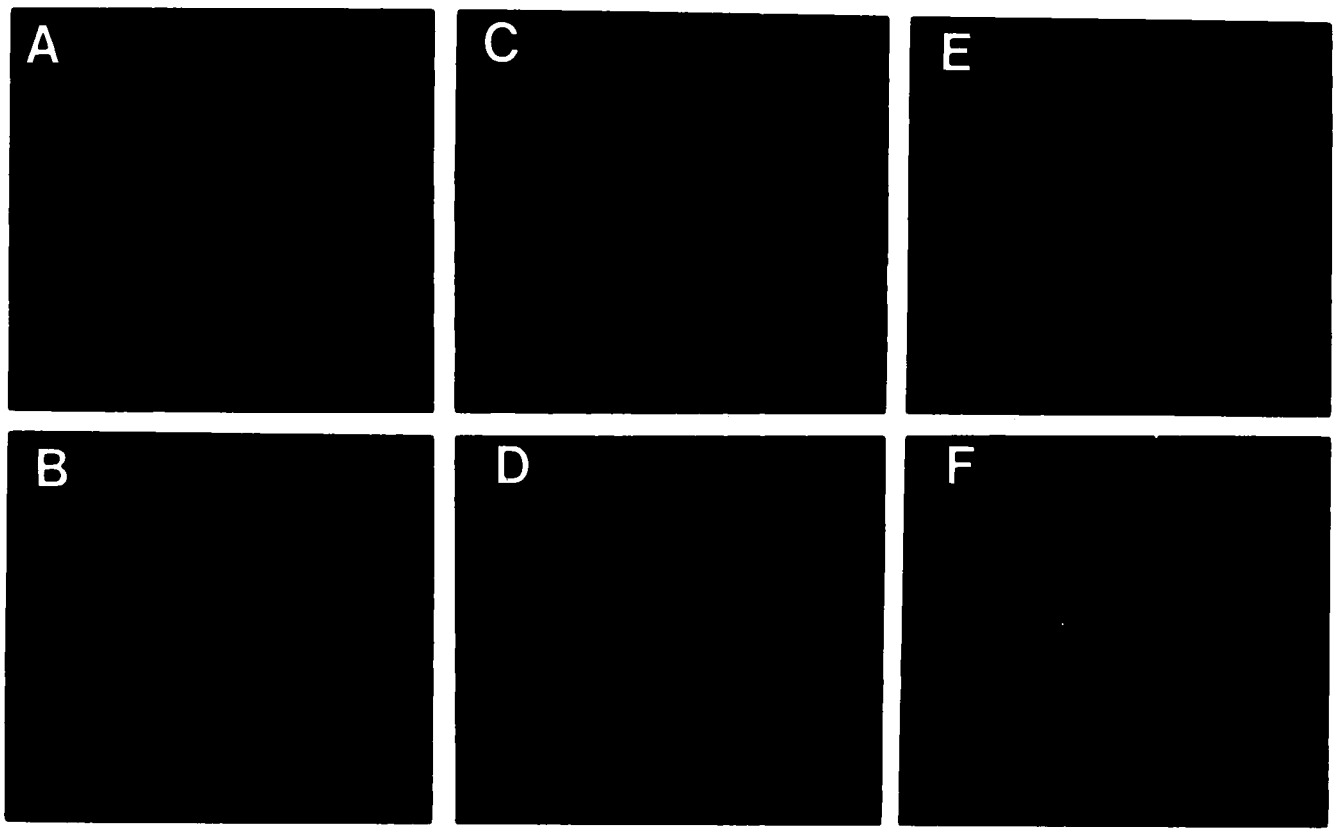

NH2
1
N-CHO
2
3
4
CHO-O
CHO-O
CHO-O
O-CHO
O-CHO
O-CHO
4
SCR
DOMAINS
O-LINKED
CARBOHYDRATE
DOMAIN
GPI
ANCHOR
CELL MEMBRANE

Figure 9. PI-PLC sensitivity of the protein recognized by EVR1. HeLa cell monolayers were dispersed by trypsin treatment, washed, and resuspended in PI-PLC buffer or in PI-PLC buffer containing 0.5 units of PI-PLC per 1 X 10^6 cells. After 1 h at 37°C, cells were washed and processed for indirect immunofluorescence analysis. Cells were incubated with either EVR1 hybridoma culture supernatant (undiluted), DAF-specific mAb 1H4 hybridoma culture supernatant (undiluted), or with MHC I-specific IgG_1 (1:10), for 30 min at room temperature. Primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:10), and cells were photographed through a FITC filter.

Panels:
A) HeLa cells; EVR1
B) PI-PLC treated HeLa cells; EVR1
C) HeLa cells; mAb 1H4
D) PI-PLC treated HeLa cells; mAb 1H4
E) HeLa cells; anti-MHC I IgG_1
F) PI-PLC treated HeLa cells; anti-MHC I IgG_1


A
C
E
B
D
F

parental CHO cell line were compared by indirect immunofluorescence (Figure 10). CHO-DAF cells (panel A), but not CHO cells (panel C) were able to bind EVR1. Identical results were obtained with mAb 1H4 (not shown). In Western blots of proteins from HeLa cell lysates, both EVR1 and mAb 1H4 reacted specifically with a protein of approximately 75 kDa (Figure 11, lanes 1 and 2).

IV. DAF is a HeLa cell receptor for EV70

Results obtained up to this point indicated that EVR1 was directed against DAF. It was necessary, however, to clearly demonstrate that DAF functioned as a receptor for EV70. To accomplish this, two complementary approaches were used: (1) antibody blockade using a DAF-specific mAb; (2) expression of DAF in a receptor-negative cell line.

A. A DAF-specific mAb protects HeLa cells against EV70 infection and inhibits virus binding

Further evidence that DAF acts as a receptor for EV70 was obtained in cell protection and virus binding inhibition assays using the DAF-specific mAb 1H4. As observed with EVR1, mAb 1H4 protected HeLa but not LLC-MK_2 cells against EV70 infection (Table 7). Figure 12 shows that mAb 1H4 was also able to interfere with ^{35}S-EV70 binding to HeLa cells. Pretreatment of monolayers with mAb 1H4 reduced virus binding by 70%, relative to control wells. Cell protection and virus binding inhibition by mAb 1H4 were concentration-dependent (Tables 9 and 10).

B. DAF expression confers EV70 binding activity to 3T3 cells

Ultimately, proof of a receptor function for DAF required the demonstration that expression of DAF was sufficient to convert a receptor-negative cell line into a cell line with

Figure 10. Specificity of EVR1 for DAF. Trypsin-dispersed CHO and CHO-DAF cells were processed for indirect immunofluorescence analysis. After incubation with EVR1 ascites fluid (1:200) for 30 min at room temperature, cells were washed, and EVR1 binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:10). Cells in panels A and C were photographed through a FITC filter.

Panels:
A) CHO-DAF cells
B) CHO cells, Nomarski optics
C) CHO cells, same field as in (B)

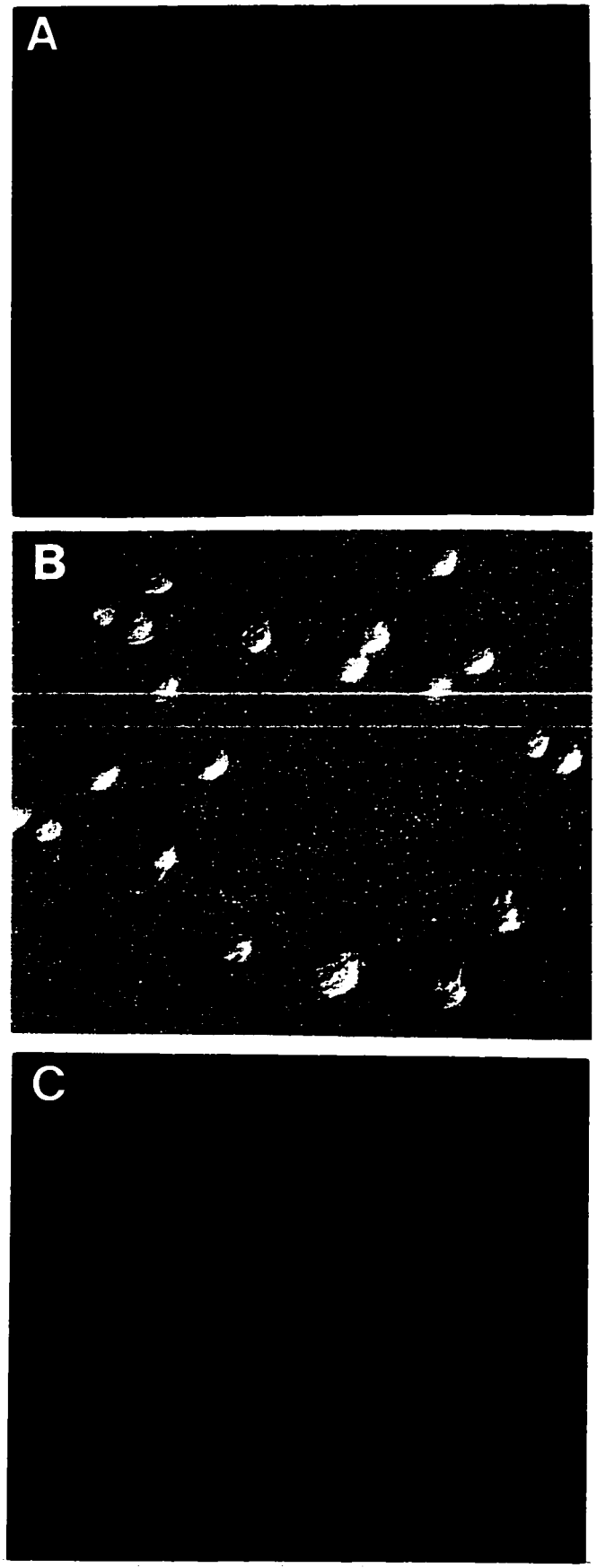

A
B
C

Figure 11. Western blot analysis of HeLa cell lysates with EVR1 and mAb 1H4. Proteins from HeLa cell lysates (25μg per lane) were separated under non-denaturing conditions on 8% polyacrylamide gels containing 0.01% SDS. Proteins were transferred to Immobilon-P, and after blocking, membrane strips were incubated with EVR1 ascites fluid (1:1000), DAF-specific mAb 1H4 hybridoma culture supernatant (1:3000), or antibody against an irrelevant antigen (LCMV-specific IgG_1, 1:300), for 1 h at 37°C. Primary antibody binding was detected with peroxidase conjugated goat anti-mouse IgG (H+L) (1:2000) and o-dianisidine solution, as described in Materials and Methods. Positions of molecular mass standards are indicated to the left of the figure.

Lanes:
1) EVR1
2) mAb 1H4
3) LCMV-specific IgG_1

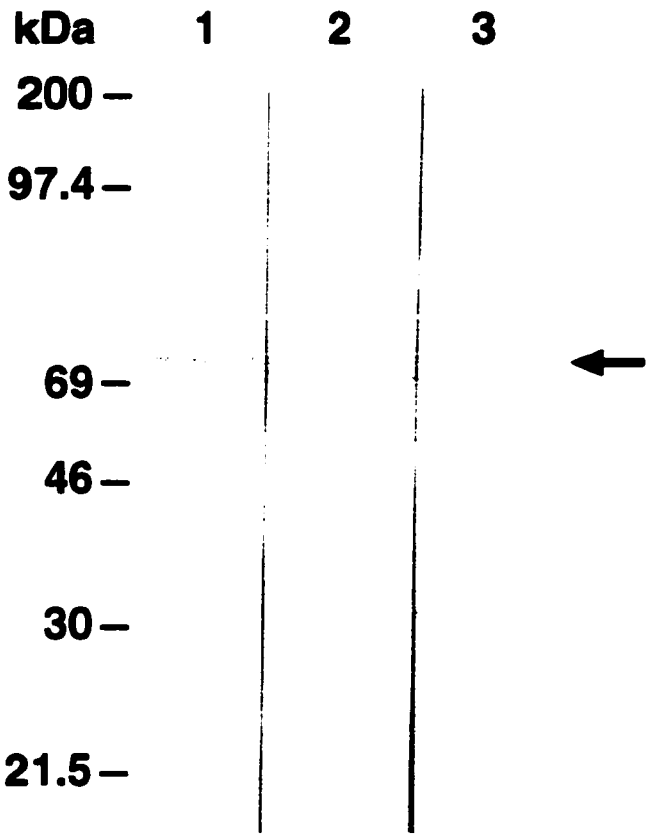

kDa
1
2
3
200
97.4
69
46
30
21.5

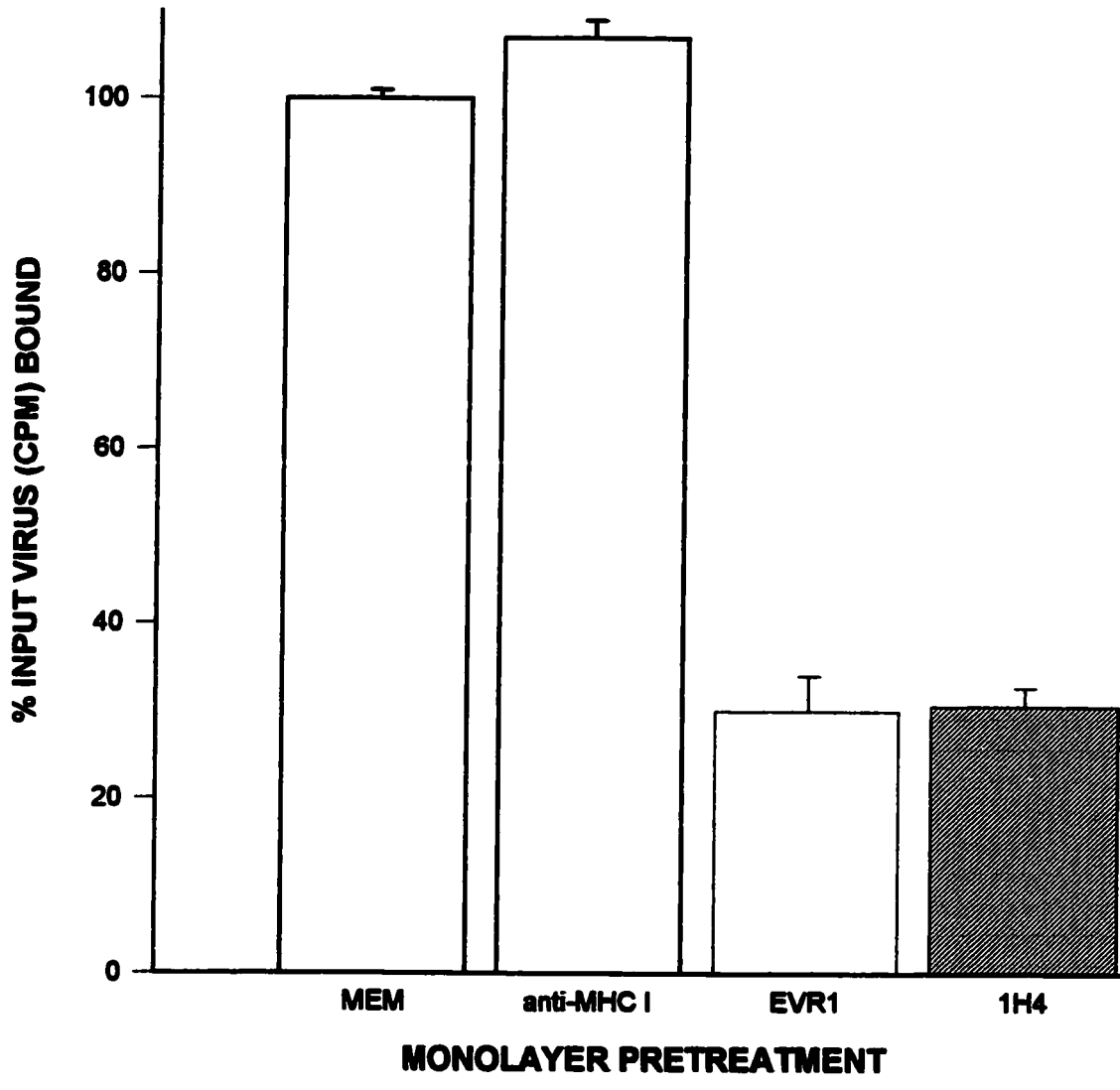
Table 7. Cell specificity of mAb 1H4 protection

	Cell protection[a]	
MAb[b]	HeLa	LLC-MK_2
1H4	+	-
EVR1	+	-

[a] EV70, MOI 1.0
+ no observable cytopathic effects for a minimum of 96 h post-infection;
- cytopathic effects observed within 45 h post-infection.
[b] MAb 1H4 used at 35μg/mL;
EVR1 used at 17.5 μg/mL.

Figure 12. Inhibition of EV70 binding to HeLa cells by mAb 1H4. Confluent HeLa cell monolayers grown in 24-well tissue culture plates were treated with 1H4 hybridoma culture supernatant (1:2), EVR1 hybridoma culture supernatant (1:2), culture medium, or antibody directed against an irrelevant antigen (anti-MHC I IgG_1, 10 μg/mL) for 1 h at 37°C. After washing, monolayers were incubated with 4,000 cpm of ^{35}S-labelled EV70 for 1 h at 33°C, and the amount of virus bound to cells was determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to the MEM control, ± standard deviation for four samples.

MEM: culture medium pretreatment
anti-MHC I: culture medium containing MHC I - specific IgG_1 (10 μg/mL)
EVR1: EVR1 hybridoma culture supernatant (9 μg/mL)
1H4: 1H4 hybridoma culture supernatant (9 μg/mL)


% INPUT VIRUS (CPM) BOUND
100
80
60
40
20
0
MEM
anti-MHC I
EVR1
1H4
MONOLAYER PRETREATMENT

a receptor-positive phenotype. CHO-DAF cells were used earlier to demonstrate the specificity of EVR1 for DAF (Figure 10). These cells could not be used to confirm that DAF acted as a receptor for EV70, since virus was able to bind to the parental CHO cell line. In virus binding assays, CHO cells bound 14 ±1% of input virus (cpm) (59±0.1% , relative to HeLa cells), while CHO-DAF cells bound 18±1% of input virus (cpm) (76±0.1% relative to HeLa cells). In view of these results, another cell line had to be found for use in DAF expression and receptor function studies.

Virus binding assays revealed that the murine fibroblast 3T3 cell line would be suitable for DAF receptor function experiments, since 3T3 cells consistently bound only 2 to 3% of input ^{35}S-EV70 (10 to 15 % relative to HeLa cell controls). A 3T3 cell line constitutively expressing human DAF (3T3-DAF; White *et al.*, 1992) was obtained and tested in virus binding assays. As shown in Figure 13, ^{35}S-EV70 binding to 3T3-DAF and to control 3T3-RDAF (3T3 cells with the DAF gene in the reverse orientation with respect to its promoter) cells was low, (28% and 14% of the amount of virus that bound to HeLa cells, or 4% and 2% of input virus, respectively). Although 3T3-DAF cells bound more virus than did 3T3-RDAF cells, the significance of this difference was difficult to interpret, since this amount of binding was at the lower limit of detection of the binding assay. When 3T3-DAF cells were examined by flow cytometry using either mAb 1H4 (Figure 14) or EVR1 (Appendix I) as primary antibody, a small shift in the fluorescence intensity profile was observed relative to the shift seen with HeLa cells, indicating that the level of DAF expression was lower on 3T3-DAF cells than on HeLa cells. Thus, the low level of EV70 binding initially detected with 3T3-DAF cells was interpreted to reflect the low level of DAF expression in these cells.

Figure 13. EV70 binding to 3T3-DAF and 3T3-RDAF cell lines. Confluent cell monolayers grown in 24-well tissue culture plates were incubated with 5,000 cpm of ^{35}S-labelled EV70 per well for 1 h at 33°C. The percent of input virus bound to cells was then determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to the MEM control, ± standard deviation for four samples.

HeLa: HeLa cell positive control monolayers
3T3-DAF: 3T3-DAF monolayers
3T3-RDAF: 3T3-RDAF negative control monolayers

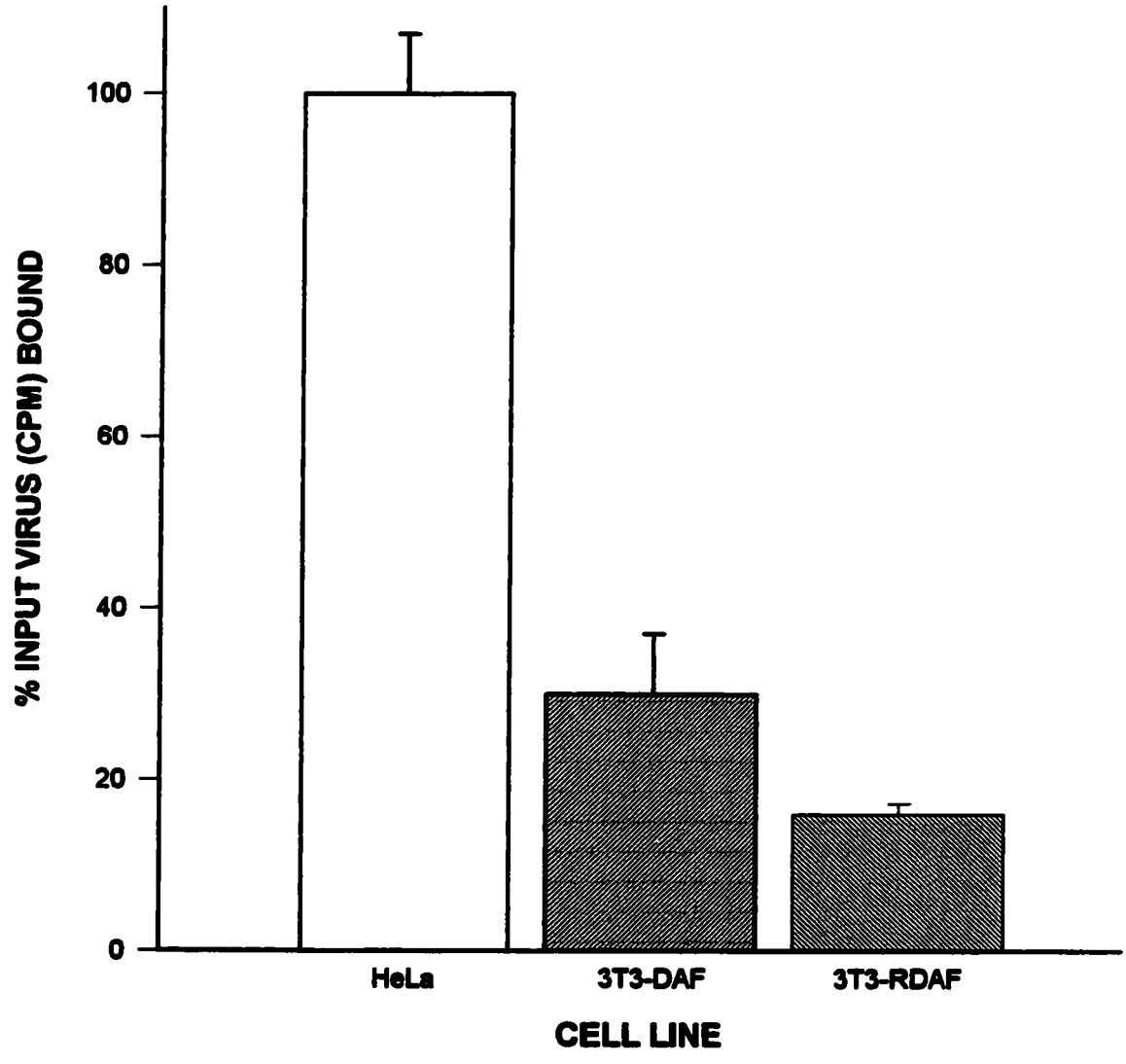
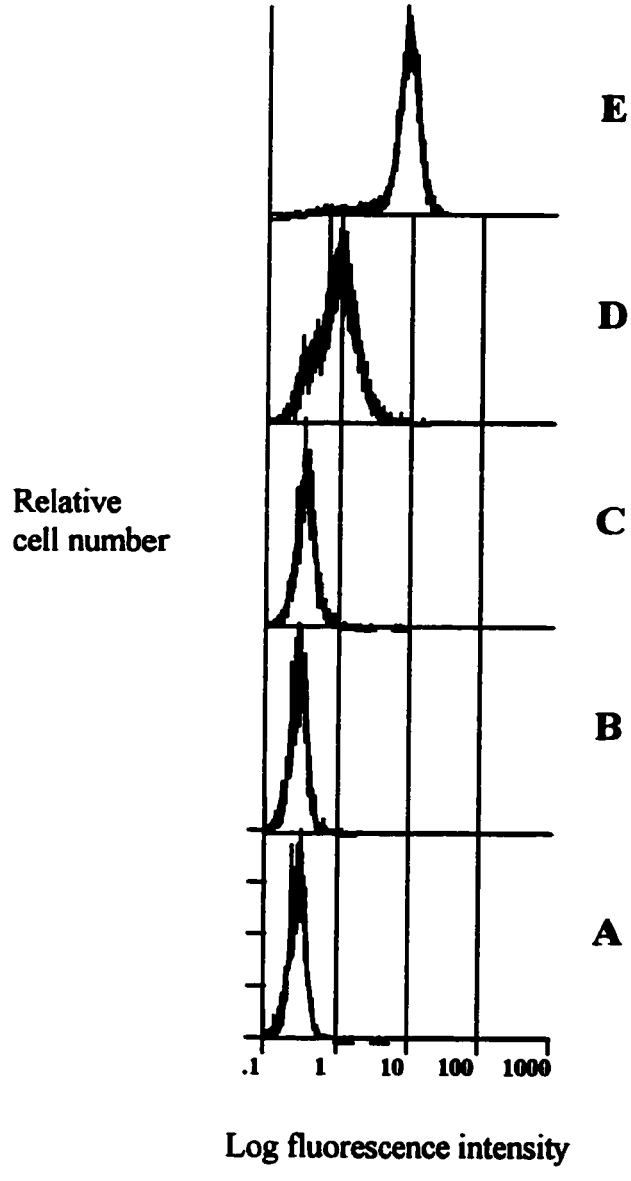

% INPUT VIRUS (CPM) BOUND
100
80
60
40
20
0
HeLa
3T3-DAF
3T3-RDAF
CELL LINE

Figure 14. Flow cytometric analysis of DAF expression in 3T3-DAF and 3T3-RDAF cell lines. Trypsin-dispersed cells were incubated with DAF-specific 1H4 hybridoma supernatant (1:5) and processed for flow cytometry. After 20 min at room temperature, cells were washed, and primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:20). Autofluorescence and secondary antibody controls for each cell line produced superimposable histograms. Panels A and B are therefore shown as representative negative control samples.

Panels:
A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) 3T3-RDAF cells; 1H4
D) 3T3-DAF cells; 1H4
E) HeLa cells; 1H4


E
D
C
B
A
Relative
cell number
.1
1
10
100
1000
Log fluorescence intensity

Since wild-type 3T3 cells did not bind appreciable amounts of ^{35}S-EV70, this cell line was used in studies to determine whether DAF expression could confer virus-binding activity to cells. For these experiments, a transient expression system was used. 3T3 cells were transfected with an expression vector containing human DAF cDNA (pcDNA3/DAF), and were subsequently infected with vaccinia virus vTF7-3, which provided T7 RNA polymerase to direct DAF expression. As shown in Figure 15, cells transfected with pcDNA3/DAF expressed DAF at the cell surface, and bound both EVR1 and mAb 1H4 (panels B and F). Cells transfected with pcDNA3 alone did not react with either antibody (panels C and G). Analysis of transfected cells by flow cytometry (Figure 16) revealed that this transient expression system generated mixed populations of cells displaying a range of DAF expression levels. For the experiments represented in Figures 15 and 17, approximately 10% of cells transfected with pcDNA3/DAF expressed DAF at higher levels than did HeLa cells, while approximately 40% of cells expressed low to moderate levels of DAF. The remaining cells were negative for DAF expression.

Monolayers of pcDNA3/DAF-transfected 3T3 cells were able to bind ^{35}S-labelled EV70 (Figure 17). Although the amount of binding varied between experiments, cells transfected with pcDNA3/DAF consistently bound 60 to 100 % of the virus that bound to HeLa cell controls, and 2 to 4 times more virus than did 3T3 cells transfected with vector alone.

These results clearly demonstrated that DAF mediated attachment of EV70 to 3T3 cells. It was now important to determine if DAF expression was sufficient to render 3T3 cells permissive for EV70. The transient expression system could not be used for this

Figure 15. Indirect immunofluorescence assay for DAF expression in transiently transfected 3T3 cells. 3T3 cell monolayers grown in 24-well tissue culture plates were transfected with vector pcDNA3 (3T3/pcDNA3), or with pcDNA3 containing the coding sequence of human DAF (3T3/DAF). Following transfection and infection with vaccinia virus vTF7-3, cells were processed for indirect immunofluorescence analysis. After incubation with DAF-specific 1H4 hybridoma supernatant (1:5) or EVR1 ascites fluid (1:100) for 20 min at room temperature, cells were washed and primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:20). Cells in panels A to C and E to G were photographed through a FITC filter.

Panels A to D: EVR1 used as primary antibody
A) HeLa cells
B) pcDNA3/DAF-transfected 3T3 cells (3T3/DAF)
C) pcDNA3-transfected 3T3 cells (3T3/pcDNA3)
D) same field as in (C), Nomarski optics

Panels E to H: MAb 1H4 used as primary antibody
E) HeLa cells
F) pcDNA3/DAF-transfected 3T3 cells (3T3/DAF)
G) pcDNA3-transfected 3T3 cells (3T3/pcDNA3)
H) same field as in (G), Nomarski optics

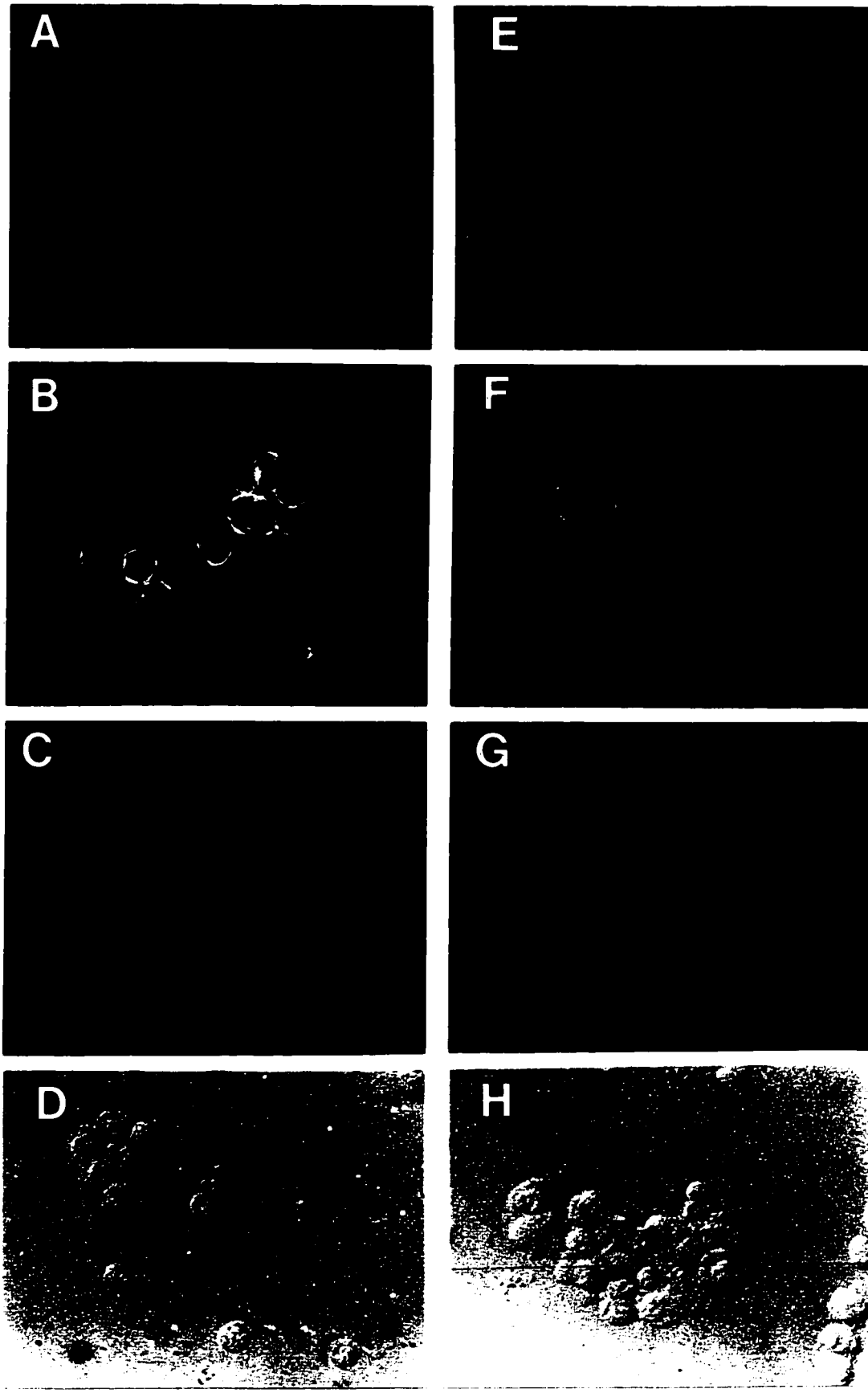

A
E
B
F
C
G
D
H

Figure 16. Flow cytometric analysis of DAF expression in transiently transfected 3T3 cells. 3T3 cell monolayers were transfected with vector pcDNA3 (3T3/pcDNA3), or with pcDNA3 containing the coding sequence of human DAF (3T3/DAF). Following transfection and infection with vaccinia virus vTF7-3, cells were processed for flow cytometry. After incubation with DAF-specific 1H4 hybridoma supernatant (1:5) or EVR1 ascites fluid (1:100) for 20 min at room temperature, cells were washed and primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:20). Cells transfected with pcDNA3 were used for autofluorescence and secondary antibody controls, and trypsin-dispersed HeLa cells were used as a positive control.

Column 1: mAb 1H4 used as primary antibody
Column 2: EVR1 used as primary antibody

Panels:
A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) pcDNA3-transfected cells
D) pcDNA3/DAF-transfected cells
E) HeLa cells

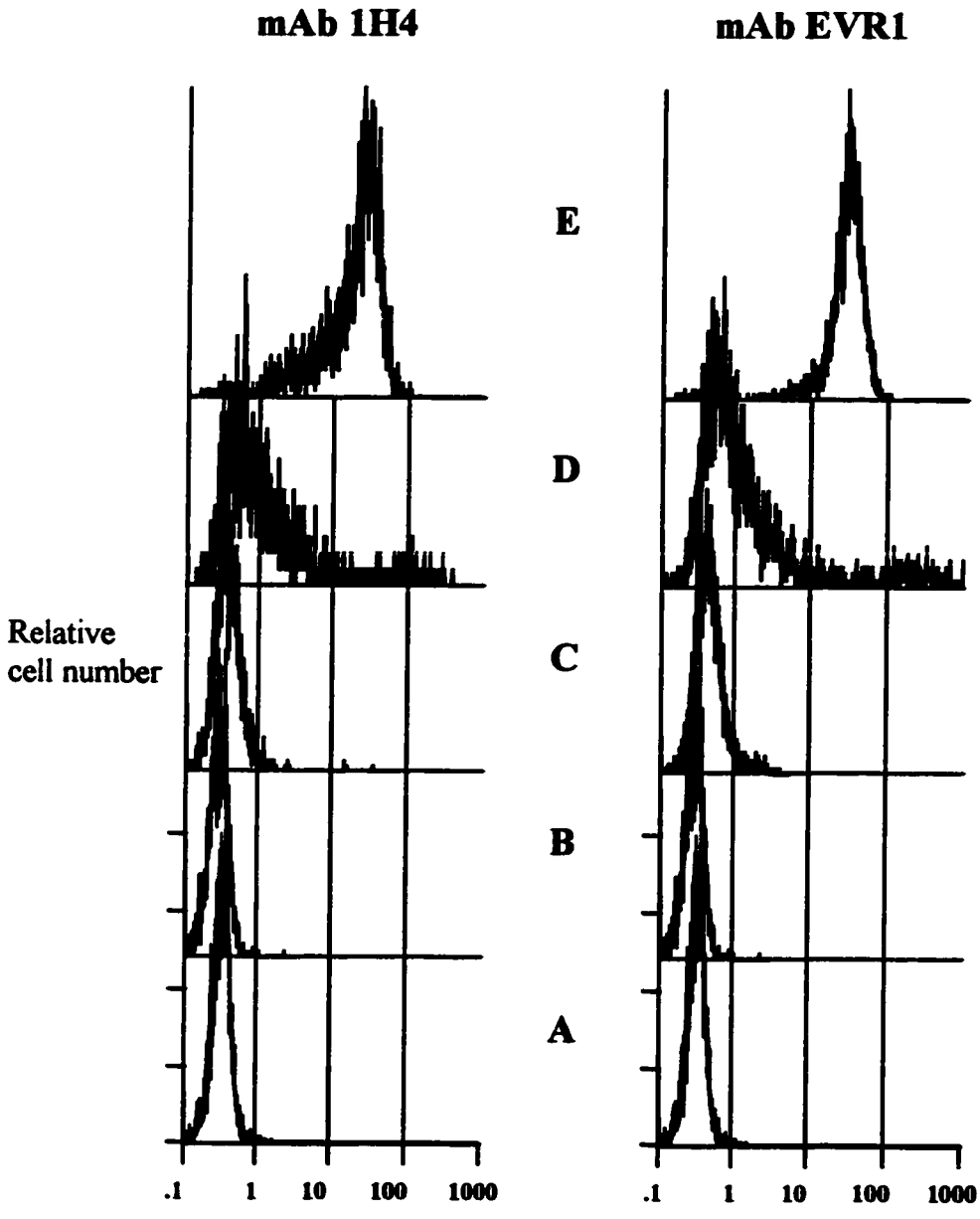
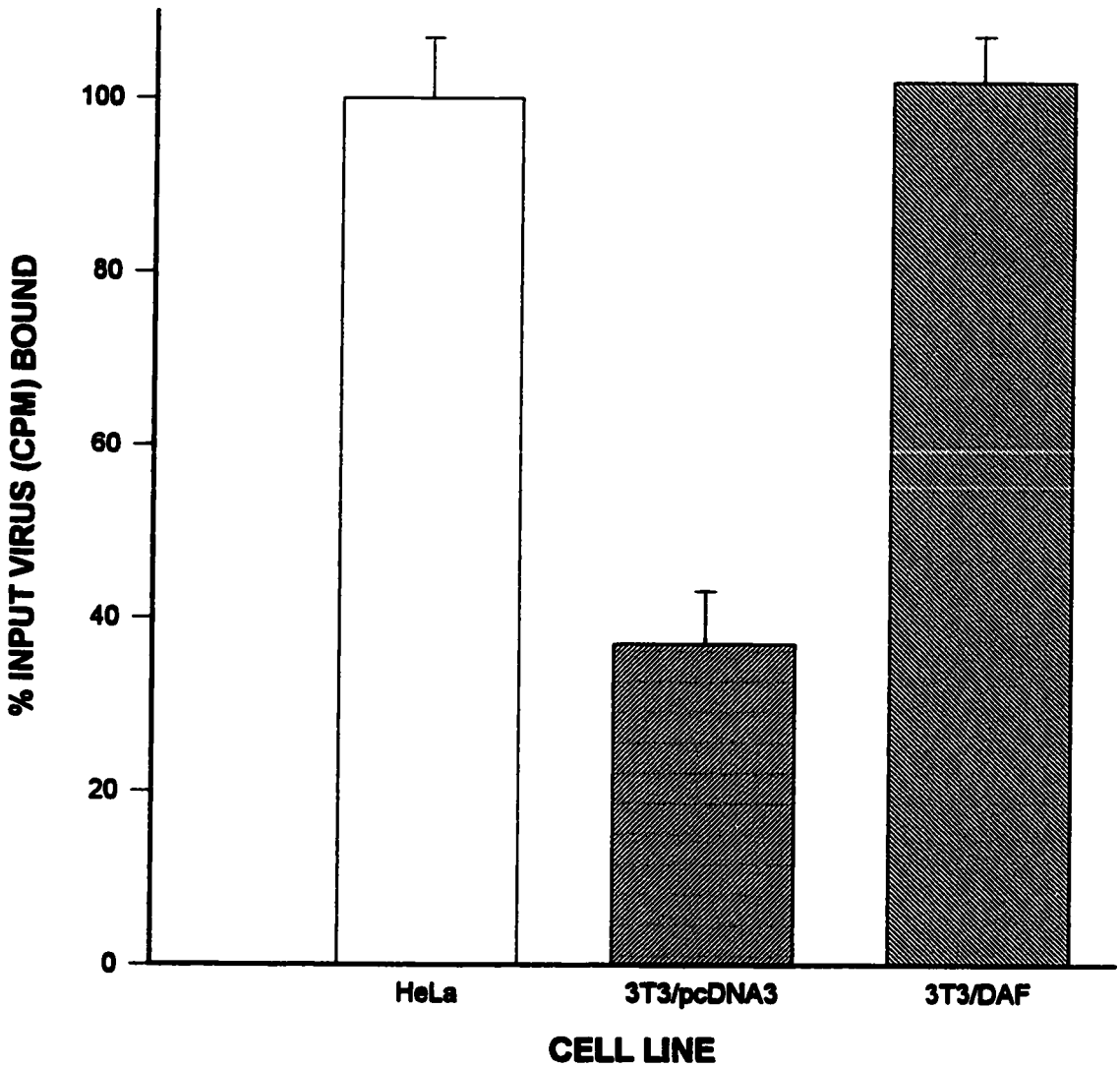

mAb 1H4
mAb EVR1
E
D
C
B
A
Relative cell number
.1
1
10
100
1000
.1
1
10
100
1000
Log fluorescence intensity

Figure 17. EV70 binding to 3T3 cells transiently expressing DAF. 3T3 cell monolayers grown in 24- well tissue culture plates were transfected with vector pcDNA3 (3T3/pcDNA3), or with pcDNA3 containing the coding sequence for human DAF (3T3/DAF). Following transfection and infection with vaccinia virus vTF7-3, monolayers were washed and incubated with 3,000 cpm of ^{35}S-labelled EV70 per well. After a 45 min incubation at 33°C, the amount of virus bound to cells was determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to HeLa cell control, ± standard deviation for three samples.

HeLa: HeLa cell control
3T3/pcDNA3: pcDNA3- transfected 3T3 cells
3T3/DAF: pcDNA3/DAF- transfected 3T3 cells


% INPUT VIRUS (CPM) BOUND
100
80
60
40
20
0
HeLa
3T3/pcDNA3
3T3/DAF
CELL LINE

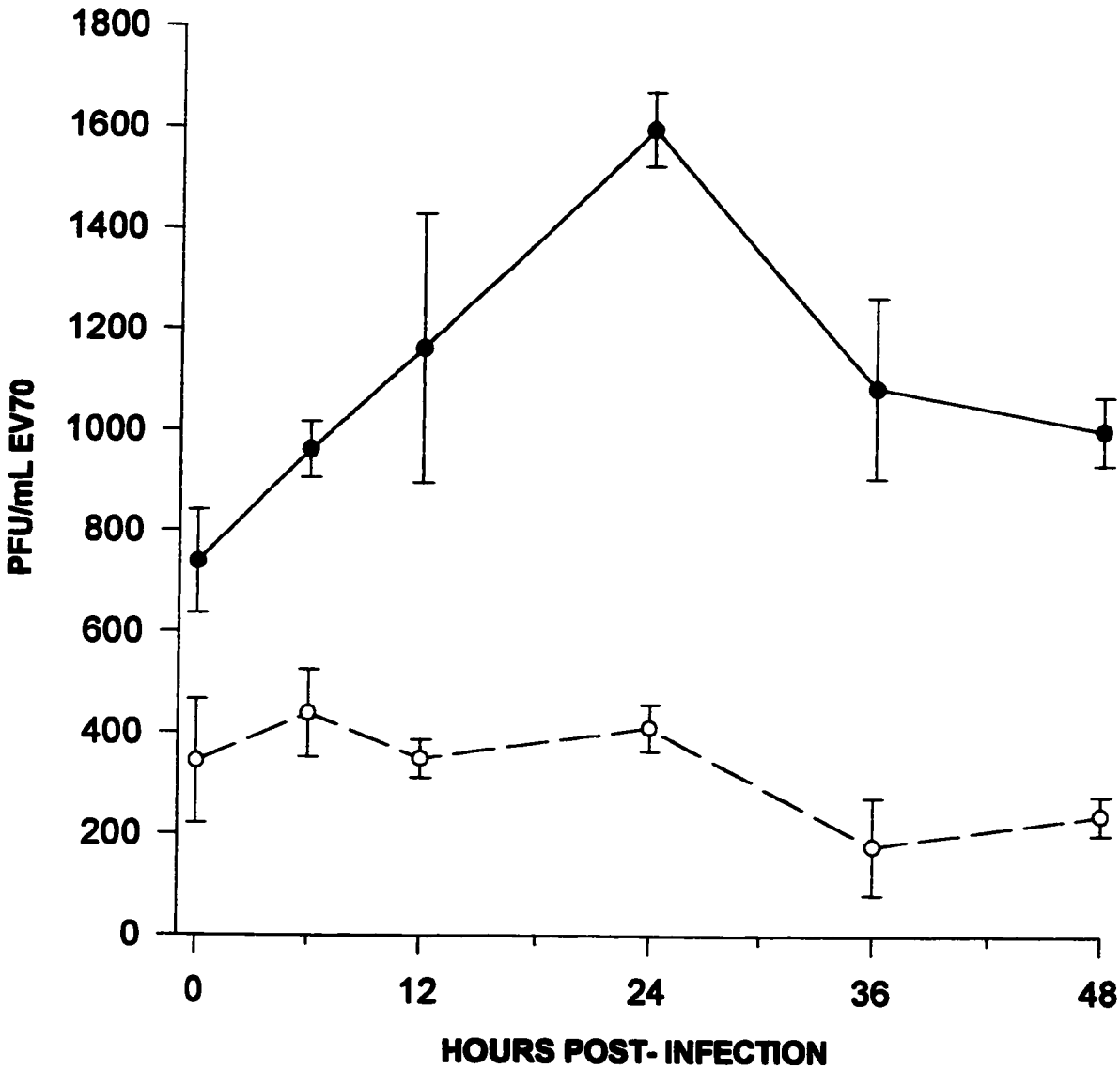
purpose, since vaccinia virus results in pronounced morphological changes in the transfected cells, and eventually kills the cells, making EV70-induced CPE and EV70 replication impossible to detect.

C. Expression of DAF renders 3T3 cells susceptible to infection by EV70

Although the low levels of DAF expressed on the 3T3-DAF cell line limited its usefulness in ^{35}S-EV70 binding assays, the ability of EV70 to infect these cells was investigated. Monolayers of cells were infected with EV70 at a MOI of 5, and assayed for the production of infectious virus at regular intervals, over a 48 h period. As shown in Figure 18, 3T3-DAF cells supported EV70 replication. Virus yield from DAF-expressing cells increased to a peak value of 1.6 X 10^3 PFU/mL by 24 h, representing a 2.5-fold increase from time 0, and began to decline thereafter. Control 3T3-RDAF cells were refractory to infection: the amount of virus detected in these cells remained low, approximately 300 PFU/mL for 24 h, and then as seen with 3T3-DAF cells, titre began to fall. Experiments in which samples were assayed at 24 h intervals over a 120 h period (data not shown) indicated that the decline in virus titres noted above continued, in all likelihood reflecting decay of progeny virus following one cycle of replication (3T3-DAF cells) and decay of input virus (3T3-RDAF cells). The difference in the amount of virus associated with the 3T3-DAF and 3T3-RDAF cells at time 0 (Figure 18) may simply reflect greater EV70 binding activity of the DAF-expressing cells.

Figure 18. EV70 replication in 3T3-DAF cells. Confluent 3T3-DAF and 3T3-RDAF cell monolayers grown in 24-well tissue culture plates were infected with EV70 at a MOI of 5, for 1 h at 33°C. After washing, fresh MEM was applied to each well. At the indicated intervals, cells plus supernatants were recovered, and frozen and thawed twice to liberate infectious virus. EV70 titers were measured by plaque assay on LLC-MK_2 monolayers. Results are expressed as means ± standard deviation for two duplicate independent determinations.

● 3T3-DAF cells
○ 3T3-RDAF control cells


1800
1600
1400
1200
1000
800
600
400
200
0
PFU/mL EV70
0
12
24
36
48
HOURS POST- INFECTION

V. Mapping the EV70 binding site(s) of DAF

It was now evident that EV70 is one of several human enteroviruses that use DAF as a cellular receptor (Bergelson *et al.*, 1995; Bergelson *et al.*, 1994; Shafren *et al.*, 1995; Ward *et al.*, 1994). Clearly, it was no longer possible to attribute the unusual host range and tropism of EV70 to the unique nature of its receptor. However, examination of how EV70 interacts with DAF may reveal differences between EV70 and other DAF-binding picornaviruses. These differences may in turn lead to the discovery of receptor - host cell interactions and post-attachment events unique to EV70, which may play roles in determining host range and tropism.

Initial mapping of the EV70 binding site(s) of DAF was performed by antibody blockade, using a number of DAF-specific mAbs. An important limitation of this mapping technique is that mAbs may non-specifically block virus binding by steric hindrance. Furthermore, mAb binding may affect the conformation of DAF, and in turn may influence EV70 binding at a distance from the actual mAb binding site. For these reasons, a second mapping technique was also used. This second method involved evaluating the EV70-binding activities of several altered DAF molecules following their transient expression in 3T3 cells.

A. Antibody blockade

To identify the regions of DAF involved in EV70 binding, mAbs directed against each of the SCR domains of DAF (Table 8) were used in cell protection and virus binding inhibition assays.

In cell protection assays, mAbs directed against SCR 1 (11D7), SCR 2 (1F7), and SCR3 (1H4), protected HeLa cells against EV70 infection, while an antibody specific for

Table 8. HeLa cell protection by DAF-specific mAbs

MAb[a]	SCR	Protection[b]
11D7	1	+
1F7	2	+
1H4	3	+
8D11	4	-
EVR1		+

[a] 11D7, 4 μg/mL; 1F7, 24 μg/mL; 1H4, 35 μg/mL; 8D11, 36 μg/mL; EVR1, 17.5 μg/mL.

[b] EV70, MOI 1.0
+ no observable cytopathic effects for a minimum of 96 h post-infection;
- cytopathic effects observed within 48 h post-infection.

SCR4 (8D11) did not (Table 8). Monolayers preincubated with mAbs EVR1 (17.5 μg/mL), 11D7 (4 μg/mL), 1F7 (24 μg/mL), and 1H4 (35 μg/mL) remained free of CPE for 96 h after infection with EV70 (MOI 1). HeLa cells treated with mAb 8D11 (36 μg/mL), and control monolayers (receiving no antibody pretreatment) began showing CPE within 48 h of infection. The protection provided by these mAbs was concentration-dependent (Table 9).

The ability of DAF-specific mAbs to prevent EV70 infection of HeLa cells correlated with their ability to inhibit virus binding (Figure 19). Relative to HeLa cell control monolayers (receiving no antibody pretreatment; represented as 100±4.7% input virus bound), EVR1 (9 μg/mL) reduced virus binding by 72%, 11D7 (8 μg/mL) by 68%, 1F7 (12 μg/mL) by 76%, and 1H4 (7 μg/mL) by 73%. Pretreatment of monolayers with mAb 8D11 (18 μg/mL) had no effect on virus binding (100±3.5% relative to HeLa control). As observed in cell protection assays, virus binding inhibition by these mAbs was concentration-dependent (Table 10). Therefore, antibody blockade experiments localized EV70 binding to within the first three SCR domains of DAF.

B. DAF/MCP hybrid protein expression

In order to more precisely localize the EV70 binding sites of DAF, a series of DAF/MCP chimeric proteins (Manchester *et al.*, 1995) were transiently expressed in 3T3 cells, and the EV70 binding properties of these hybrid molecules were analyzed.

DAF and MCP are members of the regulators of complement activation (RCA) family of proteins. Among the members of this family, MCP is the most closely related to DAF (Adams *et al.*, 1991; Clarkson *et al.*, 1995; Lublin *et al.*, 1988). Both proteins are composed of four highly conserved SCR domains of approximately 60 amino acids each, followed by

Table 9. Concentration dependent cell protection of HeLa cells by EVR1 and by DAF-specific mAbs.

		CPE[a]			
		Hours post-infection			
MAb	**Dilution[b]**	**24**	**48**	**72**	**96**
EVR1	1:2	-	-	-	+
	1:5	-	-	+	+
1H4	1:2	-	-	-	+
	1:5	-	-	+	+
8D11	1:2	-	+/-	+	+
	1:5	-	+	+	+
11D7	1:20	-	-	-	+
	1:40	-	+/-	+	+
1F7	1:20	-	-	-	-
	1:50	-	-	-	-/+
	1:100	-	-	+	+

[a] -, no CPE; +/-, evidence of CPE; + extensive CPE
[b] Stock antibody concentrations: EVR1, 17.5 µg/mL; 11D7, 40 µg/mL; 1H4, 35 µg/mL; 8D11, 36 µg/mL.

Figure 19. Inhibition of EV70 binding to HeLa cells by DAF-specific mAbs. Confluent HeLa cell monolayers grown in 24-well tissue culture plates were treated with culture medium, control antibody, or with DAF-specific mAbs as indicated, for 1 h at 37°C. After washing, monolayers were incubated with 5,000 cpm of ^{35}S-labelled EV70 per well . After a 45 min incubation at 33°C, the amount of virus bound to cells was determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to the MEM control, ± standard deviation for two samples.

MEM: culture medium pretreatment
anti-MHC I: culture medium containing MHC I - specific IgG_1 (10 μg/mL)
EVR1: hybridoma culture supernatant, 9 μg/mL
11D7: hybridoma culture supernatant, 8 μg/mL
1F7: ascites fluid, 12 μg/mL
1H4: hybridoma culture supernatant 9 μg/mL
8D11: hybridoma culture supernatant 18 μg/mL

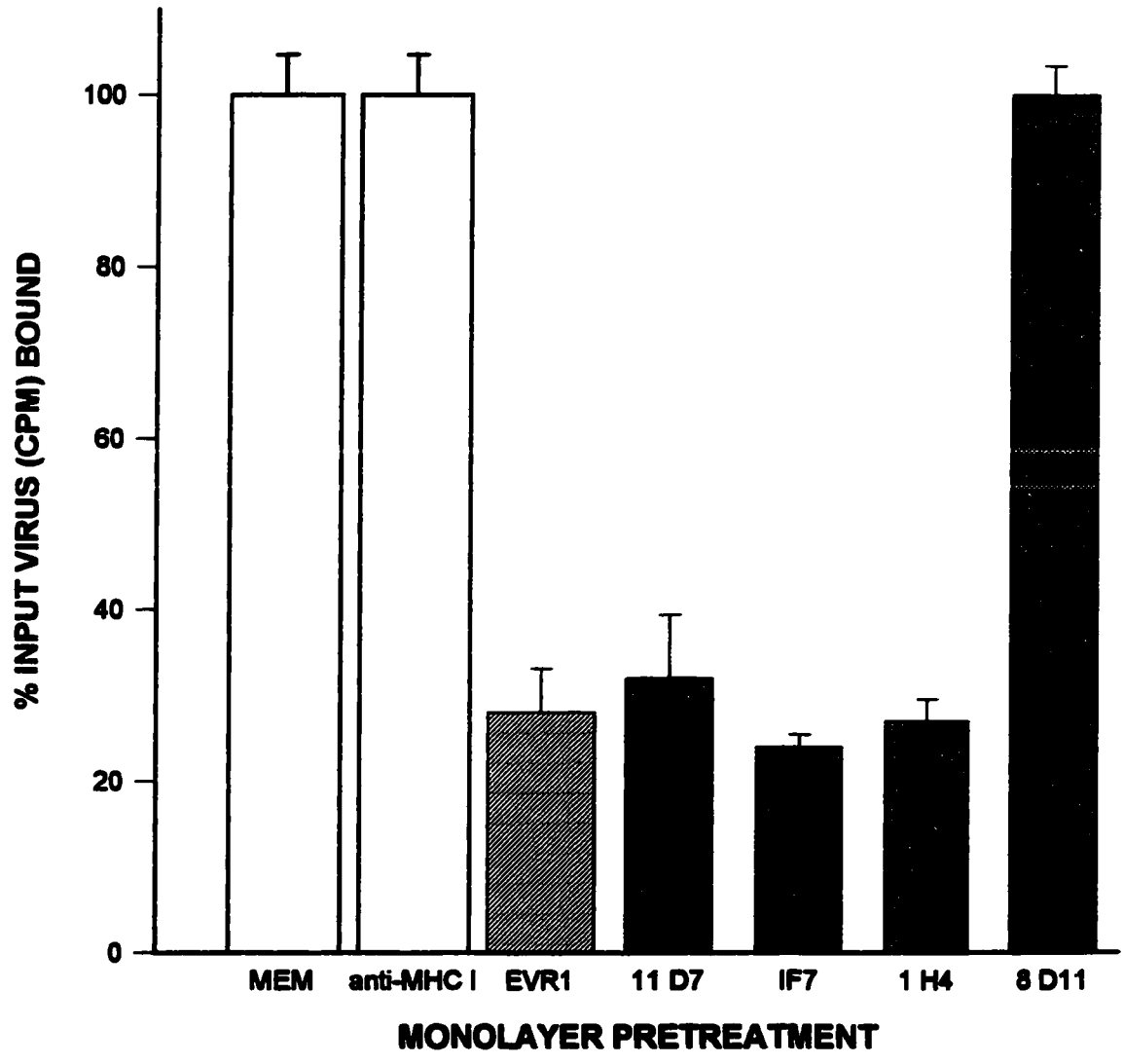

% INPUT VIRUS (CPM) BOUND
100
80
60
40
20
0
MEM
anti-MHC I
EVR1
11 D7
IF7
1 H4
8 D11
MONOLAYER PRETREATMENT

Table 10. Concentration dependent inhibition of EV70 binding to HeLa cells by EVR1 and by DAF-specific mAbs

	%Virus (cpm) bound[a]				
MAb/Dilution[b]	**1:2**	**1:5**	**1:10**	**1:20**	**1:40**
EVR1	29	34	51	65	nd
11D7	nd	32	38	70	88
1H4	30	28	34	42	nd
8D11	30	31	31	30	nd

	1:10	1:50	1:100	1:200	1:250	1:500	1:1000	1:1500
1F7	20	25	23	26	35	38	43	55

[a] Percent of input ^{35}S-EV70 bound to monolayers relative to HeLa cell control monolayers receiving no antibody pretreatment.

[b] Stock antibody concentrations: EVR1, 17.5μg/mL; 11D7, 40 μg/mL; 1H4, 35 μg/mL; 8D11, 36 μg/mL.

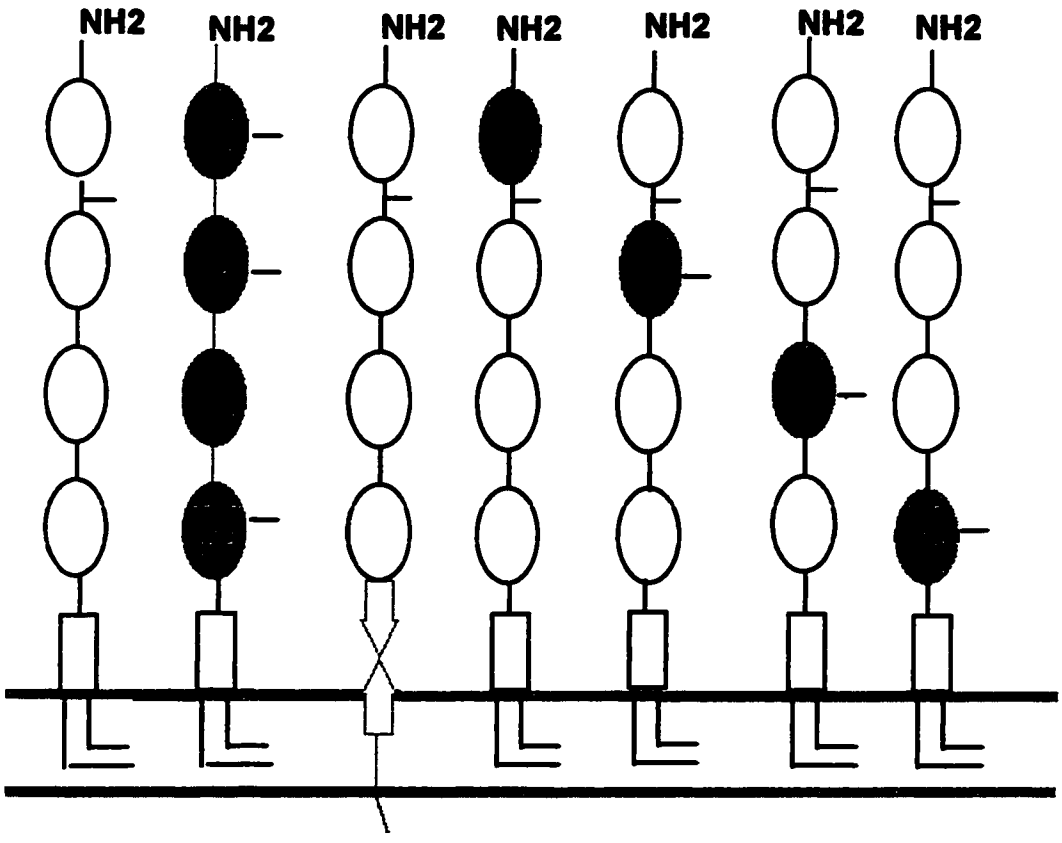
a heavily O-glycosylated serine, threonine, and proline-rich (STP) region near the carboxy terminus. The structure of MCP differs from DAF in two respects: (1) MCP has a region of unknown function following its STP region; and (2) MCP has a carboxy terminal peptide transmembrane domain (TM) and cytoplasmic domain, instead of the GPI anchor found in DAF (Lublin *et al.*, 1988). Domain-replacement molecules (in which individual DAF SCRs were substituted with the corresponding domains of MCP) and DAF SCR deletion mutants were available. DAF/MCP chimeras were chosen for these studies, since they were more likely to retain the overall conformation of the native DAF molecule, and thus minimize any undue structural/conformational effects on virus binding (Coyne *et al.*, 1992; Lublin and Coyne, 1991; Nowicki *et al.*, 1993). The different DAF/MCP chimeras used in these experiments are illustrated in Figure 20.

Before evaluating the receptor function of each of the hybrid proteins, it was necessary to: (i) confirm the specificities of each available DAF and MCP-specific mAb; (ii) confirm the SCR substitution of each chimera; and (iii) demonstrate that each of these hybrid proteins was expressed at similar levels in transfected 3T3 cells.

The cDNAs for DAF, a version of DAF with the TM domain of MCP (TM-DAF), MCP with the GPI anchor of DAF (MCP-PI), and DAF/MCP chimeras were supplied in expression vectors pcDNA3, pCRα, or pSRαEN. Therefore, for every expression experiment, negative controls consisted of 3T3 cells transfected with the appropriate vector lacking a DAF/MCP insert, and positive controls consisted of 3T3 cells transfected with the appropriate vector containing DAF.

Figure 20. DAF/MCP chimeras used in EV70 binding studies. Chimeras consisted of DAF molecules in which individual SCR domains were replaced with the corresponding SCR of membrane cofactor protein (MCP). DAF SCR domains are represented as open circles, MCP SCR domains are numbered and shaded grey. Horizontal bars indicate regions of N-linked glycosylation on the respective SCRs. Diagram adapted from Manchester *et al.*, 1995.

DAF: wild-type human DAF
MCP-PI: GPI-anchored version of MCP
TM-DAF: Transmembrane and cytoplasmic domains of MCP, SCR domains of DAF
DM1: MCP SCR1 replacing DAF SCR1
DM2: MCP SCR2 replacing DAF SCR2
DM3: MCP SCR3 replacing DAF SCR3
DM4: MCP SCR4 replacing DAF SCR4


NH2
NH2
NH2
NH2
NH2
NH2
NH2
DAF
MCP-PI
TM-DAF
DM1
DM2
DM3
DM4

(i) **Antibody specificities:** Primary antibody binding, detected with FITC-conjugated sheep anti-mouse IgG_1 ,was measured by flow cytometry. The entire complement of chimera-antibody combinations that was analyzed is summarized in Table 11. Cells transfected with pcDNA3, pCRα, or pSRαEN did not bind DAF or MCP-specific mAbs, as demonstrated by the absence of a shift in the fluorescence intensity profiles generated. Cells transfected with pcDNA3/DAF or pCRα/DAF bound each of the DAF-specific mAbs, but did not bind the MCP-specific mAb. Conversely, cells transfected with pSRαEN/MCP-PI bound MCP-specific mAb, but none of the DAF-specific mAbs. Flow cytometry histograms showing antibody reactivities for all control samples are presented in Appendix II.

(ii) **Characterization of DAF/MCP chimeras:** Each DAF/MCP chimera was characterized by flow cytometry using DAF domain-specific mAbs, to ensure that the proper SCRs of DAF were expressed. Figure 21 presents data obtained with 3T3 cells transiently expressing chimera DM4 (in which DAF SCR4 is replaced with MCP SCR4). MAbs directed against DAF SCR 1 (11D7, panel C), SCR 2 (1F7, panel D), and SCR 3 (1H4, panel E) were all able to bind to DM4-expressing cells., while a mAb directed against SCR4 (8D11) did not bind to DM4-expressing cells (panel F). Results confirming the replacement of the appropriate DAF SCR domains in DM1, DM2, and DM3 were also obtained. Flow cytometry histograms for these chimeras are included in Appendix III.

(iii) **Analysis of DAF/MCP chimera expression:** Before being able to compare the virus binding ability of each of these proteins, it was important to confirm that the chimeras and wild type DAF were expressed at similar levels on the surface of transfected cells. Cell surface expression was detected with DAF SCR-specific mAbs, and was assessed by flow

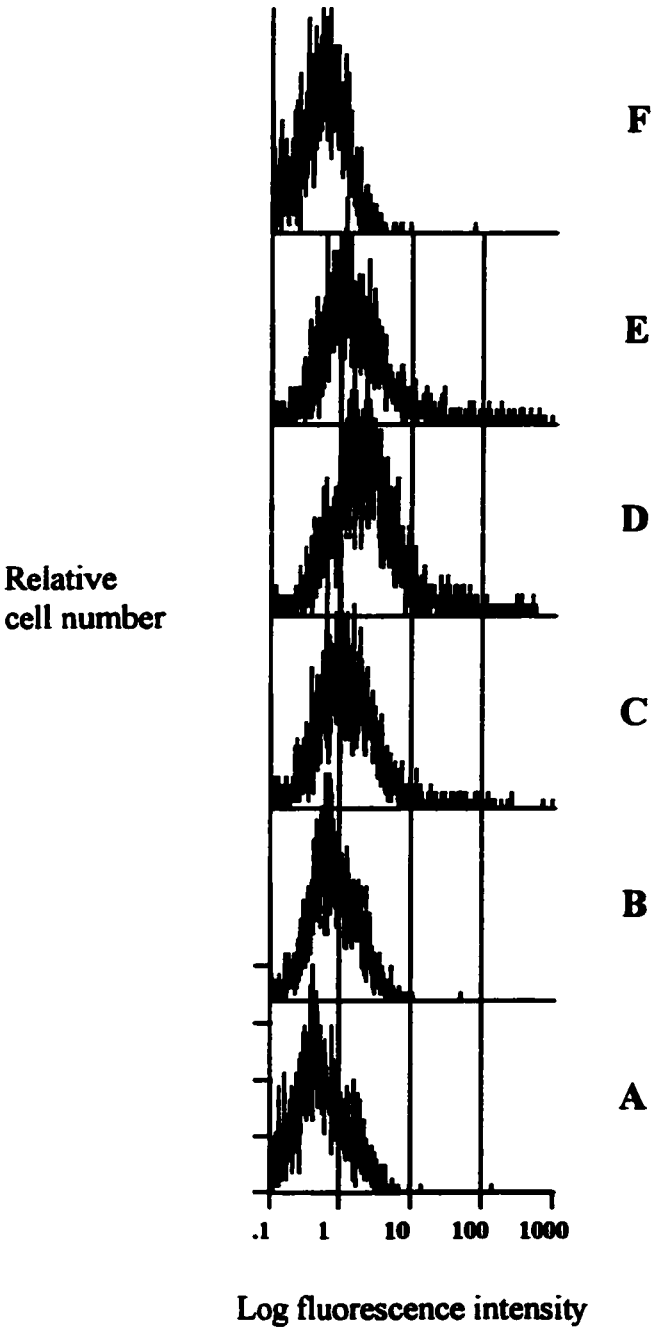
Table 11. Summary of DAF/MCP expression studies and mAb reactivities

	Primary antibody					
Construct	**EVR1**	**11D7 (SCR1)**	**1F7 (SCR2)**	**1H4 (SCR3)**	**8D11 (SCR4)**	**α-MCP**
pCRα	-	-	-	-	-	-
DAF[a]	+	+	+	+	+	-
MCP-PI[b]	-	-	-	-	-	+
DM1[a]	-	-	+	+	+	nd
DM2[b]	+	+	-	+	+	nd
DM3[a]	+	+	+	-	+	nd
DM4[a]	+	+	+	+	-	nd
pcDNA3	-	-	-	-	-	nd
pcDNA3-DAF	+	+	+	+	+	nd
pcDNA3-TM-DAF	+	+	+	+	+	nd

[a] construct in vector pCRα
[b] construct in vector pSRαEN
+ positive reaction with primary antibody
- negative reaction with primary antibody
nd not determined

Figure 21. Flow cytometric analysis of DAF-specific mAb binding to chimeric receptor DM4. 3T3 cell monolayers were transfected with vector pCRα, or with pCRα containing sequences encoding the DAF/MCP chimera DM4 (pCRαDM4). Following transfection and infection with vaccinia virus vTF7-3, cells were processed for flow cytometry. After incubation with DAF-specific mAbs 11D7 (1:200), 1F7 (1:500), 1H4 (1:5), or 8D11 (1:200) for 20 min at room temperature , cells were washed, and primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (diluted 1:20). Cells transfected with pCRαDM4 were used for autofluorescence and secondary antibody controls.

Panels:
A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) DM4; mAb 11D7 (anti-SCR1)
D) DM4; mAb 1F7 (anti-SCR2)
E) DM4; mAb 1H4 (anti-SCR3)
F) DM4; mAb 8D11 (anti-SCR4)


F
E
D
C
B
A
Relative
cell number
.1
1
10
100
1000
Log fluorescence intensity

cytometry. If the fluorescence intensity profile of cells expressing one chimera was the same as the profile generated by cells expressing another chimera, the expression levels of these proteins was considered to be equivalent.

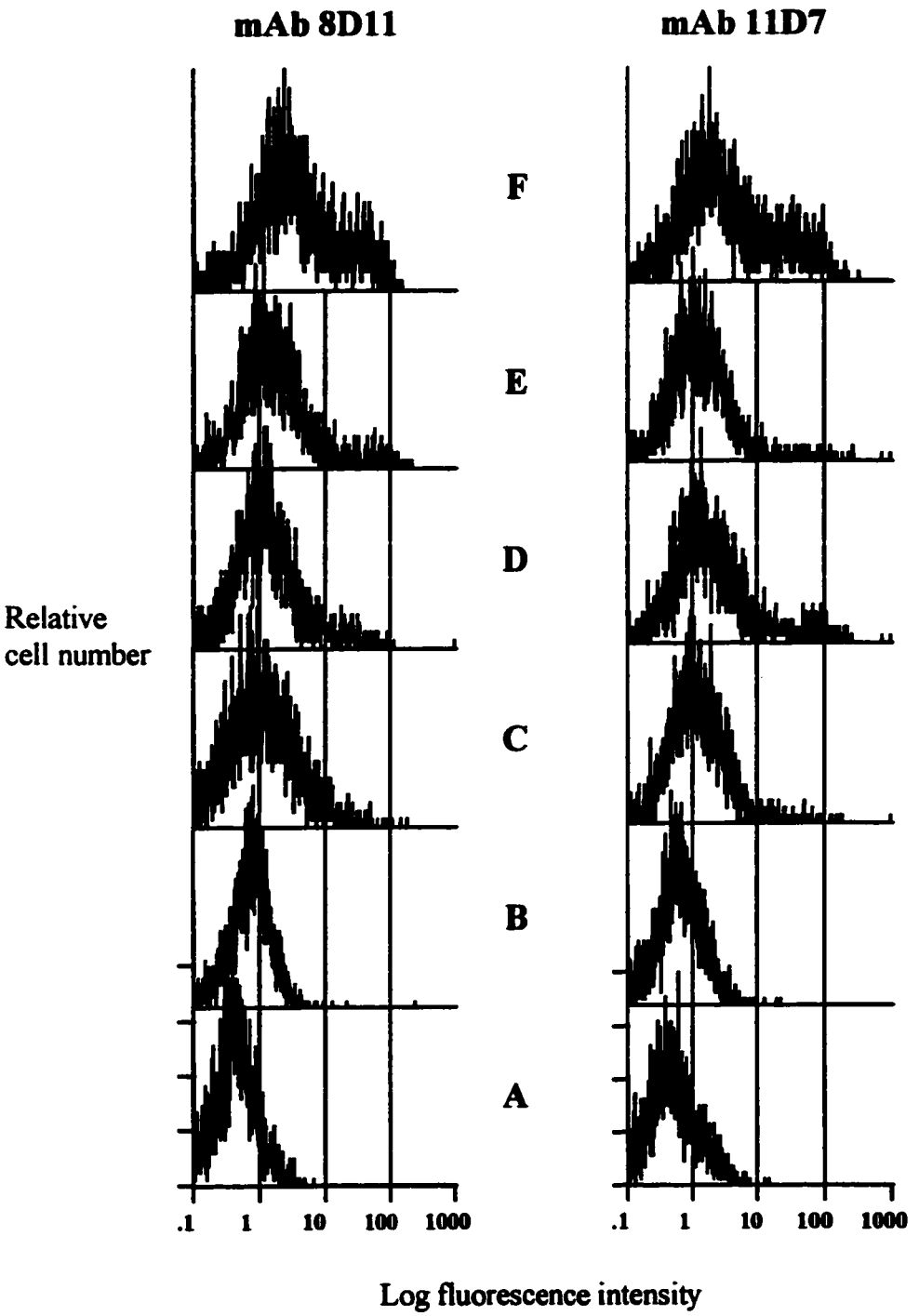
Since no single mAb recognized an epitope common to all chimeras, at least two different mAbs were required to compare expression levels of the various proteins. Results of flow cytometric analyses indicated that a given SCR-specific mAb did not necessarily exhibit the same binding activity with all DAF/MCP chimeras containing the appropriate SCR (Appendix III). This was reflected in the observation that the fluorescence intensity profiles generated with some mAbs varied considerably, depending on the SCR substitution of the chimera being analyzed. Similarly, different mAbs did not necessarily exhibit the same binding activity with the same DAF/MCP chimera. Taken together, these findings suggest that while the overall conformation of the DAF/MCP chimeras may not significantly differ from that of DAF, SCR replacement may cause sufficient local conformational change to affect the ability of certain SCR-specific mAbs to bind. This hypothesis is supported by the observation that cells transiently expressing wild type DAF consistently bound more DAF-specific antibody than did cells expressing any of the chimeras, regardless of which antibody was used (Appendix III).

Given these results, it was necessary to select DAF-specific mAbs that exhibited the same binding activity with a number of chimeras, regardless of the SCR substitution. As shown in Figure 22 (column 1), SCR4-specific mAb 8D11 displayed consistent binding activity with cells expressing chimeras DM1, DM2, and DM3, and was therefore used to compare surface expression levels of these proteins. Similarly, SCR1-specific mAb 11D7

Figure 22. Flow cytometric analysis of DAF/MCP chimera expression in transiently transfected 3T3 cells. 3T3 cell monolayers grown in 24-well tissue culture plates were transfected with vector pCRα, with vectors containing sequences encoding DAF/MCP chimeras DM1, DM2, DM3, or DM4, or with pCRαDAF. Following transfection and infection with vaccinia virus vTF7-3, cells were processed for flow cytometry. After incubation with DAF-specific mAb 8D11 (anti-SCR4; 1:200) or 11D7 (anti-SCR1, 1:200) for 20 min at room temperature, cells were washed, and primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:20). Cells transfected with each of the different constructs were used for autofluorescence and secondary antibody controls. Since these all produced superimposable histograms, panels A and B are shown as representative negative control samples.

Column 1: mAb 8D11 used as primary antibody
A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) DM1
D) DM2
E) DM3
F) DAF

Column 2: mAb 11D7 used as primary antibody
A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) DM2
D) DM3
E) DM4
F) DAF


mAb 8D11
mAb 11D7
F
E
D
C
B
A
Relative cell number
.1
1
10
100
1000
.1
1
10
100
1000
Log fluorescence intensity

displayed consistent binding activity with cells expressing chimeras DM2, DM3, and DM4, and was used to compare expression levels of these proteins (Figure 22, column 2).

The fluorescence intensity profiles presented in Figure 22 confirmed that all of the chimeric receptors were expressed to similar levels. Finally, these results (Figures 22 and 23; Appendices II and III) showed that the T7 promoter directed expression of similar amounts of each of the chimeric receptors, regardless of the vector in which the cDNAs were cloned.

In a separate experiment, SCR3-specific mAb 1H4 was used to compare expression of TM-DAF (in pcDNA3) to that of DAF (pcDNA3/DAF). Figure 23 shows that the binding of mAb 1H4 to TM-DAF expressing cells appeared to be slightly lower than binding to DAF-expressing cells. These results indicate that either less TM-DAF was expressed on the cell surface, or that replacing the GPI anchor of DAF with the transmembrane and cytoplasmic domains of MCP affected the binding ability of mAb 1H4. Finally, the expression levels of pcDNA3/DAF and pCRα-DAF in 3T3 cells were compared using mAb 1H4, and found to be similar (Appendix IV)

In view of all these data, differences in the abilities of the various DAF/MCP chimeras to bind EV70 were attributed to the presence or absence of sequences essential for virus attachment.

C. Binding activity of DAF/MCP chimeras

Virus binding to cells expressing each of the DAF/MCP chimeras was analyzed, and the percent of input virus bound relative to cells transfected with pCRαDAF was determined (Figure 24). To establish whether MCP could be used as a receptor for EV70, MCP was also compared to DAF for its ability to bind virus. Results indicated that MCP could not act as

Figure 23. Flow cytometric analysis of DAF and TM-DAF expression in transiently transfected 3T3 cells. 3T3 cell monolayers grown in 24-well tissue culture plates were transfected with vector pcDNA3, with pcDNA3 containing sequences encoding human DAF (DAF), or with pcDNA3 containing the sequences encoding the transmembrane version of DAF (TM-DAF). Following transfection and infection with vaccinia virus vTF7-3, cells were processed for flow cytometry. After incubation with DAF-specific 1H4 hybridoma supernatant (1:5) for 20 min at room temperature, cells were washed, and primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:20). Cells transfected with pcDNA3 were used for autofluorescence and secondary antibody controls.

Panels:
A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) pcDNA3- transfected cells; 1H4
D) DAF-TM transfected cells; 1H4
E) DAF-transfected cells; 1H4

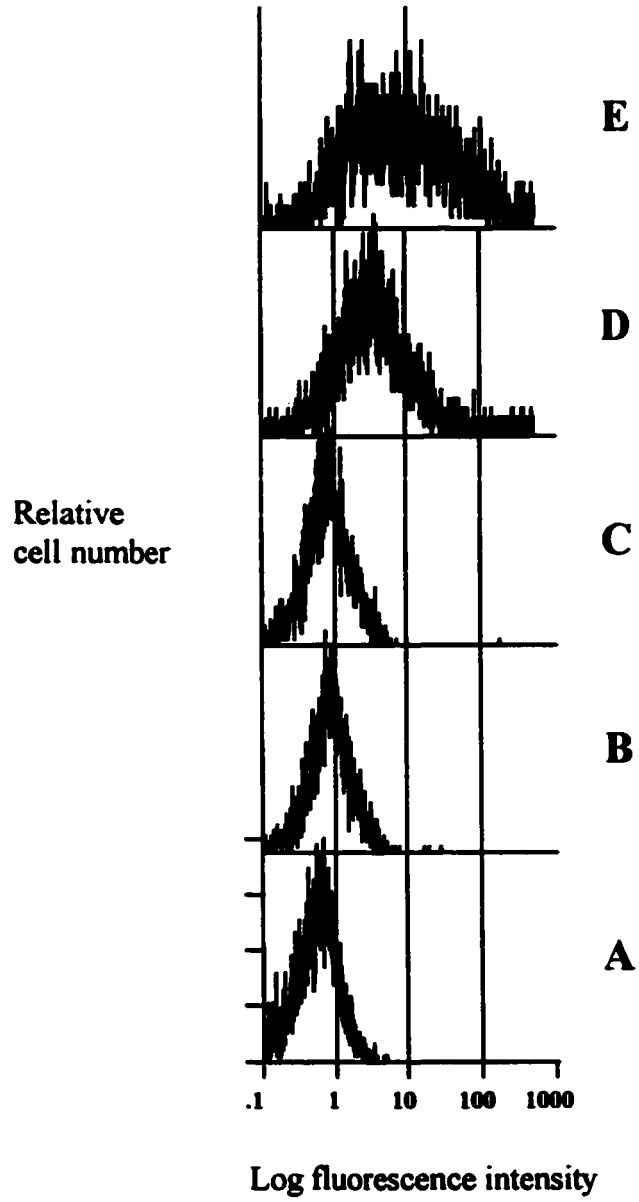
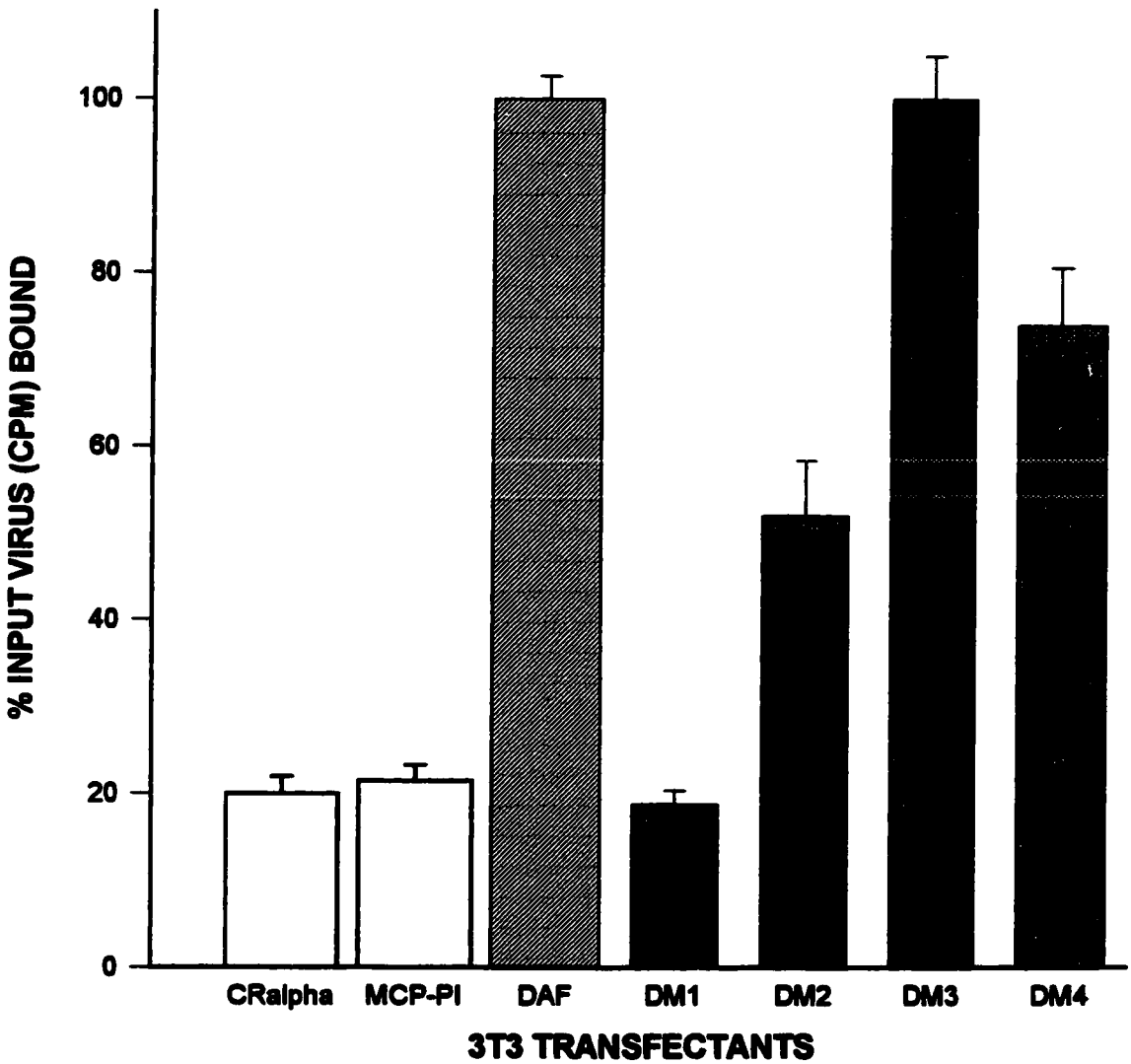

E
D
C
B
A
Relative
cell number
.1
1
10
100
1000
Log fluorescence intensity

Figure 24. EV70 binding to 3T3 cells transiently expressing DAF/MCP chimeras. 3T3 cell monolayers grown in 24-well tissue culture plates were transfected with vector pCRα , or with vectors containing the genes encoding the DAF/MCP chimeras DM1, DM2, DM3, DM4, or MCP-PI. Following transfection and infection with vaccinia virus vTF7-3, monolayers were washed and incubated with 3,000 cpm of ^{35}S-labelled EV70 per well. After 1 h at 33°C, the amount of virus bound to cells was determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to 3T3 cells transfected with pCRαDAF, ± standard deviation for three samples.

pCrα: vector alone
MCP-PI: MCP-PI in vector pSRαEN
DAF: DAF in pCRα
DM1: DM1 in pCRα
DM2: DM2 in pSRαEN
DM3: DM3 in pCRα
DM4: DM4 in pCRα


% INPUT VIRUS (CPM) BOUND
100
80
60
40
20
0
CRalpha
MCP-PI
DAF
DM1
DM2
DM3
DM4
3T3 TRANSFECTANTS

a receptor for EV70, since virus binding to MCP-expressing cells and to cells transfected with pCRα alone was the same (20 ± 2% of input virus bound). Replacement of the first SCR of DAF (DM1) reduced EV70 binding to background levels (20 ± 2% of input virus bound). Replacement of the second SCR (DM2) had a less pronounced negative effect on virus binding, with 52±6% of input virus bound. Replacement of the third SCR (DM3) had no effect on EV70 binding (100±5% of input virus bound). Replacement of either the fourth SCR of DAF (DM4), or of the STP and GPI anchor regions of DAF (TM-DAF; Figure 25) had only small negative effects on virus binding (70 ± 8%, and 74 ±7% of input virus bound, respectively).

VI. Identification of the EVR1 binding site of DAF

In a final set of experiments, the region of DAF recognized by EVR1 was determined. Antibody blockade (cell protection and virus-binding inhibition; Table 8 and Figure 19, respectively) could only exclude sequences within SCR4 as the EVR1 binding site. Therefore, 3T3 cells transiently expressing each of the DAF/MCP chimeric receptors were tested for their ability to bind EVR1. Flow cytometric analysis of each of the chimeras (Figure 26) showed that transfected cells expressing DM1 did not bind EVR1 (panel C). In contrast, EVR1 bound to cells expressing DM2, DM3 or DM4 (panels D, E, and F, respectively). The SCR1- specificity of EVR1 was confirmed when DM1-expressing cells were shown to bind all DAF-specific mAbs except for mAb 11D7 (directed against SCR1), and EVR1 (see Appendix III).

Figure 25. EV70 binding to 3T3 cells transiently expressing TM-DAF. 3T3 cell monolayers grown in 24-well tissue culture plates were transfected with vector pcDNA3 , with pcDNA3 containing sequences encoding human DAF (DAF), or with pcDNA3 containing the sequences encoding the transmembrane version of DAF (TM-DAF). Following transfection and infection with vaccinia virus vTF7-3, monolayers were washed and incubated with 3,000 cpm of ^{35}S-labelled EV70 per well. After 45 min at 33°C, the amount of virus bound to cells was determined by scintillation counting. Results are shown as the mean percentage of virus bound relative to pcDNA3/DAF transfected cellsl, ± standard deviation for three samples.

pcDNA3: pcDNA3 - transfected cells
DAF: pcDNA3/DAF - transfected cells
TM-DAF: pcDNA3/TM-DAF - transfected cells

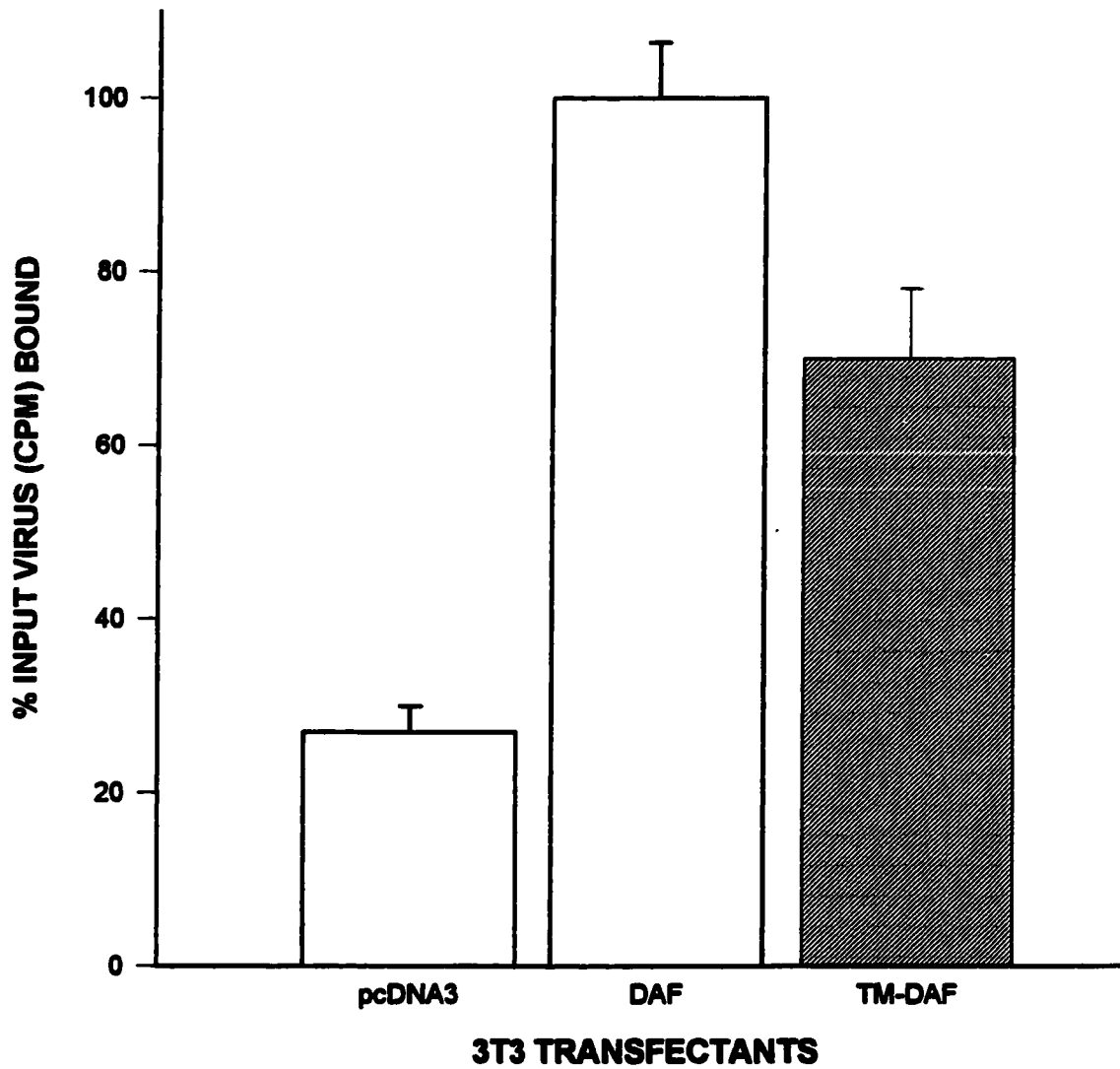
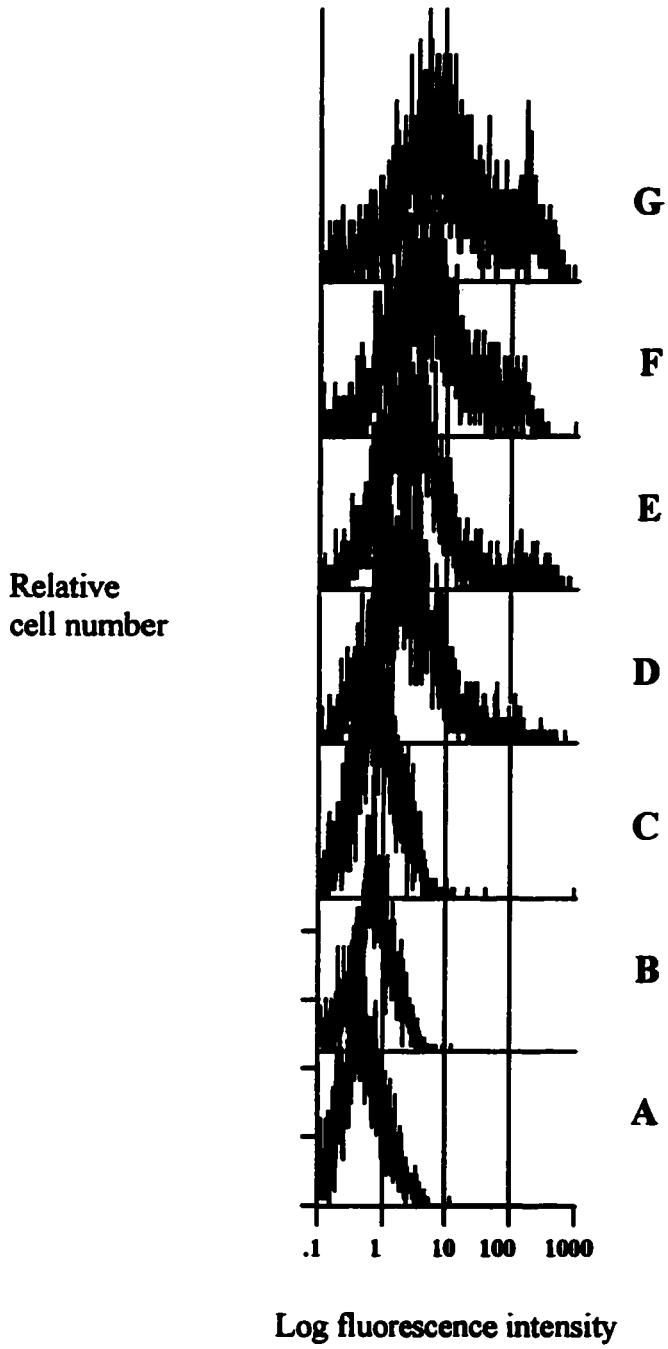

% INPUT VIRUS (CPM) BOUND
100
80
60
40
20
0
pcDNA3
DAF
TM-DAF
3T3 TRANSFECTANTS

Figure 26. Identification of the EVR1-binding site of DAF. 3T3 cell monolayers grown in 24-well tissue culture plates were transfected with vector pCRα, with pCRα containing the sequences encoding DAF/MCP chimeras DM1, DM2, DM3, or DM4, or with pCRαDAF. Following transfection and infection with vaccinia virus vTF7-3, cells were processed for flow cytometry. After incubation with EVR1 (1:100) for 20 min at room temperature, cells were washed, and primary antibody binding was detected with FITC-conjugated sheep anti-mouse IgG_1 (1:20). Cells transfected with each of the different constructs were used for autofluorescence and secondary antibody controls. Since these all produced superimposable histograms, panels A and B are shown as representative negative control samples.

Panels:
A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) DM1 in pCRα; EVR1
D) DM2 in pSRαEN; EVR1
E) DM3 in pCRα; EVR1
F) DM4 in pCRα DM1; EVR1
G) DAF in pCRα; EVR1


Relative
cell number
G
F
E
D
C
B
A
.1
1
10
100
1000
Log fluorescence intensity

DISCUSSION

Most viruses cause disease in only a small number of species, and within the infected host, symptoms of viral disease can often be correlated with infection of a small number of specific target tissues. In order to initiate infection, viruses must bind to specific molecules expressed on the surface of susceptible cells (Bass and Greenberg, 1992; Norkin, 1995). This interaction is not only critical for infection, but for many viruses is also considered to be a key determinant of host range and of tropism (Crowell and Landau, 1983; Haywood, 1994; Norkin, 1995). Over twenty years ago it was shown that for picornaviruses, pathogenesis could be correlated with the presence of virus-specific receptors on affected tissues (Lonberg-Holm *et al.*, 1976; Crowell and Landau, 1983). Although there was evidence that attachment to cells was not always sufficient for infection (Angel and Burness, 1977; Colonno *et al.*, 1990; Maddon *et al.*, 1986; Yoshii *et al.*, 1977), when this project was undertaken, it was generally accepted that the specificity of the virus-host cell receptor interaction was an important determinant of picornavirus tropism (Crowell and Landau, 1983; Lentz, 1990).

Two intriguing characteristics of EV70 are its tropism and host range. *In vivo*, EV70 replication is restricted to the conjunctival epithelium, and on rare occasions involves the central nervous system, while *in vitro*, EV70 infects cells derived from a variety of mammalian species (Yoshii *et al.*, 1977). This ability to replicate in cells of non-primate origin is, with few exceptions, a unique characteristic among human enteroviruses. In the context of what was known about the nature and distribution of virus receptors, and the influence these molecules exert on viral pathogenesis, it was hypothesized that the unusual

tropism and host range of EV70 reflected use of a host cell receptor that was distinct from the picornavirus receptors recognized at the time this project was initiated.

I. Analysis of EV70 binding to cells

At the onset of this work , the lack of information regarding either the nature of the host cell receptor for EV70 (Kono *et al.*, 1978) or the binding behaviour of this virus (Yoshii *et al.*, 1977), made it necessary to establish a binding assay for EV70. This virus binding assay allowed rapid and reproducible measurement of ^{35}S-EV70 binding to LLC-MK$_2$ cells, which were used in initial experiments. Traditionally, studies of the kinetics of virus binding have been used as a starting point in defining the characteristics of receptor molecules (Colonno, 1987; Crowell and Landau, 1983). Virus binding competition experiments have established that the picornaviruses can be grouped into families according to specificities for distinct receptors (Abraham and Colonno, 1984; Lonberg-Holm *et al.*, 1976; Quirsin-Thiry and Nihoul, 1961). To determine the specificity of the receptor for EV70, the ability of PV and of CVB3 to interfere with EV70 binding to LLC-MK$_2$ cells was examined. These viruses were chosen for several reasons. Earlier studies showed that PV and CVB3 belonged to separate receptor families, and it was of interest to determine if EV70 could be assigned to either of these receptor groups. In addition, phylogenetic analyses (Pöyry *et al.*, 1996; Stanway, 1990) showed that EV70 was closely related to both PV and CVB3. Finally, the identity of the poliovirus receptor was known (Mendelsohn *et al.*, 1989), and candidate CVB3 receptor proteins had been identified (Hsu *et al.*, 1990; Mapoles *et al.*, 1985). Binding assays showed that the LLC-MK$_2$ cell receptor for EV70 was distinct from the receptor used

by PV, and that CVB3 exerted a partial inhibitory effect on EV70 binding. This suggested that either EV70 and CVB3 compete for the same receptor, share a component of a receptor complex, or that CVB3 attaches to the cell at a site near enough to the receptor for EV70 to sterically hinder EV70 binding.

II. Identification of a cellular receptor for EV70

Initial attempts at identifying a virus binding protein in LLC-MK_2 cells using the affinity based VOPBA method were unsuccessful. Although this technique has successfully been used to identify virus binding proteins for adenoviruses (Defer *et al.*, 1990), rhinoviruses (Mischack *et al.*, 1988) reovirus (Verdin *et al.*, 1989), and LCMV (Borrow and Oldstone, 1992), among others, it is not broadly applicable. In principle, membrane components are denatured in the process of electrophoresis and blotting, and thus receptors that are functional multimers or that have binding sites sensitive to these procedures may not retain binding activity (Bass and Greenberg, 1992). However, in VOPBAs, ^{35}S-EV70 adsorbed nonspecifically to several species of LLC-MK_2 membrane proteins. To expedite the identification of the receptor for EV70, this method was abandoned in favour of an immunological approach, based on the production of a monoclonal antibody specific for the receptor for EV70.

Antireceptor antibodies are useful and increasingly popular tools for the identification of host cell receptors for viruses. The majority of antibodies with specificity for picornavirus receptors have been generated against HeLa cell epitopes (Campbell and Cords, 1983; Crowell *et al.*, 1986; Greve *et al.*, 1989; Hsu *et al.*, 1988; Minor *et al.*, 1984; Nobis *et al.*,

1985; Tomassini *et al.*, 1989a, 1989b). Therefore, to be able to eventually take advantage of picornavirus receptor-specific and other human cell-specific immunological reagents that were already available, HeLa cells were used to generate an EV70 receptor-specific antibody. HeLa cells support EV70 replication (Mirkovic *et al.*, 1973), and in binding assays, bound EV70 to levels comparable to those seen with LLC-MK_2 cells.

A. Antireceptor mAb EVR1

Immunization of BALB/c mice with HeLa cell membrane preparations led to the production of a mAb, EVR1, directed against a HeLa cell receptor for EV70. The anti-receptor activity of this monoclonal antibody was confirmed through a series of physical and biological assays. EVR1 protected HeLa cells against infection by EV70 in a concentration-dependent manner, and virus binding inhibition assays showed that EVR1 exerted this protective effect at the level of the virus-receptor interaction. Since antibodies may block virus binding through non-specific masking of the cell surface, or by steric hindrance (Campbell and Cords 1983; Crowell *et al.*, 1986), the specificity of EVR1 protection was confirmed in cell protection assays, which showed that EVR1 could not protect HeLa cells against infection by either PV or CVB3. EVR1 did not protect LLC-MK_2 cells against EV70 infection, and indirect immunofluorescence analysis revealed that EVR1 did not bind to LLC-MK_2 cells. These observations suggested: (1) that the receptor for EV70 on LLC-MK_2 cells is not the same as the receptor on HeLa cells; or (2) that the LLC-MK_2 receptor is a simian homolog which does not contain the epitope recognized by EVR1. EVR1 was then used to define the characteristics of the EV70-binding component of HeLa cells. As strategies to isolate the candidate receptor were being considered, Bergelson *et al.* (1994),

and shortly afterwards Ward *et al.* (1994) reported that the complement-regulatory protein decay-accelerating factor (DAF/CD55) served as a cellular receptor for several echovirus serotypes. These viruses, like EV70, are human pathogens belonging to the genus enterovirus.

B. EVR1 recognizes DAF

DAF is a member of the regulators of complement activation (RCA) family of proteins. (Hourcade *et al.*, 1989; Lublin and Atkinson, 1989; Nicholson-Weller, 1992). This is a family of functionally related, structurally homologous and genetically linked proteins which act to protect host tissues against autologous complement mediated lysis. The RCA family consists of the serum proteins factor H and C4 binding protein (C4-bp), and the membrane-bound proteins C3b receptor (CR1), C3d receptor (CR2), membrane cofactor protein (MCP), and decay-accelerating factor (DAF), all of which are encoded by genes located on the long arm of human chromosome 1 (Hourcade *et al.*, 1989; Lublin *et al.*, 1987). Each of these proteins either prevent the formation, or participate in the proteolytic inactivation of, the C3 convertases of the complement system, thereby halting the complement cascade and preventing formation of mediators of the inflammatory response and of the cytolytic terminal membrane attack complex (Lublin and Atkinson, 1989; Nicholson-Weller, 1992).

The structural hallmark of the RCA family is the presence of a variable number of contiguous, conserved repeated domains (short consensus repeat; SCR). Each SCR domain is composed of approximately 60 amino acids, and contains four conserved cysteine residues, as well as conserved glycine, tryptophan, phenylalanine and tyrosine residues. The SCRs

display 18 to 30% amino acid identity to each other within a given protein and between RCA proteins (Coyne *et al.*, 1992, Clarkson *et al.*, 1995).

Human DAF is a 70 to 75 kDa integral membrane glycoprotein that regulates C3 convertase activity by preventing its formation and also by dissociating preformed enzyme on the surface of cells. In HeLa cells, the most abundant DAF-specific mRNA encodes a polypeptide of 381 amino acids (Medof *et al.*, 1987a). DAF is composed of four contiguous SCR domains, followed by a heavily O-glycosylated serine and threonine rich juxtamembrane domain. Between SCRs 1 and 2 is a single N-glycosylation site. Unique among members of the RCA family, the carboxy terminus of DAF is covalently linked to a glycophospholipid structure (GPI anchor) through which the protein is attached to the plasma membrane. (Medof *et al.*, 1986). The functional domains of DAF have been identified (Lublin and Coyne, 1991; Coyne *et al.*, 1992). SCR1 and the GPI anchor do not appear to be necessary for the complement regulating function of DAF. Only SCR domains 2, 3, and 4 appear to be specifically required for DAF funtion, and the O-glycosylated domain fulfills a critical, but non-specific role in projecting the functional domains of DAF above the plasma membrane (Coyne *et al.*, 1992).

In addition to its complement regulating and echovirus-binding activities, DAF has also been shown to act as a receptor for several adhesin-bearing strains of *E. Coli* (Bernet-Camard, 1996; Nowicki *et al.*, 1993; Pham *et al.*, 1995). DAF is found on most peripheral blood cells and on many epithelial and endothelial cells exposed to serum (Lublin and Atkinson, 1989; Nicholson-Weller 1992). DAF is also expressed at high levels on the conjunctival epithelium, and DAF antigen has been detected in the cerebrospinal fluid (Lass

et al., 1990; Medof *et al.* 1987b), and is therefore found in tissues involved in the pathogenesis of acute haemorrhagic conjunctivitis (Higgins, 1982; Uchida , 1989b).

The biochemical characterization of the protein identified by EVR1 provided sufficient evidence to suggest that this protein, and therefore the receptor for EV70, was DAF. Both glycoproteins have a molecular weight of approximately 70 to 75 kDa in HeLa cells, and are resistant to hydrolysis by trypsin (Lublin and Atkinson, 1989). The sensitivity of the EVR1-protein interaction to heat and to reducing agents was consistent with EVR1 recognizing an epitope on one of the four disulphide bond-containing SCR domains of DAF. The fact that DAF was first isolated from human and guinea pig erythrocytes (Nicholson-Weller *et al.*, 1981; Nicholson-Weller *et al.*, 1982), and the observation that EV70 was able to agglutinate human and guinea pig erythrocytes (Kono *et al.*, 1978), further strengthened the possible link between DAF and the ligand of EVR1.

The identity of the protein recognized by EVR1 was confirmed by showing that: (1) the ligand of EVR1 was sensitive to PI-PLC; (2) EVR1 reacted specifically with DAF-expressing CHO cells, and (3) DAF-specific mAb protected HeLa cells against EV70 infection and inhibited virus binding. It was also observed that parental (DAF^-) CHO cells and DAF-expressing CHO cells bound equivalent amounts of EV70. This was consistent with the findings of Yoshii *et al.* (1977), who showed that the hamster-derived cell line BHK21 adsorbed high levels of EV70 and supported virus replication. These results suggest that a CHO cell surface molecule has the ability to bind EV70, but does not exclude the possibility that EV70 was also binding to human DAF. Since the CHO-DAF cell line used here is known to express low levels of DAF (D. Lublin, personal communication), more abundant expression

of DAF may be necessary to detect EV70 binding above the levels seen with the parental CHO cell line.

C. DAF is a receptor for EV70

Conclusive evidence that DAF acts as a receptor for EV70 was provided by direct demonstration that in 3T3 cells, DAF mediates EV70 binding and that EV70 binding leads to infection. When the gene encoding human DAF was transiently expressed in 3T3 cells, these cells acquired the ability to bind EV70. When 3T3 cells constitutively expressing human DAF (White *et al.*, 1992) were infected with EV70, low amounts of progeny virus, consistent with a single round of replication, were detected. The low level of virus replication observed in 3T3-DAF cells may reflect poor efficiency of infection due to the small number of receptor molecules expressed on the cell surface (Koike *et al.*, 1991a), however, it is more likely that EV70 replication is poorly supported in 3T3 cells. As yet unidentified cellular factors required for efficient growth of EV70 in these cells may be lacking, or murine counterparts of these factors may function poorly (Tomko *et al.*, 1997), resulting in a partial block in the viral replication cycle. No CPE was observed in 3T3-DAF cells following infection with EV70. This may be due to the poor replication of virus in these cells, however, growth of EV70 in the absence of CPE has also been observed in murine (L), hamster (BHK21), and porcine (IB-RS-2; ESK) cell lines (Yoshii *et al.*, 1977). Adaptation of EV70 to 3T3 cells by repeated passage may also result in more productive infection.

The original hypothesis of this thesis, that the host range and tropism of EV70 are dictated by the unique nature and distribution of its host cell receptor is not supported by the evidence presented here and by others. DAF expression *in vivo* is widespread (Lublin and

Atkinson, 1989), and human enteroviruses that do not share the replication characteristics of EV70 also bind to DAF (Bergelson *et al.*, 1994, 1995; Shafren *et al.*, 1995, 1997b). It was reasoned that examination of how EV70 interacts with DAF may reveal differences between EV70 and the other DAF-binding human enteroviruses. It was further reasoned that these differences may eventually lead to the identification of receptor-host cell interactions and post-binding events that influence EV70 tropism and host range.

III. Mapping the EV70 binding site of DAF

The EV70 binding sites of DAF were initially examined by antibody blockade. However, mapping virus binding sites using this method alone has certain limitations. The large size of antibodies relative to the DAF molecule, and the absence of information regarding both the sequences of the epitopes recognized by the antibodies and the precise location of these epitopes in the folded DAF molecule make interpretation of these data difficult. Antibody blockade experiments implicated SCRs 1, 2, and 3 in EV70 binding (Table 12). Other researchers have noted differences in mapping results when antibody blockade and the virus-binding properties of mutant DAF proteins were compared (Table 12). Both deletion mutants and DAF/MCP chimeras were available to more precisely determine the EV70 binding sites of DAF (Bergelson *et al.*, 1994, 1995; Clarkson *et al.*, 1995; Manchester *et al.*, 1995). A concern with interpreting results of binding assays using deletion mutants is that function may be lost as a result of conformational changes to the molecule, rather than the removal of regions critical for binding (Coyne *et al.*, 1992; Friestadt and Racaniello, 1991; Koike *et al.*, 1991a; Nowicki *et al.*, 1993; Selinka *et al.*, 1991; Staunton *et al.*, 1990). For

Table 12. Picornavirus-binding domains of DAF

Virus	SCR[a]				Reference
CVA21	1	2	3	4	Shafren *et al.* (1997)
CVB3	1	2*	3	4	Bergelson *et al.* (1995)
CVB5	1	2	3	4	Shafren *et al.* (1994)
ECHO7	1	2*	3*	4*	Bergelson *et al.* (1994)
EV70	1*	2	3	4	

[a] Shading indicates binding domains identified by antibody blockade; asterisks denote binding domains identified by expression of SCR deletion mutants or DAF/MCP chimeras.

this reason, the more conservative approach, analysis of the binding properties of DAF/MCP chimeras, was chosen. MCP is the most closely related RCA family member to DAF (Adams *et al.*, 1991; Coyne *et al.*, 1992; Lublin *et al.*, 1988). Both proteins have complementary functions in regulating the activity of C3 convertases, and are structurally similar, with four SCR domains and a serine/threonine rich, O-glycosylated domain proximal to the cell membrane (Lublin *et al.* 1988). The similarity of these molecules would, in theory, minimize any overt conformational changes to the receptor molecule, while allowing entire domains to be replaced.

DAF/MCP chimeras were transiently expressed in 3T3 cells, and the surface expression of each molecule was verified using DAF SCR-specific mAbs. Virus binding assays revealed that the first SCR of DAF contains sequences that are essential for EV70 binding. This result is consistent with observations that the amino-terminal extracellular domain of PVR (Koike *et al.*, 1991a; Selinka *et al.*, 1992) and ICAM-1 (McClelland *et al.*, 1991; Staunton *et al.*, 1990), are essential for PV and major group HRV virus binding, respectively. The requirement of SCR1 for EV70 binding is also consistent with the canyon hypothesis of virus binding (Rossman *et al.*, 1985; Rossman 1989) which proposes that residues within the most distal domain of the receptor molecule interact with residues located on the floor of canyon-like depressions formed by the capsid proteins of certain picornaviruses. Replacement of the second SCR of DAF (DM2) had an intermediate effect on EV70 binding. This might indicate that optimal binding of EV70 to DAF involves the interaction of sequences on the viral capsid with residues present in SCR2 (Friestadt and Racaniello, 1991), or, alternatively, that DAF SCR2 ensures optimal presentation of SCR1

for binding (Bernhardt *et al.*, 1994, Racaniello 1996; Staunton *et al.*, 1990). It is also possible that the N-glycosylation of SCR2 in the chimera DM2 (Clarkson *et al.*, 1995; Lublin *et al.*, 1988) interferes with the optimal interaction of SCR1 sequences with EV70. Virus binding results also indicate that sequences present in SCR3 (DM3) or SCR4 (DM4) of DAF are not specifically required for virus binding. Although DM4 expressing cells displayed a slightly reduced ability to bind virus, these data together with the inability of SCR4-specific mAb 8D11 to block EV70 attachment and infection indicate that this region of DAF is not specifically involved in virus binding. Similarly, sequences in the serine/threonine rich domain and in the GPI anchor region do not appear to be specifically required for virus binding. As seen with chimera DM4, TM-DAF expressing cells displayed slightly reduced EV70 binding activity. The decreased virus binding observed with cells expressing chimeras with carboxy terminal modifications (DM4, TM-DAF), may indicate that DAF molecules with changes in this region are not optimally positioned for virus binding (Bernhardt *et al.*, 1994).

Before discussing the implications of the observations that (1) EV70 is a receptor for EV70, and (2) that the EV70 binding site is located within SCR1, it is important to discuss how the original view of virus-receptor interactions has evolved since this project began.

IV. Virus receptors and co-receptors

Within the last six years, the sucessful application of immunologic and molecular techniques has led to the identification of several new virus receptors (Haywood, 1994; Norkin, 1995). These discoveries provide a new perspective on the virus-receptor relationship. The original hypothesis, that virus attachment and subsequent delivery of viral

genomes into the cell involves a simple interaction between the virus and a receptor molecule on the surface of a target cell, has been superceded by a model which for many viruses, can involve receptors, co-receptors, and multi-step binding processes for infection to take place (Haywood, 1994; Norkin, 1995).

Outside of the family *Picornaviridae*, there are several examples that illustrate the complexity of virus-receptor interactions, including herpes simplex virus (Montgomery *et al.*, 1996) and measles virus (Buckland and Wild, 1997), but the best characterized of these are HIV and Adenovirus.

A. HIV

The long-standing observation that CD4 supports viral entry only when expressed on human cells (Maddon *et al.*, 1986) led to speculation that the expression of a human cell-specific co-factor was required for infection. In 1996, Berger and colleagues (Feng *et al.*, 1996) identified a transmembrane protein belonging to the chemokine receptor family as the first HIV-1 co-receptor. Fusin (now CXCR4), in conjunction with CD4, mediates infection of T-cell tropic laboratory strains of HIV-1, but not primary isolates of macrophage-tropic (M-tropic) HIVs (Alkhatib *et al.*, 1996). Subsequently, CCR5, a member of a second chemokine receptor family, was shown to be a major co-receptor for M-tropic primary HIV isolates (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996, Dragic *et al.*, 1996). The precise role of these and other (Doranz *et al.*, 1996; Moser, 1997) chemokine receptors in determining the tropism of HIV-1, and their participation in post-entry events is being investigated (Cheng-Mayer *et al.*, 1997; Moser, 1997; Rucker *et al.*, 1996).

Genetic analyses have shown that regions of the viral envelope gp120 glycoprotein contain major determinants of the cell tropism of HIV. HIV binding and entry is now believed to be a multi-step process, in which the viral envelope glycoprotein complex gp120-gp41 binds to CD4, leading to conformational changes in the oligomeric complex of CD4-gp120-gp41 necessary for a high affinity interaction between regions of gp120 and the co-receptor (Lapham *et al.*, 1996; Rucker *et al.*, 1996; Trkola *et al.*, 1996; Wu *et al.*, 1996). This co-receptor / viral glycoprotein interaction then leads to conformational changes in the virus fusion pepide, resulting in the fusion of cell and viral membranes, and the introduction of HIV nucleoprotein into the cell.

B. Adenovirus

Studies of human adenovirus serotypes 2 (Ad2) and 5 (Ad5) revealed that entry of these non-enveloped DNA viruses into susceptible cells is also a step-wise process, consisting of two experimentally dissociable events: initial attachment to the surface of cells, and internalization (Shenk, 1996; Wickham *et al.*, 1993). The Ad capsid is an icosahedron with an extended fibre molecule projecting from each of its 12 vertices. Attachment to cells is mediated by the distal portion of the fibre protein, referred to as the knob or head (Philipson *et al.*, 1968; Defer *et al.*, 1990). Following attachment, virus is internalized by receptor-mediated endocytosis. The observation that Ad binds to but does not enter some cells (Silver and Anderson, 1988) suggested that a host cell-specific factor was necessary for entry. The amino terminus of the fibre protein is attached to the capsid via a pentamer of coat protein referred to as the penton base. Each subunit of this structure contains an RGD tripeptide

motif, and Wickham and colleagues (1993) showed that the interaction of the penton base with cell surface integrins $\alpha_v\beta_3$ / $\alpha_v\beta_5$ mediates Ad internalization.

The attachment receptor for Ad has only recently been identified. Virus binding competition assays originally showed that Ad2 and Ad5 share a receptor with CVB (Lonberg-Holm, *et al.*, 1976). Using the CVB-blocking antibody RmcB described by Hsu *et al.* (1988,1990) two groups isolated and partially characterized a 46 kDa transmembrane glycoprotein referred to as HCAR (human coxsackievirus and adenovirus receptor). Expression of HCAR mediates Ad binding and enhances virus entry (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). A model is proposed whereby Ad attaches to cells via HCAR, which through an unknown mechanism, facilitates entry in conjunction with secondary interactions with integrins expressed on the cell surface (Bergelson *et al.*, 1997). Northern blot analysis revealed that only low levels of HCAR mRNA were detected in liver and lung, and none was found in kidney, placenta or skeletal muscle, which is inconsistent with the common sites of Ad2 and Ad5 infection (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). This suggests that HCAR is not the main fibre-binding receptor for these viruses, but that in different cells, different proteins may fulfill the same function.

V. Co-receptors for picornaviruses

A list of the picornavirus receptors identified as of this writing is presented in Table 13. As previously recognized, these molecules are integral membrane glycoproteins. What is newly apparent is that molecules from a variety of protein families can serve as receptors for picornaviruses: the immunoglobulin gene superfamily (PVR; ICAM-1), the integrin family

Table 13. Picornavirus receptors identified to date (1997)

Virus	Receptor	Reference
Poliovirus	PVR	Mendelsohn, *et al.*, 1989
	CD44	Shepley and Racaniello, 1994
Human Rhinovirus (major group)	ICAM-1	Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989
Human Rhinovirus (minor group)	LDL-R; α_2 MR/RLP	Hofer *et al.*, 1994
Encephalomyocarditis virus	Integrin VCAM-1	Huber, 1994
	70 kDa sialoglycoprotein	Jin *et al.*, 1996
Foot-and-mouth disease virus	Integrin $\alpha_v\beta_3$	Mason *et al.*, 1994; Berinstein *et al.*, 1995; Liepert *et al.*, 1997
	F_cR	Mason *et al.*, 1993
	Heparan sulphate	Jackson *et al.*, 1996
Echovirus 1,8	Integrin VLA-2	Bergelson *et al.*, 1992, 1993
Echovirus 3,6,7,11,12,20,21	DAF	Bergelson *et al.*, 1994; Ward *et al.*, 1994
Coxsackievirus A9	Integrin $\alpha_v\beta_3$	Roivainen *et al.*, 1994
Coxsackievirus A21	ICAM-1	Colonno *et al.*, 1986; Lonberg-Holm *et al.*, 1976
	DAF	Shafren *et al.*, 1997a
	HCAR	Shafren *et al.*, 1997b
Coxsackievirus B3-RD; B1,3,5	DAF	Bergelson *et al.*, 1995
		Shafren *et al.*, 1995
Coxsackievirus B1-6	HCAR	Mapoles *et al.*, 1985
		Bergelson *et al.*, 1997; Tomko *et al.*, 1997
	100 kDa nucleolin-like	Raab deVerdugo *et al.*, 1995
Hepatitis A	70 kDa mucin-like	Kaplan *et al.*, 1996

(VCAM-1, VLA-2, $\alpha_v\beta_3$), as well as low density lipoprotein receptor (LDLR)-related glycoproteins, and complement regulatory proteins (DAF). Observations that in many cases, receptor expression alone does not correlate with infection of cells has led to the search for potential co-receptors / co-factors / accessory factors for these viruses. In recent months, two picornavirus co-receptors have been identified.

A. CVB

The binding of CVB3 to HeLa cells has long been thought to involve interaction with a receptor complex consisting of at least two components (Hsu *et al.*, 1990). MAb RmcA, directed against a 70 kDa HeLa cell glycoprotein, protected cells against infection with odd-numbered CVB, as well as by Echovirus 6 (Crowell *et al.*, 1986). Bergelson (Bergelson *et al.*, 1995) and Shafren (Shafren *et al.*, 1995), demonstrated that this protein was DAF, but while CVB1, B3, and B5 bound to DAF, murine cells engineered to express DAF were resistant to infection. An additional HeLa cell receptor for CVBs was identified using mAb RmcB, which protected HeLa cells against infection by all six CVB serotypes, as well as by Ad 2 and Ad5 (Hsu *et al.*, 1988). The 46 kDa protein recognized by RmcB is HCAR (Bergelson *et al.*, 1997; Tomko *et al.*, 1997), and when cDNA encoding HCAR was introduced into CHO or 3T3 cells, these transfectants bound and supported replication of three CVB serotypes (CVB2, 3, and 4). In contrast with Ad2 and Ad5, the tissue distribution of HCAR mRNA correlated well with the tropism of CVBs (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). These results suggest that HCAR is the major CVB receptor. Of additional interest were the observations that several DAF- binding strains of CVB3 and CVB5 were able to infect CAR-expressing CHO cells (Bergelson *et al.*, 1997), and that infection of

human RD cells by a DAF-binding CVB3 required interaction with HCAR (Shafren, personal communication). These findings suggest that in some strains, DAF acts in initial virus attachment, but that HCAR is required for internalization and uncoating (Bergelson *et al.*, 1997).

Crystallographic analysis of CVB3 supports the proposition that there may be two distinct receptor-binding regions on the virus capsid. Study of the surface topology of CVB3 (Muckelbauer *et al.*, 1995) reveals the presence of a canyon structure surrounding the vertices of the icosahedron, which is proposed to serve as the binding site for the major receptor (CAR). A second , shallow depression, lined with residues from VP2 and VP3 may be the secondary receptor (DAF) -binding site .

B. CVA21

Early competition experiments indicated that CVA21 and the major group human rhinoviruses competed for a common receptor (Colonno *et al.*, 1986; Lonberg-Holm *et al.*, 1976). Shafren *et al.* (1997a) have recently shown that ICAM-1 expression in normally CVA21-resistant murine L cells rendered these cells susceptible to infection. CVA21 binding to ICAM-1 was also shown to induce the formation of conformationally-altered A particles, thought to be a requirement for human enterovirus infection (Rueckert, 1996). Subsequently, Shafren showed that CVA21 also binds to DAF, but that this interaction is not sufficient to infect cells. Thus, it has been proposed that as with CVBs, DAF acts as an attachment protein for CVA21, serving to sequester virus for optimal presentation to its primary receptor. It has also been proposed that since CVA21 binding to ICAM-1 leads to the formation of A particles, that ICAM-1 may bind to residues in the floor of a major canyon

structure, while DAF may bind (through SCR1) to a second minor capsid depression, as described for CVB3 (Shafren *et al.*, 1997b).

C. HRV

While ICAM-1 is the primary receptor for HRV, ICAM-1 expression is not a guarantee of infection in monkey (Greve *et al.*, 1990) or rodent (Shafren *et al.*, 1997a) cells. Furthermore, *in vivo* expression of ICAM-1 includes many cells and tissues that are not known to support RV replication (Dustin *et al.*, 1996), indicating that ICAM-1 is not the determinant of HRV tropism (Greve *et al.*, 1989). The identity of the additional cellular factor(s) specifying susceptibility to the major group HRV is still not known.

D. PV

Similar to the situation with HRV, the susceptibility of tissues to PV infection does not appear to be determined merely by the presence of PVR. Analysis of PVR mRNA distribution and protein expression reveal that PVR is expressed in many tissues that are not considered to be replication sites for PV (Friestadt *et al.*, 1990; Mendelsohn *et al.*, 1989; Koike *et al.*, 1991b). The PV blocking mAb produced by Shepley *et al.* (1988) recognized a 100 kDa glycoprotein found only in cells and tissues permissive for PV, and whose expression *in vivo* precisely corresponded with PV tropism. This protein, subsequently identified as the lymphocyte homing receptor CD44, does not bind PV (Shepley and Racaniello, 1994), and was proposed to be required, in conjunction with PVR, for entry of PV into susceptible cells (Shepley and Racaniello, 1994). Recent work (Racaniello, 1996) suggests that cells expressing PVR in the absence of CD44 permit normal replication of virus in cell culture, implying that something other than CD44 is a PVR cofactor.

E. Echoviruses

Expression of the integrin VLA-2 is sufficient to render rodent cells permissive to infection by Echoviruses 1 and 8 (Bergelson *et al.*, 1992; 1993). In contrast, expression of DAF is not sufficient for rodent cell infection by the echoviruses shown to bind DAF (Bergelson *et al.*, 1994; Ward *et al.*, 1994). No accessory factors for these viruses have yet been identified.

VI. Multiple receptor use by picornaviruses

Further complicating the search for definitive receptors (or co-receptors), is the fact that some viruses, including picornaviruses, may use different molecules to infect different cell types (Bergelson *et al.*, 1997; Harouse *et al.*, 1991; Haywood, 1994; Hong *et al.*, 1997; Mason *et al.*, 1993). For example, CVA9 binds to cells via RGD motifs to the integrin $\alpha_v\beta_3$ (Roivainen *et al.*, 1991, 1994), however, when the RGD motif is cleaved from the virion surface, CVA9 is still able to to infect certain cell lines (Roivainen *et al.*, 1996). The ability of CVA9 to use this unidentified alternative receptor(s) is dependent on the target cell line and the phenotype of the virus (+/- RGD, Roivainen *et al.*, 1996). The minor group HRV use the low density lipoprotein receptor (LDLR) for binding and entry of cells, however, in LDLR-deficient fibroblasts, are also able to use α_2 macroglobulin receptor (Hofer *et al.*, 1994). Also, type A FMDV binds and enters via interaction with the integrin $\alpha v\beta 3$, and the RGD motif was found to be essential for infectivity (Baxt and Becker, 1990; Berinstein *et al.*, 1995). Several receptor molecules have been identified for EMCV. Glycophorin A was identified as a receptor for EMCV on erythrocytes (Allaway and Burness, 1986), but these

cells do not support virus replication. The D strain of EMCV (D-EMCV) binds to the integrin VCAM-1 on cardiac vascular endothelial cells, and this interaction leads to infection (Huber, 1994). The EMCV receptor on other nucleated cells is thought to be a 70 kDa sialoglycoprotein (Jin *et al.*, 1994). Finally, in addition to DAF and HCAR, a 100 kDa nucleolin-like protein has been identified in cell lines permissive for all CVB serotypes (Raab de Verdugo *et al.*, 1995). The role of this protein in CVB infection iis not known.

VII. EV70 and DAF

In view of this information, several models for EV70 binding and infection may be considered.

A. DAF as the primary receptor for EV70

It is important to acknowledge that the experiments described above identify regions of DAF involved in virus binding, and do not address the question of whether or not DAF expression is sufficient and / or necessary for EV70 infection. The poor replication of EV70 in 3T3-DAF cells does not preclude DAF from serving as a primary receptor for EV70. ICAM-1 has unequivocally been identified as the receptor for major group HRV. However, Greve *et al.* (1990) observed that HRV displays a serotype-dependent ability to infect Vero cells engineered to express ICAM-1. While some serotypes readily infected these cells and yielded high titres of progeny virus, others replicated poorly, and some did not replicate at all. Therefore, it was concluded that for different HRV serotypes, different host-cell factors may be required. To determine the role of DAF in EV70 infection, it would be important to identify a cell line that does not naturally bind EV70 but that is able to support EV70

replication. Once such a cell line is found, the constitutive expression of DAF and various DAF/MCP chimeras would be required to determine if binding to DAF is sufficient to effect entry, and to determine the minimal DAF domain requirements for infection.

Early events in virus infection involve alterations to the virion, as well as changes to the target cell. The early post-binding events of the picornavirus replication cycle are poorly understood (Ansardi *et al.*, 1996; Rueckert, 1996). Studies of enterovirus-receptor binding have shown that this interaction leads to the formation of A particles, and that the production of these conformationally altered virions is a prerequisite for cell infection (Rueckert, 1996).

The model of A particle formation is based primarily on studies of PV and of the major group HRV (Casanovas and Springer, 1994; Crowell and Philipson, 1971; Fricks and Hogle, 1990; Hoover-Litty and Greve, 1993). It is proposed that receptor binding induces conformational changes in the viral capsid, resulting in the loss of VP4, and the externalization of parts of VP1 which normally reside on the interior of the capsid. Exposure of the hydrophobic extensions of VP1 permit the virus to insert into the lipid bilayer of the cell, and form a pore through which viral RNA passes into the cytoplasm.

The cellular location of picornavirus uncoating is unresolved. Some evidence suggests a role for receptor-mediated endocytosis, and that uncoating takes place within the acidified endosome (FMDV, Carrillo *et al.*, 1984; Mason *et al.*, 1993, PV, Madshus *et al.*, 1984). Others have shown that uncoating of PV and HRV occurs at neutral pH, and propose that following receptor binding, viral RNA is introduced into cells at the plasma membrane (PV, Fricks and Hogle, 1990, Gromeier and Wetz, 1990; HRV, Casanovas and Springer, 1994; Greve *et al.*, 1991). Thus a uniform mechanism for the uncoating of all picornaviruses

appears unlikely. Rueckert (1996) suggests that the site of uncoating may be determined by the uncoating sensitivity of the virion. If virus-receptor complexing initiates uncoating at neutral pH, uncoating may occur at the plasma membrane, but if an additional stimulus (low pH) is required, then uncoating would be delayed until the virion is transported into the endosome. It has not been determined if EV70 undergoes conformational alteration following binding to DAF.

DAF, like some other molecules used as virus receptors or co-receptors (chemokine receptors, Atchison *et al.*, 1997; ICAM-1, Holland and Owens, 1997; integrins, Hynes 1992) has been shown to transmit signals across cell membranes (Parolini *et al.*, 1996; Shenoy-Scaria *et al.*, 1992). Crosslinking of DAF with SCR3-specific mAb results in the phosphorylation of several cytoplasmic proteins in T cells (Shenoy-Scaria *et al.*, 1992), and the GPI anchor of DAF has been shown to interact with src family protein tyrosine kinases (Parolini *et al.*, 1996; Shenoy-Scaria *et al.*, 1992). In addition, there is controversial evidence suggesting that GPI anchored proteins concentrate in specific microdomains in the plasma membrane known as caveolae, and that these structures may play a role in signal transduction pathways (Parolini *et al.*, 1996; Mayor *et al.*, 1994; Parton, 1996; Schnitzer *et al.*, 1995), and perhaps in endocytic processes as well (Parton, 1996). This also raised the possibiltity that EV70 binding might trigger a signal through DAF, which may participate in virus entry.

Receptor ligation by the virus may trigger signals through the receptor that are advantageous for the virus interaction with the host (Staunton *et al.*, 1992). SV40 binding to cells has been shown to activate signal transduction events that promote virus entry

(Dangoria *et al.*, 1997), while Echovirus 1 binding to its cellular receptor is not sufficient to trigger signalling pathways (Huttunen *et al.*, 1997).

Can EV70 binding to DAF initiate signalling? The significance of the observation that EV70 binds to DAF SCR1 is not clear. One might assume that since SCR3 (together with the GPI anchor) is involved in intracellular signalling (Shenoy-Scaria *et al.*, 1992), the interaction of EV70 with DAF may not lead to DAF-mediated signal transduction events that might influence EV70 uptake and infection of specific cell types. However, the possibility that EV70 binding to DAF can initiate signal transduction has not been assessed. It is also possible that virus binding does not directly trigger signalling, but that this interaction might alter the conformation of DAF, permitting it to interact with other cell surface molecules which trigger signalling.

B. EV70 infection requires DAF and a co-receptor

As with the DAF-binding echoviruses and coxsackieviruses, successful infection by EV70 may require the concomitant expression of DAF and additional cell surface molecule(s).

(i) **HCAR**: Virus competition binding experiments suggested that there may be a relationship between the receptor or receptor complexes of CVB3 and EV70. However, mAb RmcB, which is now known to be directed against HCAR, failed to protect HeLa cells against EV70 infection. These cell protection experiments have subsequently been repeated, and confirm earlier results (K. Dimock, personal communication). Several other observations suggest that HCAR is not a co-receptor for EV70. The tissue distribution of HCAR does not correspond with known sites of EV70 replication (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). Furthermore, murine L cells do not express CAR, and are refractory to CVB infection

(Tomko *et al.*, 1997), while this cell line is known to bind EV70 and support its replication (Yoshii *et al.*, 1977). Experiments in which HCAR alone, and HCAR plus DAF are expressed in 3T3 cells could resolve whether or not HCAR is required for EV70 infection.

(ii) **ICAM-1**: Both CVA21 and EV70 bind to the first SCR domain of DAF. This might indicate that these viruses share similar mechanisms for interacting with multiple receptor molecules. However, several lines of evidence indicate that ICAM-1 is not a receptor or co-receptor for EV70. ICAM-1 is not expressed in the normal conjunctiva (Vorkauf *et al.*, 1993), and therefore, would not be expected to play a role in the tropism of EV70. In addition, murine L cells and hamster BHK cells, which do not express ICAM-1 (Shafren *et al.*, 1997a), are both able to bind sigificant amounts of EV70, and support EV70 replication (Yoshii *et al.*, 1977).

VIII. EV70 host range and DAF

The determinants of EV70 host range are not known. The ability of EV70 to bind to and replicate in a variety of non-primate-derived cell lines raises the possibility that if DAF is the primary receptor for EV70, DAF homologs may be used in the cells of other species.

A number of picornavirus receptor homologs are known. A murine poliovirus receptor has been identified, but is not able to bind PV, even strains adapted to grow in mice (Morrison and Racaniello, 1992). Similarly, a rat PVR homolog is not able to function as a receptor for PV (Aoki *et al.*, 1994). In contrast, African green monkey, cynomolgus monkey and tamarin PVR homologs have been identified, and are able to bind PV (Koike *et al.*, 1992; Aoki *et al.*, 1994). Residues in the first domain of PVR that are critical for virus binding have

been identified, and it is the conservation of these key amino acids that allows certain homologs to retain PV binding activity (Aoki *et al.*, 1994). A murine homolog of ICAM-1 has been identified, and does not bind HRV (McClelland *et al.*, 1991). Recently, an avian homolog of the minor group HRV receptor has been identified in chicken oocytes (Hofer *et al.*, 1994). While this molecule is able to bind virus, binding does not lead to infection of chicken cells. A murine homolog (MCAR) of the newly described HCAR has been described, and it is a functional receptor for CVB3 and CVB4 (Tomko *et al.*, 1997).

Several homologs of DAF have been identified, and show a range of homologies to human DAF (overall identity at the amino acid level shown in parentheses): guinea pig, (58%; Nonaka *et al.*, 1995) ; orangutan, (95%; Nickells *et al.*, 1994); rhesus monkey, (95%; D. Lublin, personal communication); and mouse, (47%; Fukuoka *et al.*, 1996; Spicer *et al.*, 1995). DAF/MCP chimera expression studies localized EV70 binding to SCR1 of DAF, and further analyses will be required to determine the precise sequence requirements for binding. However, the primary cellular function of DAF is the regulation of complement activation, and this activity has been mapped to SCR 3, with a requirement for SCRs 2 and 4 (Coyne *et al.*, 1992). This would suggest that if complement regulating function is preserved in DAF homologs, sequence conservation will be greatest among these domains. This is supported by the data of Spicer *et al.*, (1995) who compared the sequences of each SCR domain of murine and human DAF, and found the lowest homology between SCR1 (44%) (compared to 57% for SCR2, 56% for SCR3 , and 59% for SCR4). Some clones of these homologs are available, and could be tested for EV70 receptor function. In addition, more detailed mapping of the EV70 binding site of human DAF may be useful to determine which amino

acids are critical for binding, and whether or not these are conserved in DAF homologs. Again, it is not known if DAF by itself can function as a receptor for EV70 in non-human cells, or if additional cell surface or cytoplasmic proteins are required for EV70 infection.

IX. Concluding remarks

Recent developments in the field of virus receptor research have led to tremendous change in the way virus-receptor interactions are viewed. A situation in which a virus particle attaches to a single specific cell surface molecule and subsequently enters and infects the cell is likely to be exceptional. For many viruses, interaction with the host cell receptor or receptors can be complex, involving multiple distinct components of the virus and on the cell.

The data presented in this thesis clearly demonstrate that EV70 uses DAF as an attachment protein on HeLa cells. However, several observations argue against DAF being the determinant of host range and tropism of this virus. Most significantly, DAF has an extremely wide distribution *in vivo*. While DAF is expressed in tissues involved in the pathogenesis and clinical manifestations of EV70 infection, it is also expressed on many cells and in many tissues that are not recognized as sites of EV70 replication. Furthermore, several human enteroviruses that share neither the restricted *in vivo* tropism nor the extended *in vitro* host range of EV70, also bind to DAF on the surface of cells.

EV70 has clearly adapted to the environmental conditions of the eye: it has a relatively short incubation period, and replicates preferentially at 33 to 35°C. It seems logical that in this fluid-bathed environment that EV70 would also adapt to bind to an abundantly expressed

molecule on conjunctival epithelium cells. The work presented here has not conclusively resolved whether or not DAF expression is necessary or sufficient to initiate EV70 infection of cells. To address this question, additional experiments will have to be performed. Given our results and the current state of knowledge regarding virus receptors, it is plausible that DAF serves as an initial attachment molecule, tethering EV70 to the cell in order to present the virus to one or several other cell surface molecules which ultimately permit virus entry and infection of the conjunctival epithelium.

Thus, at the completion of this thesis, the precise role of DAF in EV70 infection remains to be elucidated. Whether or not it is the concomitant expression of DAF and specific cell surface co-factors that determines EV70 host range and tropism, or if post-entry events, perhaps triggered by virus binding, regulate the specificity of EV70 infection, awaits further investigation.

REFERENCES

Abraham, G., and R.J. Colonno. 1984. Many rhinovirus serotypes share the same cellular receptor. J. Virol. **51:**340-345.

Acharya, R., E. Fry, B. Stuart, G. Fox, D. Rowlands, and F. Brown. 1989. The three-dimensional structure of foot-and -mouth disease virus at 2.9Å resolution. Nature **337:**709-715.

Adams, E.M., M.C. Brown, M. Nunge, M. Krych, and J.P. Atkinson. 1991. Contribution of the repeating domains of membrane cofactor protein (CD46) of the complement system to ligand binding and cofactor activity. J. Immunol. **147:**3005-3011.

Alexander, H.E., G. Koch, I. Mountain, K. Sprunt, and O. Van Damme. 1958. Infectivity of ribonucleic acid of poliovirus on HeLa cell monolayers. Virology **5:** 172-173.

Alkhatib, G., C. Combadiere, C.C. Broder, Y. Feng, P.E. Kennedy, P.M. Murphy, and E.A. Berger. 1996. CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1855-1958.

Allaway, G.P., and A.T.H. Burness. 1986. Site of attachment of encephalomyocarditis virus on human erythrocytes. J. Virol. **59:**768- 770.

Ambros, V., and D. Baltimore. 1978. Protein is linked to the 5' end of poliovirus RNA by a phosphodiester linkage to tyrosine. J. Biol. Chem. **253**:5263-5266.

Angel, M.A., and A.T.H. Burness. 1977. The attachment of encephalomyocarditis virus to erythrocytes from several animal species. Virology **83:** 428-432.

Ansardi, D.C., D.C. Porter, M.J. Anderson, and C.D. Morrow. 1996. Poliovirus assembly and encapsidation of genomic RNA. Adv. Vir. Res. **48:**1-68.

Aoki, J., S. Koike, I. Ise, Y. Sato-Yoshida, and A. Nomoto. 1994. Amino acid residues on human poliovirus receptor involved in interaction with poliovirus. J. Biol. Chem. **269:**8431-8438.

Arthos, J., C.K. Deen, M.A. Chailkin, A. Fornwald, Q.J. Sattentau, P.R. Clapham, R.A. Weiss, J.S. McDougal, C. Pietropaolo, P.J. Maddon, A. Truneh, R. Axel, and R.W. Sweet. 1989. Identification of the rsidues in human CD4 critical for the binding of HIV. Cell **57:**469-481.

Asbell, P.A., W. de la Pena, D. Harms, M. Hatch, and H.E. Kaufman. 1985. Acute hemorrhagic conjunctivitis in Central America: first enterovirus epidemic in the Western hemisphere. Ann. Ophthal. 17:205-210.

Atchison, R.W., J. Gosling, F.S. Monteclaro, C. Franci, L. Digilio, I.F. Charo, and M.A. Goldsmith. 1996. Multiple extracellular elements of CCR5 and HIV-1 entry: dissociation from response to chemokines. Science 274:1924-1926.

Axler, D.A., and R.L. Crowell. 1968. Effect of anticellular serum on the attachment of enteroviruses to HeLa cells. J. Virol. 2:813-821.

Babalola, O.E., S.S. Amoni, E. Samaila, U. Thaker, and S. Darougar. 1990. An outbreak of acute haemorrhagic conjunctivitis in Kaduna, Nigeria. Br. J. Ophthalmol. 74:89-92.

Badger, J., I. Minor, M.J. Kremer, M.A. Oliveira, T.J. Smith, J..P. Griffith, D.M.A. Guerin, S. Krishnaswamy, M. Luo, M.G. Rossmann, M.A. McKinlay, G.D. Diana, F.J. Dutko, M. Fancher, R.R. Rueckert, and B.A. Heinz. 1988. Structural analysis of a series of antiviral agents complexed with human rhinovirus 14. Proc. Natl. Acad. Sci. USA 85:3304-3308.

Baltimore, D. 1968. Structure of the poliovirus replicative intermediate RNA. J. Mol. Biol. 32:359-368.

Bass, D.M.,and H.B. Greenberg. 1992. Strategies for the identification of icosahedral virus receptors. J. Clin. Invest. 89:3-9.

Baxt, B., and Y. Becker. 1990. The effect of peptides containing the arginine-glycine-aspartic acid sequence on the adsorption of foot and mouth disease virus to tissue culture cells. Virus Genes 4:73-83.

Bergelson, J.M., M. Chan, K.R. Solomon, N.F. St. John, H. Lin, and R.W. Finberg. 1994. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc. Natl. Acad. Sci. USA 91:6245-6248.

Bergelson, J.M., J.A. Cunningham, G. Droguett, E.A. Kurt-Jones, A. Krithivas, J.S. Hong, M.S. Horwitz, R.L. Crowell, and R.W. Finberg. 1997. Isolation of a common receptor for Coxsackie B viruses and Adenoviruses 2 and 5. Science. 275:1320-1323.

Bergelson, J.M., and R.W. Finberg. 1993. Integrins as receptors for virus attachment and cell entry. TIM 1:287-288.

Bergelson, J.M., J.G. Mohanty, R.L. Crowell, N.F. St. John, D.M. Lublin, and R.W. Finberg. 1995. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). J. Virol. **69:**1903-1906.

Bergelson, J.M., N. St. John, S. Kawaguchi, M. Chan, H. Stubal, J. Modlin, and R.W. Finberg. 1993. Infection by echovirus 1 and 8 depends on the α_2 subunit of human VLA-2. J. Virol. **67:**6847-6852.

Bergelson, J.M., M.P. Shepley, B.M.C. Chan, M.E. Hemler, and R.W. Finberg. 1992. Identification of the integrin VLA-2 as a receptor for echovirus 1. Science **255:**1718-1720.

Berinstein, A., M. Roivainen, T. Hovi, P.W. Mason, and B. Baxt. 1995. Antibodies to the vitronectin receptor (integrin $\alpha_v\beta_3$) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J. Virol. **69:**2664-2666.

Bern, C., M.A. Pallansch, H.E. Gary, Jr., J.P. Alexander, T.J. Torok, R.I. Glass, and L.J. Anderson. 1992. Acute hemorrhagic conjunctivitis due to enterovirus 70 in American Samoa: serum-neutralizing antibodies and sex-specific protection. Am. J. Epidemiol. **136:**1502-1506.

Bernet-Camard, M.-F., M.-H. Coconnier, S. Hudault, and A.L. Servin. 1996. Pathogenecity of the diffusely adhering strain *Escherichia coli* C1845: F1845 adhesin-decay accelerating factor interaction, brush border microvillus injury, and actin disassembly in cultured human intestinal epithelial cells. Infect. Immun. **64:**1918-1928.

Bernhardt, G., J. Harber, A. Zibert, M. deCrombrugghe, and E. Wimmer. 1994. The poliovirus receptor: identification of domains and amino acid residues critical for virus binding. Virology **203:**344-356.

Bhat, S., S.L. Spitalnik, F. Gonzalez-Scarano, and D.H. Silberberg. 1991. Galactosyl ceramide or a derivative is an essential component of the neural receptor for human immunodeficiencey virus type 1 envelope glycoprotein gp120. Proc. Natl. Acad. Sci. USA **88:**7131-7134.

Bhide, V.S., S.R. Prasad, and S.S. Gogate. 1994. Isolation of a variant of enterovirus 70 from a patient during an epidemic of acute haemorrhagic conjunctivitis in Pune in 1991. Acta Virol. **38:**245-246.

Bienz, K., D. Egger, T. Pfister, and M. Troxler. 1992. Structural and functional characterization of poliovirus replication complex. J. Virol. **66:**2740-2747.

Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. **7:**1513-1523.

Borrow, P., and M.B.A. Oldstone. 1992. Characterization of lymphocytic choriomeningitis virus-binding proteins: a candidate cellular receptor for the virus. J. Virol. **66:**7270-7281.

Buckland, R., and T.F. Wild. 1997. Is CD46 the cellular receptor for measles virus? Virus Res. **48:1-9.**

Burness, A.T.H., and I.U. Pardoe. 1983. A sialoglycopeptide from human erythrocytes with receptor-like properties for encephalomyocarditis and influenza viruses. J. Gen. Virol. **64:**1137-1148.

Cambbell, B.A., and C.E. Cords. 1983. Monoclonal antibodies that inhibit attachment of group B coxsackieviruses. J. Virol. **48:**561-564.

Carrillo, E.C., C. Giachetti, and R.H. Campos. 1984. Effect of lysosomotropic agents on foot-and-mouth diseases virus replication. Virology **135:**542-545.

Casasnovas, J.M., and T.A. Springer. 1994. Pathway of rhinovirus disruption by soluble intercellular adhesion molecule 1 (ICAM-1): an intermediate in which ICAM-1 is bound and RNA is released. J. Virol. **68:**5882-5889.

Chapman, M.S., and M.G. Rossman. 1993. Comparison of surface properties of picornaviruses: strategies for hiding the receptor site from immune surveillance. Virology **195:**745-756.

Chatterjee, S., C.O. Quarcoopombe, and A. Apenteng. 1970. An epidemic of acute conjunctivitis in Ghana. Ghana Med. J. **9:**9-11.

Chen, C.-W. 1989. Acute hemorrhagic conjunctivitis due to enterovirus 70 in China (Taiwan). In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations.* Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger AG, Basel. pp. 161-166.

Chen, C.-W., W.-L. Huang, and Y.-F. Hsie. 1989. Clinical features of CA 24v acute hemorrhagic conjunctivitis. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations.* Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger AG, Basel. pp.225-234.

Cheng-Mayer, C., R. Liu, N.R. Landau, and L. Stamatatos. 1997. Macrophage tropism of human immunodeficiency virus type 1 and utilization of the CC-CKR5 corecptor. J. Virol. **71:**1657-1661.

Cherry, J.D. 1987. Enteroviruses: the forgotten viruses of the 80's. In *Medical Virology VII*. Edited by L.M. de la Maza, and E.M. Peterson. Elsevier Science Publishers B.V., Amsterdam. pp.1-32.

Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P.D. Ponath, L. We, C.R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135-1148.

Clarkson, N.A., R. Kaufman, D.M. Lublin, T. Ward, P.A. Pipkin, P.D. Minor, D.J. Evans, and J.W. Almond. 1995. Characterization of the echovirus 7 receptor: Domains of CD55 critical for virus binding. J. Virol. **69:**5497-5501.

Co, M.S., B.N. Fields, and M.I. Greene. 1986. Viral receptors serving host functions. In *Concepts in Viral Pathogenesis II*. Edited by A.L. Notkins, and M.B.A. Oldstone. Springer-Verlag, New York. pp. 126-131.

Colonno, R.J. 1987. Cell surface receptors for picornaviruses. BioEssays **5:**270-274.

Colonno, R.J., P.L. Callahan, and W.J. Long. 1986. Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinoviruses. J. Virol. **57:**7-12.

Colonno, R.J., J.H. Condra, S. Mizutani, P.L. Callahan, M.-E. Davies, and M.A. Murcko. 1988. Evidence for the direct involvement of the rhinovirus canyon in receptor binding. Proc. Natl. Acad. Sci. USA. **85:**5449-5453.

Colonno, R.J., R.L. LaFemina, C.M. DeWitt, and J.E. Tomassini. 1990. The major-group rhinoviruses utilize the intercellular adhesion molecule 1 ligand as a cellular receptor during infection. In *New Aspects of Positive-strand RNA Viruses*. Edited by M.A. Brinton, and F.X. Heinz. American Society for Microbiology, Washington. pp. 257-261.

Coyne, K.E., S.E. Hall, E.S. Thompson, M.A. Arce, T. Kinoshita, T. Fujita, D.J. Anstee, W. Rosse, and D.M. Lublin. 1992. Mapping of epitopes, glycosylation sites, and complement regulatory domains in human decay accelerating factor. J. Immunol. **149:** 2906-2913.

Crowell, R.L. 1966. Specific cell-surface alteration by enteroviruses as reflected by viral-attachment interference. J. Bacteriol. **912:**198-204.

Crowell, R.L., A.K. Field, W.A Schlief, W. Long, R.J. Colonno, J.E. Mapoles, and E.A. Emini. 1986. Monoclonal antibody that inhibits infection of HeLa cells and rhabdomyosarcoma cells by selected enteroviruses thriugh receptor blockade. J. Virol. **57:**438-445.

Crowell R.L., and K.-H.L.Hsu. 1986. Isolation of cellular receptors for viruses. 1986. In *Concepts in Viral Pathogenesis II*. Edited by A.L. Notkins, and M.B.A. Oldstone. Springer-Verlag, New York. pp. 117-125.

Crowell, R.L., and B.J. Landau. 1983. Receptors in the initiation of picornavirus infections. In *Comprehensive Virology*. Edited by H. Fraenkel-Conrat, and R.R. Wagner. Plenum Press, New York. pp. 1-42.

Crowell, R.L., and L. Philipson. 1971. Specific alterations of coxsackievirus B3 eluted from HeLa cells. J. Virol. **8**:509-515.

Dalgleish, A.G., P.C.L. Beverley, P.R. Clapham, D.H. Crawford, M.F. Greaves, and R.A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312**:763-767.

Dangoria, N.S., W.C. Breau, H.A. Anderson, D.M. Cishek, and L.C. Norkin. 1996. Extracellular simian virus 40 induces and ERK/MAP kinase-independent signalling pathway that activates primary response genes and promotes virus entry. J. Gen. Virol. **77**:2173-2182.

Darougar, S., M.A. Monnickendam, and R. M. Woodland. 1989. Management and prevention of ocular viral and chlamydial infections. CRC Crit. Rev. Microbiol. **16**:369-418.

Davitz, M.A., M.G. Low, and V. Nussenzweig. 1986. Release of decay-accelerating factor (DAF) from the the cell membrane by phosphatidylinositol-specific phospholipase C (PIPLC). Selective modification of a complement regulatory protein. J. Exp. Med. **163**:1150-1161.

Defer, C., M.-T. Belin, M.-L. Caillet-Boudin, and P. Boulanger. 1990. Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. J. Virol. **64**:3661-3673.

Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. DiMarzio, S. Marmon, R.E. Sutton, C.M. Hill, C.B. Davis, S.C. Peiper, T.J. Schall, D.R. Littman, and N.R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381**:661-666.

Diamond, M.S., D.E. Staunton, S.D. Marlin, and T.A. Springer. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third Ig-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell **65**:961-971.

Doranz, B.J., J. Rucker, Y. Yi, R.J. Smyth, M. Samson, S.C. Peiper, M. Parmentier, R.G. Collman, and R.W. Doms. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the β-chemokine receptors DKR-5, DKR-3, and CKR-2b as fusion cofactors. Cell **85**:1149-1158.

Dragic, T., V. Litwin, G.P. Allaway, S.R. Martin, Y. Huang, K.A. Nagashima, C. Cayanan, P.J. Maddon, R.A. Koup, J.P. Moore, and W.A. Paxton. 1996. HIV-1 entry itno CD4+ cells is mediated by the chemokine receptor CC-CKR5. Nature **381**:667-673.

Dustin, M.L., R. Rothlein, A.K. Bhan, C.A. Dinarello, and T.A. Springer. 1986. Induction by IL-1 and interferon, tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. **137**:245-254.

Eisner, R. 1992. Research news: Finding out how a viral hitchhiker snags a ride. Science **255**:1647.

Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochem. **8**:871-874.

Feng, Y., C. Broder, P. Kennedy, and E. Berger. 1996. HIV-1 entry cofactor: functional cDNA cloning of seven-transmembrane, G protein-coupled receptor. Science **272**:872-877.

Fields, B.N. 1993. Pathogenesis of viral infections. In *Emerging Viruses*. Edited by S.S. Morse. Oxford University Press, New York. pp.69-78.

Fox, G., N.R. Parry, P.V. Barnett, B. McGinn, D.J. Rowlands, and F. Brown. 1989. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J. Gen. Virol. **70**:625-637.

Freistadt, M.S., G. Kaplan, and V.R. Racaniello VR. 1990. Heterogeneous expression of poliovirus receptor-related proteins in human cells and tissues. Mol. Cell. Biol. **10**:5700-5706.

Freistadt, M.S., and V.R. Racaniello. 1991. Mutational analysis of the cellular receptor for poliovirus. J. Virol. **65**:3873-3876.

Fricks, C.E., and J.M. Hogle. 1990. Cell-induced conformational change in poliovirus: externalizatiion of the amino terminus of VP1 is responsible for liposome binding. J. Virol. **64**:1934-1945.

Fuerst, T.R., E.G. Niles, F.W. Studier, and B. Moss. 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA. **83**:8122-8126.

Fukuoka, Y., A. Yasui, N. Okada, and H. Okada. 1996. Molecular cloning of murine decay accelerating factor by immunoscreening. Int. Immunol. **8**:379-385.

Gershoni, J.M., M. Lapidot, N. Zakai, and A. Loyter. 1986. Protein blot analysis of virus receptors: identification and characterization of the Sendai virus receptor. Biochem. Biophys. Acta. **856:**19-26.

Ghendon, Y. 1989. Ocular enterovirus infections in the world. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations.* Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp. 3-9.

Goh, K.T., P.L. Ooi, K. Miyamura, T. Ogino, and S. Yamazaki. 1990. Acute haemorrhagic conjunctivitis:seroepidemiology of coxsackievirus A24 variant and enterovirus 70 in Singapore. J. Med. Virol. **31:**245-247.

Graves, J.H. 1973. Serological relationship of swine vesicular disease virus and coxsackie B5 virus. Nature (London) **245:** 314-315.

Greve, J.M., G. Davis, A.M. Meyer, C.P. Forte, S.C. Yost, C.W. Marlor, M.E. Kamarck, and A. McClelland. 1989. The major human rhinovirus receptor is ICAM-1. Cell **56:**839-847.

Greve, J.M., C.P. Forte, C.W. Marlor, A.M. Meyer, H. Hoover-Litty, D. Wunderlich, and A. McClelland. 1991. Mechanisms of receptor-mediated rhinovirus neutralization defined by two soluble forms of ICAM-1. J. Virol. **65:**6015-6023.

Gromeier, M., and K. Wertz. 1990. Kinetics of poliovirus uncoating in HeLa cells in a nonacidic environment. J. Virol. **64:**3590-3597.

Gruenberger, M., R. Wandl, J. Nimpf, T. Hiesberger, W.J. Schneider, E. Kuechler, and D. Blaas. 1995. Avian homologs of the mammalian low-density lipoprotein receptor family bind minor receptor group human rhinovirus. J. Virol. **69:**7244-7247.

Hansen, M.B., S.E. Nielsen, and K. Berg. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods. **119:**203-210.

Harouse, J.M., S. Bhat, S.L. Spitalnik, M. Laughlin, K. Stefano, D.H. Silberberg, and F. Gonzalez-Scarano. 1991. Inhibition of entry of HIV-1 in neural cells by antibodies against galactosyl ceramide. Science **253:**320-323.

Hierholzer, J.C., and M.H. Hatch. 1985. Acute hemorrhagic conjunctivitis. In *Viral Diseases of the Eye.* Edited by R.W. FaDarrell. Lea and Febiger Publishers, Philadelphia. pp. 165-196.

Higgins, P.G. 1982. Enteroviral conjunctivitis and its neurological complications. Arch. Virol. **73:**91-101.

Hofer, F., M. Gruenberger, H. Kowalski, H. Machat, M. Huettinger, E. Kuechler, and D. Blaas. 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor group common cold virus. Proc. Natl. Acad. Sci. USA **91:**1839-1842.

Hogle, J.M., M. Chow, and D.J. Filman. 1985. Three-dimensional structure of poliovirus at 2.9 Å resolution. Science **229:**1358-1365.

Holland, J., and T. Owens. 1997. Signaling through intercellular adhesion molecule 1 (ICAM-1) in a B cell lymphoma line. J. Biol. Chem. **272:**9108-9112.

Holland, J.J., and L.C. McLaren. 1959. The mammalian cell-virus relationship II. Adsorption, reception, and eclipse by HeLa cells. J Exp Med **109:**487-504.

Holland, J.J., and L.C. McLaren. 1961. The location and nature of enterovirus receptors in susceptible cells. J. Exp. Med. **114:**161-171.

Holland, J.J., L.C. McLaren, and J.T. Syverton.1959a. The mammalian cell-virus relationship III. Production of infections poliovirus by non-primate cells exposed to poliovirus ribonucleic acid. Proc. Soc. Exp. Biol. Med. **100:** 843-845.

Holland,J.J., L.C. McLaren, and J.T. Syverton. 1959b. The mammalian cell-virus relationship IV. Infection of naturally insusceptible cells with enterovirus nucleic acid. J. Exp. Med. **110:**65-80.

Hong, S.S., L. Karayan, J. Tournier, D.T. Curiel, and P.A. Boulanger. 1997. Adenovirus type 5 fiber knob binds to MHC class I α2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J. **16:**2294-2306.

Hoogenbrand, N., T. Helman, and J. Hoogenbrand. 1983. The effect of pre-injection of mice with pristane on ascites tumor formation and monoclonal antibody production. J. Immunol. Methods. **61:** 317-320.

Hoover-Litty, H., and J.M. Greve. 1993. Formation of rhinovirus-soluble ICAM-1 complexes and conformational changes in the virion. J. Virol. **67:**390-397.

Hornbeck, P. 1991. Antibody-sandwich ELISA to detect soluble antigens. In *Current Protocols in Immunology*, Volume 1. Edited by J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober. pp. 2.1.9-2.1.11.

Hourcade, D., V.M. Holers, and J.P. Atkinson. 1989. The regulators of complement activation (RCA) gene cluster. Adv. Immunol. **45:**381-416.

Hsiung, G.D., and J.L. Melnick. 1958. Adsorption, multiplication and cytopathogenicity of enteroviruses (poliomyelitis, coxsackie and ECHO groups) in susceptible and resistant monkey kidney cells. J. Immunol. **80**:45-50.

Hsu, K.-H.L., S. Paglini, B. Alstein, and R.L. Crowell. 1990. Identification of a second receptor protein for a coxsackievirus B3 variant, CB3-RD. In *New Aspects of Positive-strand RNA Viruses*. Edited by M. Brinton, and F. Heinz. American Society for Microbiology, Washington, D.C. pp. 271-277.

Hsu, K.-H.L., K. Lonberg-Holm, B. Alstein, and R.L. Crowell. 1988. A monoclonal antibody specific for the cellular receptor for the group B coxsackieviruses. J. Virol. **62**:1647-1652.

Huber, S.A. 1994. VCAM-1 is a receptor for encephalomyocarditis virus on murine vascular endothelial cells. J. Virol. **68**:3453-3458.

Hung, T.-P. 1989. Central nervous system complications of enterovirus type 70 infection: Epidemiological and clinical features. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations*. Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp. 235-250.

Hung, T.-P., and R. Kono. 1979. Neurological complications of acute hemorrhagic conjunctivitis (a polio-like syndrome in adults). In *Handbook of Clinical Neurology*, Vol. **38**. Edited by P.J. Vinken and G.W. Bruyn. North Holland Publishing Company, New York. pp. 595-623.

Huttunen, P., J. Heino, and T. Hyypiä. 1997. Echovirus 1 replication, not only virus binding to its receptor, VLA-2, is required for the induction of cellular immediate-early genes. J. Virol. **71**:4176-4180.

Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell **69**:11-25.

Hyypiä, T., T. Hovi, N.J. Knowles, and G. Stanway. 1997. Classification of enteroviruses based on molecular and biological properties. J. Gen. Virol. **78**:1-11.

Jenkins, O., J.D. Booth, P.D. Minor, and J.W. Almond. 1987. The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the picornaviridae. J. Gen. Virol. **68**:1835-1848.

Jin, Y.-M., I.U. Pardoe, A.T.H. Burness, and T.I. Michalak. 1994. Identification and characterization of the cell surface 70-kilodalton sialoglycoprotein(s) as a candidate receptor for encephalomyocarditis virus on human nucleated cells. J. Virol. **68**:7308-7319.

John, T.J., S. Christopher, and J. Abraham. 1980. Neurological manifestation of acute haemorrhagic conjunctivitis due to enterovirus 70. Lancet ii:1283-1284.

Johnson, J.E. 1996. Functional implications of protein-protein interactions in icosahedral viruses. Proc. Natl. Acad. Sci. USA. **93:**27-33.

Johnson, R.T.. 1994. The Soriano Award Lecture. Emerging infections of the nervous system. J. Neurol. Sci. **124:**2-14.

Kaplan, G., M.S. Freistadt, and V.R. Racaniello. 1990. Neutralization of poliovirus by cell receptors expressed in insect cells. J. Virol. **64:**4697-4702.

Kaplan, G., D. Peters, and V.R. Racaniello. 1990. Poliovirus mutants resistant to neutralization with soluble cell receptors. Science **250:**1596-1599.

Kennet, R.H. 1979. Cell Fusion. In *Methods in Enzymology, Vol. LVIII. Cell Culture*. Edited by W.B. Jakoby, and I. H. Paston. Academic Press, NY. pp. 345-359.

Kennet, R.J., K.A. Denis, A.S. Tung, and N.R. Klinman. 1978. Hybrid plasmocytoma production: Fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. Curr. Top. Microbiol. Immunol **81:** 77-91.

Kew, O.M., B.K. Nottay, M.H. Hatch, J.C. Hierholzer, and J.F. Obijeski. 1983. Oligonucleotide fingerprinting analysis of isolates from 1980 to 1981 pandemic of acute haemorrhagic conjunctivitis: evidence for a close genetic relationship among Asian and American strains. Infect. Immun. **41:**631-635.

Kilpatrick, D.R., and H.L. Lipton. 1991. Predominant binding of Theiler's viruses to a 30-kilodalton receptor protein on susceptible cell lines. J. Virol. **65:**5244-5249.

Kishore, J., N. Manjunath, U. Bareja, L. Verma,S. Broor, and P. Seth. 1989. Study of an outbreak of epidemic conjunctivitis in Delhi in 1986. Indian J. Path. Microbiol. **32:**266-269.

Klatzman, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312:**767-768.

Koch, F., and G. Koch. 1985. *The Molecular Biology of Poliovirus*. Springer Verlag, New York.

Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibodies of predefined specificity. Nature (London) **256:**495-497.

Koike S., H. Horie, I. Ise, A. Okitsu, M. Yoshida, N. Iizuka, K. Takeuchi. T. Takegami, and A. Nomoto. 1990. The poliovirus receptor protein is produced both as membrane-bound and secreted forms. EMBO J. **9**:3217-3224.

Koike S., I. Ise, and A. Nomoto. 1991a. Funtional domains of the poliovirus receptor. Proc. Natl. Acad. Sci. USA. **88**:4104-4108.

Koike, S., I. Ise, Y. Sato, H. Yonekawa, O. Gotoh, and A. Nomoto. 1992. A second gene for the African green monkey poliovirus receptor that has no putative N-glycosylation site in the functional N-terminal immunoglobulin-like domain. J. Virol. **66**:7059-7066.

Koike, S., C. Toya, T. Kurata, S. Abe, I. Sie, H. Yonekawa, and A. Nomoto. 1991b. Transgenic mice susceptible to poliovirus. Proc. Natl. Acad. Sci. USA **88**:951-955.

Kono, R. 1978. Etiology of acute hemorrhagic conjunctivitis. In *Concilium Ophthalmologicum*. Edited by K. Shimizu, and J.A. Oosterhuis. Elsevier, Amsterdam. pp. 470-477.

Kono, R. 1985. Acute hemorrhagic conjunctivitis (letter). Am. J. Epidemiol. **121**:775-776.

Kono, R., A. Sasagawa, K. Ishii, M. Ochi, S. Sugiura, H. Matsumiya, Y. Uchida, K. Kameyama, M. Kaneko, and N. Sakurai. 1972. Pandemic of a new type of conjunctivitis. Lancet **i**:1191-1194.

Kono, R., A. Sasagawa, H. Kodama, N. Uchida, Y. Akao, J. Mukoyama, and T. Fujiwara. 1973. Neurovirulence of acute-haemorrhagic-conjunctivitis virus in monkeys. Lancet **1**:61-63.

Kono, R., A. Sasagawa, K. Miyamura, and E. Tajiri. 1975. Serologic characterization and sero-epidemiology studies on acute hemorrhagic conjunctivitis (AHC) virus. Amer. J. Epidemiol. **101**:444-457.

Kono, R., A. Sasagawa, S. Yamazaki, N. Nakazono, K. Minami, S. Otatsume, Y. Robin, J. Renaudet, M. Cornet, S.N. Afoakwa, J.A.A. Mingle, J.K. Obinim, and A. Huros. 1981. Seroepidemiologic studies of acute hemorrhagic conjunctivitis virus (enterovirus type 70) in West Africa. III. Studies with animal sera from Ghana and Sengal. Amer. J. Epidemiol. **114**:362-368.

Kono, R., E. Tajiri, K. Miyamura, A. Sasagawa, and T. Tsuruhara. 1978. Hemagglutination and hemagglutination inhibition tests with Enterovirus type 70. J. Clin. Microbiol. **7**:595-598.

Krah, D.L., and R.L. Crowell. 1985. A solid-phase assay of solubilized HeLa cell membrane receptors for binding group B coxsackieviruses and polioviruses. Virology **118**:148-156.

Krishnaswamy, S., and M.G. Rossman. 1990. Structural refinement and analysis of Mengo virus. J. Mol. Biol. **211**:803-844.

Ku, F.-C. 1989. Epidemiological and etiological studies of acute hemorrhagic conjunctivitis in China. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations*. Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp.151-156.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227**:680-685.

Landau, N.R., M. Wharton, and D.R. Littman. 1988. The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. Nature. **334**:159-162.

Langford, M.P., and G.C. Stanton. 1980. Replication of acute hemorrhagic conjunctivitis viruses in conjunctival-corneal cell cultures of mice, rabbits, and monkeys. Invest. Ophthalmol. Vis. Sci. **19**: 1477-81.

Lapham, C.K., J. Ouyang, B. Chandrasekhar, N.Y. Nguyen, D.S. Dimitrov, and H. Golding. 1996. Evidence for cell-surface association between fusi and the CD4-gp120 complex in human cell lines . Science **274**:602-605.

Lass, J.H., E.I. Walter, T.E. Burris, H.E. Grossniklaus, M.I. Roat, D.L. Skelnik, L. Needham, M. Singer, and M.E. Medof. 1990. Expression of two molecular forms of the complement decay-accelerating factor in the eye and lacrimal gland. Invest. Ophthalmol. Vis. Sci. **36**:1136-1148.

Lenahan, M. F., and H. A. Wenner. 1960. Propagation of entero- and other viruses in renal cells obtained from other hosts. J. Infect. Dis. **107**: 203-212.

Lentz, T.L. 1990. The recognition event between virus and host cell receptor: a target for antiviral agents. J. Gen. Virol. **71**:751-766.

Levitt, N.H., and R.L. Crowell. 1967. Comparative studies of the regeneration of HeLa cell receptors for poliovirus T1 and coxsackievirus B3. J. Virol. **1**:693-700.

Lin, K.-H., H.-L. Wang, M.-M. Sheu, W.-L. Huang, C.-C. Chen, C.-S. Yang, N. Takeda, K., N. Kato, Miyamura, and S. Yamazaki. 1993. Molecular epidemiology of a variant of coxsackievirus A24 in Taiwan: two epidemics caused by phylogenetically distinct viruses from 1985 to 1989. J. Clin. Micro. **31**:1160-1166.

Linhares, A.C., E.C.O. Santos, R.B. Freitas, and C.M. Nakauchi. 1989. Acute hemorrhagic conjunctivitis caused by EV70 in Brazil. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations*. Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp.201-207.

Lonberg-Holm, K. 1975. The effects of concanavalin A on the early events of infection by rhinovirus type 2 and poliovirus type 2. J. Gen. Virol. **28**:313-327.

Lonberg-Holm K., and B.D.Korant. 1972. Early interactions of rhinoviruses with host cells. J. Virol. **9**:29-40.

Lonberg-Holm K., R.J. Crowell, and L.Philipson. 1976. Unrelated animal viruses share receptors. Nature **259**:679-681.

Low, M.G., J. Stiernberg, G.L. Waneck, R.A. Flavell, and P.W. Kincade. 1988. Cell-specific heterogeneity in sensitivity of phosphatidylinositol-anchored membrane antigens to release by phospholipase C. J. Immunol. Methods **113**:101-111.

Lublin, D.M., and J.P. Atkinson. 1989. Decay-accelerating factor: biochemistry, molecular biology, and function. Ann. Rev. Immunol. **7**:35-58.

Lublin, D.M., and K.E. Coyne. 1991. Phospholipid-anchored and transmembrane versions of either decay-accelerating factor or membrane cofactor protein show equal efficiency in protection from complement-mediated cell damage. J. Exp. Med. **174**:35-44.

Lublin, D.M., R.S. Lemons, M.M. LeBeau, V.M. Holers, M.L. Tykocinski, M.E. Medof, and J.P. Atkinson. 1987. The gene encoding decay-accelerating factor (DAF) is located in the complement-regulatory locus on the long arm of chromosome 1. J. Exp. Med. **165**:1731-1736.

Lublin, D.M., M.K. Liszewski, T.W. Post, M.A. Arce, M.M. LeBeau, M.B. Rebentisch, R.S. Lemons, T. Seya, and J.P. Atkinson. 1988. Molecular cloning and chromosomal localization of human membrane cofactor protein (MCP). Evidence for inclusion in the multigene family of complement-regulatory proteins. J. Exp. Med. **168**:181-194.

Luo, N., G. Vriend, G. Kamer, I. Minor, E. Arnold, M.G. Rossmann, U. Boege, D.G. Scraba, G.M. Duke, and A.C. Palmenberg. 1987. The atomic structure of mengovirus at 3.0 Å resolution. Science **235**:182-191.

Maddon, P.J., A.G. Dalgleish, J.S. McDougal, P.R. Clapham, R.A. Weiss, and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333-348.

Madshus, I.H., S. Olsnes, and K. Sandvig. 1984. Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. J. Cell. Biol. **98:**1194-1200.

Malison, M.D., R.A. Gunn, M.H. Hatch, K.W. Bernard, and M.C. White. 1984. Acute hemorrhagic conjunctivitis, Key West, Florida. Am. J. Epidemiol. **120:**717-726.

Manchester, M., A. Valsamakis, R. Kaufman, M.K. Liszewski, J. Alvarez, J.P. Atkinson, D.M. Lublin, and M.A. Oldstone. 1995. Measles virus and C3 binding sites are distinct on membrane cofactor protein (CD46). Proc. Natl. Acad. Sci. USA. **92:**2303-2307.

Mannweiler K., P. Nobis, H. Hohenberg, and W. Bohn. 1990. Immunoelectron microscopy on the topographical distribution of the poliovirus receptor. J Gen. Virol. **71:**2737-2740.

Mapoles, J.E., D.L. Krah, and R.J. and Crowell. 1985. Purification of aHeLa cell receptor protein for group B coxsackieviruses. J. Virol. **55:**560-566.

Marsh, M., and A. Helenius. 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107-151.

Mason, P.W., B. Baxt, F. Brown, J. Harber, A. Murdin, and E. Wimmer. 1993. Antibody-complexed foot and mouth disease virus, but not poliovirus, can infect normally insusceptible cells via the Fc receptor. Virology **192:**568-577.

Mayor, S., K.G. Rothberg, and F.R. Maxfield. 1994. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. Science **264:**1948-1951.

McClelland, A., J. deBear, S.C. Yost, A.M. Meyer, C.W. Marlor, and J.M. Greve. 1991. Identification of monoclonal antibody epitopes and critical residues for rhinovirus binding in domain 1 of intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. USA **88:**7993-7997.

McLaren, L.C., J.J. Holland, and J.T. Syverton. 1959. The mammalian cell-virus relationship I. Attachment of poliovirus to cultivated cells of primate and non-primate origin. J. Exp. Med. **109:**475-485.

Medof, M.E., D.M. Lublin, V.M. Holers, D.J. Ayers, R.R. Getty, J.F. Leykam, J.P. Atkinson, and M.L. Tykocinski. 1987a. Cloning and characterization of cDNAs encoding the complete sequence of decay-accelerating factor of human complement. Proc. Natl. Acad. Sci. USA **84:**2007-2011.

Medof, M.E., E.I. Walter, W.L. Roberts, R. Haas, and T.L. Rosenberry. 1986. Decay accelerating factor of complement is anchored to cells by a C-terminal glycolipid. Biochemistry **25:**6740-6747.

Medof, M.E., E.I. Walter, J.L. Rutgers, D.M. Knowles, and V. Nussenzweig. 1987b. Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. J. Exp. Med. **165**:848-864.

Melnick, J.L. 1996. Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and the newer Enteroviruses. In *Fields Virology*, 3rd edition. Edited by B.N. Fields, D.M. Knipe, and P.M. Howley. Lippincott-Raven Publishers, New York. pp. 655-712.

Mendelsohn, D.L., B. Johnson, K.A.Lionetti, P. Nobis, E. Wimmer, and V.R. Racaniello. 1986. Transformation of a human poliovirus receptor gene into mouse cells. Proc. Natl. Acad. Sci. U S A **83**:7845-7849.

Mendelsohn, D.L., E. Wimmer, and V.R. Racaniello. 1989. Cellular receptor for poliovirus: Molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56**:855-865.

Mims, C.A. 1986. Virus receptors and cell tropisms. J. Infect. **12**:199-203.

Minor, P.D., P.A. Pipkin, D. Hockley, G.C. Schild, and J.W. Almond. 1984. Monoclonal antibodies which block cellular receptors of poliovirus. Virus Res. **1**:203-212.

Mirkovic, R.R., R. Kono, M. Yin-Murphy, R. Sohier, N.J. Schmidt, and J.L. Melnick. 1973. Enterovirus 70: the etiologic agent of pandemic acute haemmorrhagic conjunctivitis. Bull. WHO **49**:341-346.

Mirkovic, R.R., N.J. Schmidt, M. Yin-Murphy, and J.L. Melnick. 1974. Enterovirus etiology of the 1970 Singapore epidemic of acute conjunctivitis. Intervirol. **4**:119-127.

Mischak, H., C. Neubauer, B. Berger, E. Kuechler, and D. Blaas. 1988a. Detection of the human rhinovirus minor group receptor on renaturing Western blots. J. Gen. Virol. **69**:2653-2656.

Mischak, H., C. Neubauer, E. Kuechler, and D. Blaas. 1988b. Characteristics of the minor group receptor of human rhinoviruses. Virology **163**:19-25.

Miyamura, K. 1989. Seroepidemiology of enterovirus 70 and coxsackievirus A24 variant. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations.* Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger AG, Basel. pp. 67-85.

Miyamura, K., A. Sasagawa, E. Tajiri, and R. Kono. 1976. Growth characteristics of Acute Hemorrhagic Conjunctivitis (AHC) virus in monkey kidney cells. II. Temperature sensitivity of the isolares obtained at various epidemic areas. Intervirol. **7**:192-200.

Miyamura, K., N. Takeda, M. Tanimura, and S. Yamazaki. 1983. Evolution of enterovirus 70 and coxsackievirus A24 variant. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations.* Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger AG, Basel. pp. 399-418.

Miyamura, K., M. Tanimura, N. Takeda, R. Kono, and S. Yamazaki. 1986. Evolution of enterovirus 70 in nature: all isolates were recently derived from a common ancestor. Arch. Virol. **89:**1-14.

Miyamura, K., S. Yamazaki, E. Tajiri, and R. Kono. 1974. Growth characteristics of Acute Hemorrhagic Conjunctivitis (AHC) virus in monkey kidney cells. I. Effect of temperature on viral growth. Intervirol. **4:**279-286.

Montgomery, R.I., M.S. Warner, B.J. Lum, and P.G.Spear. 1996. Hespes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87:**427-436.

Morgan, R.A., M.B. Enriquez, and R.K. Forster. 1981. Acute hemorrhagic conjunctivitis-Florida, North Carolina. Morbid. Mortal. Wkly. Rep. **30:**501-502.

Morrison, M.E., Y.-J. He, M.W. Wien, J.M. Hogle, and V.R. Racaniello. 1994. Homolog-scanning mutagenesis reveals poliovirus receptor residues important for virus binding and replication. J. Virol. **68:**2578-2588.

Morrison, M.E., and V.R. Racaniello. 1992. Molecular cloning and expression of a murine homolog of the human poliovirus receptor gene. J. Virol. **66:**2807-2813.

Moser, B. 1997. Chemokines and HIV: a remarkable synergism. TIM **5:**88-90.

Moustafa, O., L. Saleh, K. Abdel-Wahab, and M. El-Gammal. 1989. An outbreak of caused by enterovirus 70 in Jeddah during 1985. J. Egypt. Public Health Assoc. **64:**55-75.

Muckelbauer, J.K., M. Kremer, I. Minor, G. Diana, F.J. Dutko, J. Groarke, D.C. Pevear, and M.G. Rossmann. 1995. The structure of coxsackievirus B3 at 3.5 Å resolution. Structure **3:**653-667.

Nakazono, N., and K. Kondo. 1989. Virus isolation of enterovirus 70. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations.* Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger AG, Basel. pp.295-306.

Naniche, D., T.F. Wild, C. Rabourdin-Combe, and D. Gerlier. 1992. A monoclonal antibody recognizes a humn cell surface glycoprotein involved in measles virus binding. **73:**2617-2624.

Natori, K., S. Yamazaki, K. Miyamura, and R. Kono. 1984. Genetic relationship between two enteroviruses causing the acute hemorrhagic conjunctivitis syndrome. Intervirol. **97**:97-103.

Nicholson-Weller, A. 1992. Decay accelerating factor (CD55). Curr. Top. Microbiol. Immunol. **178**:8-30.

Nicholson-Weller, A., J. Burge, and K.F. Austen. 1981. Purification from guinea pig erythrocyte stroma of a decay-accelerating factor for the classical C3 convertase, C4b2a. J. Immunol. **127**:2035-2039.

Nicholson-Weller, A., J. Burge, D.T. Fearon, P.F. Weller, and K.F. Austen. 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. J. Immunol. **129**:184-189.

Nicholson-Weller, A., and C.E. Wang. 1994. Structure and function of decay accelerating factor CD55. J. Lab. Clin. Med. **123**:485-491.

Nickells, M.W., J.I. Alvarez, D.M. Lublin, and J.P. Atkinson. 1994. Characterization of DAF-2, a high molecular weight form of decay-accelerating factor (DAF; CD55), as a covalently cross-linked dimer of DAF-1. J. Immunol. **152**:676-685.

Nobis, P., R. Zibirre, G. Meyer, J. Kuhne, G. Warnecke, and G. Koch. 1985. Production of a monoclonal antibody against an epitope on HeLa cells that is the functional poliovirus binding site. J. Gen. Virol. **66**:2563-2569.

Nomoto, A., N. Kitamura, and E. Wimmer. 1977. The 5' terminal structures of poliovirion RNA and mRNA differ only in the genome-linked protein VPg. Proc. Natl. Acad. Sci. USA **74**:5345-5349.

Nomoto, A., S. Koike, and J. Aoki. 1994. Tissue tropism and species specificity of poliovirus infection. TIM **2**:47-51.

Nonaka, M., T. Miwa, N. Okada, M. Nonaka, and H. Okada. 1995. Multiple isoforms of guinea pig decay-accelerating factor (DAF) generated by alternative splicing. J. Immunol. **155**:3037-3048.

Norkin, L.C. 1995. Virus receptors: Implications for pathogenesis and the design of antiviral agents. Clin. Microbiol. Rev. **8**:293-315.

Nowicki, B., A. Hart, K.E. Coyne, D.M. Lublin, and S. Nowicki. 1993. Short consensus repeat-3 domain of recombinant decay-accelerating factor is recognized by *Escherichia coli* recombinant Dr adhesin in a model of cell-cell interaction. J. Exp. Med. **178**:2115-2121.

Olson, N.H., P.R. Kolatkar, M.A. Oliviera, R.H. Cheng, J.M. Greve, A. McClelland, T.S. Baker, and M.G. Rossman. 1993. Structure of a human rhinovirus complexed with its receptor molecule. Proc. Natl. Acad. Sci. USA. **90**:507-511.

Palmenberg, A.C. 1990. Proteolytic processing of picornaviral polyprotein. Annu. Rev. Microbiol. **44**:603-623.

Parolini, I., M. Sargiacomo. M.P. Lisanti, and C. Peschle. 1996. Signal transduction and glycophosphatidylinositol-linked proteins (LYN, LCK, CD4, CD45, G proteins, and CD55) selectively localize in triton-insoluble plasma membrane domains of human leukemic cell lines and normal granulocytes. Blood **87**:3783-3794.

Parton, R.G. 1996. Caveolae and caveolins. Curr. Opin. Cell Biol. **8**:542-548.

Patriarca, P.A. 1989. Clinical experiences with acute hemorrhagic conjunctivitis in the United States. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations*. Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp.285-289.

Pevear, D.C., M.J. Fancher, P.J. Felock, M.G. Rossmann, M.S. Miller, G. Diana, A.M. Treasurywala, M.A. McKinlay, and F.J. Dutko. 1989. Conformational change in the floor of the human rhinovirus canyon blocks adsorption to HeLa cell receptors. J. Virol. **63**:2002-2007.

Pham, T., A. Kaul, A. Hart, P. Goluszko, J. Moulds, S. Nowicki, D.M. Lublin, and B.J. Nowicki. 1995. *dra*-related X adhesins of gestational pyelonephritis-associated *Escherichia coli* recognize SCR-3 and SCR-4 domains of recombinant decay-accelerating factor. Infect. Immun. **63**:1663-1668.

Philipson, L., K. Lonberg-Holm, and U. Pettersson. 1968. Virus-receptor interaction in an adenovirus system. J. Virol. **2**:1064-1075.

Pöyry, T., L. Kinnunen, T. Hyypiä, B. Brown, C. Horsnell, T.Hovi, and G. Stanway. 1996. Genetic and phylogenetic clustering of enteroviruses. J. Gen. Virol. **77**:1699-1717.

Quersin-Thiry, L., and E. Nihoul. 1961. Interaction between cellular extracts and animal viruses II. Evidence for the presence of different inactivators corresponding to different viruses. Acta Virol. **5**:283-293.

Raab de Verdugo, U., H.-C. Selinka, M. Huber, B. Kramer, J. Kellermann, P.H. Hofschneider, and R. Kandolf. 1995. Characterization of a 100 kilodalton binding protein for the six serotypes of coxsackie B viruses. J. Virol. **69**:6751-6757.

Racaniello, V.R. 1996. Early events in poliovirus infection: virus-receptor interactions. Proc. Natl. Acad. Sci. USA **93:**11378-11381.

Reagan, K.J., B. Goldberg, and R.L. Crowell. 1984. Altered receptor specificity of coxsackievirus B3 after growth in Rhabdomyosarcoma cells. J. Virol. **49:**635-640.

Reeves, W.C., M.M. Brenes, E. Quiroz, J. Palacios, G. Campos, and R. Centeno. 1986. Acute hemorrhagic conjunctivitis epidemic in Colon, Republic of Panama. Am. J. Epidemiol. **123:**325-335.

Ren, R., F. Costantini, E.J. Gorgacz, J.J. Lee, and V.R. Racaniello VR. 1990. Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. Cell **63:**353-362.

Ressig, M., D.W. Howes, and J.L. Melnick. 1956. Sequence of morphologic changes in epithelial cell cultures infected with picornaviruses. J. Exp. Med. **104:**289-304.

Reuer, Q., R.J. Kuhn, and E. Wimmer. 1990. Characterization of poliovirus clones containing lethal and nonlethal mutations in the genome-linked protein Vpg. J. Virol. **64:**2967-2975.

Rodrigo, M.J., and J. Dopazo. 1995. Evolutionary analysis of the picornavirus family. J. Mol. Evol. **40:**362-371.

Roivainen, M., T. Hyypiä, L. Piirainen, N. Kalkkinen, G. Stanway, and T. Hovi. 1991. RGD-dependent entry of coxsackievirus A9 into host cells and its bypass after cleavage of VP1 protein by intestinal proteases. J. Virol. **65:**4735-4740.

Roivanen. M., L. Piirainen, and T. Hovi. 1996. Efficient RGD-independent entry process of coxsackievirus A9. Arch. Virol. **141:**1909-1919.

Roivainen, M., L. Piirainen, T. Hovi, I. Virtanen, T. Riikonen, J. Heino, and T. Hyypiä. 1994. Entry of coxsackievirus A9 into host cells: Specific interactions with $\alpha_v\beta_3$ integrin, the vibronectin receptor. Virology **203:**357-365.

Rosse, W. F. 1990. Phosphatidylinositol-linked proteins and paroxysmal nocturnal hemoglobinuria. Blood **75:**1595-1601.

Rossman, M.G. 1989. The canyon hypothesis. Viral Immunol. **2:**143-161.

Rossman, M.G., E. Arnold, J.W. Erickson, E.A. Frankenberger, J.P. Griffith, H.J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A.G. Mosser, R.R. Rueckert, B. Sherry, and G. Vriend. 1985. Structure of a human common cold virus and functional relationship to other pricornaviruses. Nature **317:**145-153.

Rossman, M.G., N.H. Olson, P.R. Kolatkar, M.A. Oliviera, R.H. Cheng, J.M. Greve, A. McClelland, and T.S. Baker. 1994. Crystallographic and cryo EM analysis of virion-receptor interactions. Arch. Virol. Suppl. **9**:531-541.

Rotbart, H.A., and K. Kirkegaard. 1992. Picornavirus pathogenesis: viral access, attachment and entry into susceptible cells. Semin. Virol. **3**:483-499.

Rucker, J., M. Samson, B.J. Doranz, F. Libert, J.F. Berson, Y. Yi, R.J. Smyth, R.G. Collman, C.C. Broder, G. Vassart, R.W. Doms, and M. Parmentier. 1996. Regions in β-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. Cell **87**:437-446.

Rueckert, R. 1996. Picornaviridae: The viruses and their replication. In *Fundamental Virology*, 3rd edition. Edited by B.N. Fields, D.M. Knipe, and P.M. Howley. Lippincott-Raven Publishers, New York. pp. 477-522.

Ryan, M.D., O. Jenkins, P.J. Hughes, A. Brown, N.J. Knowles, D. Booth, P.D. Minor, and J.W. Almond. 1990. The complete nucleotide sequence of enterovirus type 70: relationships with other members of the Picronaviridae. J. Gen. Virol. **71**:2291-2299.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning. A Laboratory Manual.* 2nd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Sasagawa, A., K. Miyamura, and R. Kono. 1982. Enterovirus 70 neutralizing IgM in animal sera. Jpn. J. Med. Sci. Biol. **35**:63-73.

Satpathy, G., S. Mohanty, and N. Nyak. 1996. An epidemic of viral acute haemorrhagic conjunctivitis in Delhi in 1994. Indian J. Ophthalmol. **44**:19-21.

Sattar, S.A., K.D. Dimock, S.A. Ansari, and V.S. Springthorpe. 1988. Spread of acute hemorrhagic conjunctivitis due to enterovirus 70: effect of air temperature and relative humidity on virus survival on fomites. J. Med. Virol. **25**:289-296.

Schnitzer, J.E., D.P. McIntosh, A.M. Dvorak, J. Liu, and P. Oh. 1995. Separation of caveolae from associated microdomains of GPI-anchored proteins. Science **269**:1435-1439.

Schulman, M., C.D. Wilde, and G. Kohler. 1978. A better cell line for making hybridomas secreting specific antibodies. Nature (London) **276**:269-271.

Scott, E.J., and R.A. Killington. 1972. Haemagglutination by rhinoviruses. Lancet **i**:1369-1370.

Sekiguchi, K., Franke, A.J., and Baxt, B. 1982). Competition for cellular receptor sites among selected aphthoviruses. Arch. Virol. 74:53-64.

Selinka, H.-C., A. Zibert, and E. Wimmer. 1991. Poliovirus can enter and infect mammalian cells by way of an intercellular adhesion molecule 1 pathway. Proc. Natl. Acad. Sci. USA **88**:3598-3602.

Selinka, H.-C., A. Zibert, and E. Wimmer. 1992. A chimeric poliovirus/CD4 receptor confers susceptibility to poliovirus on mouse cells. J. Virol. **66**:2523-2526.

Shafren, D.R., R.C. Bates, M.V. Agrez, R.L. Herd, G.F. Burns, and R.D. Barry. 1995. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. J. Virol. **69**:3873-3877.

Shafren, D.R., D.J. Dorahy, S.J. Greive, G.F. Burns, and R.D. Barry. 1997a. Mouse cells expressing human intercellular adhesion molecule-1 are susceptible to infection by coxsackievirus A21. J. Virol. **71**:785-789.

Shafren, D.R., D.J. Dorahy, R.A. Ingham, G.F. Burns, and R.D. Barry. 1997b. Coxsackievirus A21 binds to decay-accelerating factor but requires intracellular adhesion molecule 1 for entry. J. Virol. **71**:4736-4743.

Shenk. T. 1996. Adenorividae: the viruses and their replication. In *Fundamental Virology*, 3rd Edition. Edited by B.N. Fields, D.M. Knipe, and P.M. Howley. Lippincott-Raven Publishers, New York. pp. 979-1016.

Shenoy-Scaria, A.M., J. Kwong, T. Fujita, M.W. Olszowy, A.S. Shaw, and D.M. Lublin. 1992. Signal transduction through decay-accelerating factor: interaction of glycosyl-phosphatidylinositol anchor and protein tyrosine kinases $p56^{lck}$ and $p59^{fyn\ 1}$. J. Immunol. **149**:3535-3541.

Shepley, M.P., and V.R. Racaniello. 1994. A monoclonal antibody that blocks poliovirus attachment recognizes the lymphocyte homing receptor CD44. J. Virol. **68**:1301-1308.

Shepley, M., B. Sherry, and H. Weiner. 1988. Monoclonal antibody identification of a 100-kDa membrane protein in HeLa cells and human spinal cord involved in poliovirus attachment. Proc. Natl. Acad. Sci. USA **85**:7743-7747.

Silver, L., and C.W. Anderson. 1988. Interaction of human adenovirus serotype 2 with human lymphoid cells. Virology **165**:377-387.

Spence, L., and H. Vellend. 1982. Conjunctivitis outbreak in a hospital-Ontario. Can. Dis. Wkly. Rep. **8**:37.

Spicer, A.P., M.F. Seldin, and S.J. Gendler. 1995. Molecular cloning and chromosomal localization of the mouse decay-accelerating factor genes. J. Immunol. **155**:3079-3091.

Stanton, G.J., J.P. Langford, and S. Baron. 1977. Effect of interferon, elevated terperature, and cell type on replication of acute hemorrhagic conjunctivitis viruses. Infect. Immun. **18**:370-376.

Stanway, G. 1990. Structure, function and evolution of picornaviruses. J. Gen. Virol. **71**:2483-2501.

Stanway, G., N. Kalkkinen, M. Roivainen, F. Ghazi, M. Khan, M. Smyth, O. Meurman, and T. Hyypiä. 1994. Molecular and biological characteristics of echovirus 22, a representative of a new picornavirus group. J. Virol. **68**:8232-8238.

Staunton, D.E., M.L. Dustin, H.P. Erikson, and T.A. Springer. 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites of LFA-1 and rhinovirus. Cell **61**:243-254.

Staunton, D.E., A. Gaur, P.-Y. Chan, and T.A. Springer. 1992. Internalization of a major group human rhinovirus does not require cytoplasmic or transmembrane domains of ICAM-1. J. Immunol. **148**:3271-3274.

Staunton, D.E., V.J. Merluzzi, R. Rothlein, R. Barton, S.D. Marlin, and T.A. Springer. 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56**:849-853.

Sugita, Y., M. Uzawa, and M. Tomita. 1987. Isolation of decay-accelerating factor (DAF) from rabbit erythrocyte membranes. J. Immunol. Methods **104**:123-130.

Takeda, N., R.J. Kuhn, C.-F. Yang, T. Takegami, and E. Wimmer. 1986. Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells. J. Virol. **60**:43-53.

Takeda, N., K. Miyamura, T. Ogino, K. Natori, S. Yamazaki, N. Sakurai, N. Nakazono, K. Ishii, and R. Kono. 1984. Evolution of enterovirus type 70: Oligonucleotide mapping analysis of RNA genome. Virology **134**:375-388.

Takegami, T., Kuhn, R.J., Anderson, C.W., Wimmer, E. 1983. Membrane-dependent uridylylation of the genome-linked protein of poliovirus. Proc. Natl. Acad. Sci. USA **80**:7447-7451.

Tanimura, M., K. Miyamura, and N. Takeda. 1985. Construction of a phylogenic tree of enterovirus 70. Jap. J. Genetics **60**:137-150.

Tardieu, M., R.L. Epstein, and H.L.Weiner. 1982. Interaction of viruses with cell surface receptors. Int. Rev. Cytol. **80**:27-61.

Tomassini, J.E., D. Graham, C.M. deWitt, D.W. Lineberger, J.A. Rodkey, and R.J. Colonno. 1989a. cDNA cloning reveals that the major group rhinovirus receptor on HeLa cells in intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. USA **86:**4907-4911.

Tomassini, J.E., T.R. Maxson, and R.J. Colonno. 1989b. Biochemical characterization of a glycoprotein required for rhinovirus attachment. J. Biol. Chem. **264:**1656-1662.

Tomko, R.P., R. Xu, and L. Philipson. 1997. HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. USA. **94:**3352-3356.

Towbin, J.,T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350-4354.

Trkola, A., T. Dragic, J. Arthos, J.M. Binley, W.C. Olson, G.P. Allaway, C. Cheng-Mayer, J. Robinson, P.J. Maddon, and J.P. Moore. 1996. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. Nature **384:**184-187.

Uchida, N. 1989a. Neurovirulence of Enterovirus 70 in monkeys. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations*. Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp.359-371.

Uchida, Y. 1989b. Clinical features of acute hemorrhagic conjunctivitis due to Enterovirus 70. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations*. Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp. 213-223.

Uchio, E., K. Yamazaki, K. Aoki, amd S. Ohno. 1996. Detection of enterovirus 70 by polymerase chain reaction in acute haemorrhagic conjunctivitis. Am. J. Ophthalmol. **122:**273-275.

Uncapher, C.R., C.M. DeWitt, and R.J. Colonno. 1991. The major and minor group receptor families contain all but one human rhinovirus serotype. Virology **180:**814-817.

Utagawa, E.T., K. Miyamura, A. Mukoyama, and R. Kono. 1982. Neuraminidase-sensitive erythrocyte receptor for Enterovirus type 70. J. Gen. Virol. **63:**141-148.

Vejjajiva, A. 1989. Acute hemorrhagic conjunctivitis with nervous system complications. In *Handbook of Clinical Neurology Vol. 12.* Edited by R.R. McKendall. Elsevier Science Publishers, New York. pp. 349-354.

Verdin, E.M., G.L. King, and E. Maratos-Flier. 1989. Characterization of a common high-affinity receptor for reovirus serotypes 1 and 3 on endothelial cells. J. Virol. **63:**1318-1325.

Vorkauf, M., G. Duncker, B. Nolle, and W. Sterry. 1993. Adhesion molecules in normal human conjunctiva. An immunohistological study using monoclonal antibodies. Graefes Arch. Clin. Exp. Ophthalmol. **231:**323-330.

Wadia, N.H., P.N. Wadia, S.M. Katrak, and V.P. Misra. 1983. A study of the neurological disorder associated with acute haemorrhagic conjunctivitis due to enterovirus 70. J. Neurol. Neurosurg. and Psych. **46:**599-610.

Ward, T., P.A. Pipkin, N.A. Clarkson, D.M. Stone, P.D. Minor, and J.W. Almond. 1994. Decay-accelerating factor CD55 is identified as the receptor for echovirus 7 using CELICS, a rapid immuno-focal cloning method. EMBO J. **13:**5070-5074.

Waterman, S.H., R. Casas-Benabe, M.H. Hatch, R.E. Bailey, R. Munoz-Jiminez, R. Ramirez - Ramirez, and M. Rodriguez-Bigas. 1984. Acute hemorrhagic conjunctivitis in Puerto Rico, 1981-1982. Am. J. Epidemiol. **120:**395-403.

White, D.J.G., T. Oglesby, M.K. Liszewski, I. Tedja, D.Hourcade, M.-W. Wang, L. Wright, J.Wallwork, and J.P. Atkinson. 1992. Expression of human decay accelerating factor or membrane cofactor protein genes on mouse cells inhibits lysis by human complement. Transplant. Proc. **24:**474-476.

White, J.M., and D.R. Littman. 1989. Viral receptors of the immunoglobulin superfamily. Cell **56:**725-728.

Wickham, T.J., P. Mathias, D.A. Cheresh, and G.R. Nemerow. 1993. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. Cell **73:**309-319.

Wimmer, E. 1994. Introduction and overview. In *Cellular receptors for animal viruses.* Edited by E. Wimmer. Cold Spring Harbor Laboratory Press, New York. pp.1-14.

Wimmer, E., C.U.T. Hellen, and X. Cao. 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353-436.

Wright, P.D., G.H. Strauss, and M.P. Langford. 1992. Acute hemorrhagic conjunctivitis. Am. Fam. Phys. **45:**173-178.

Wu, L., N.P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A.A. Cardoso, E. Desjardin, W. Newman, C. Gerard, and J. Sodroski. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. Nature **384:**179-183.

Yamazaki, K., I. Oishi, and Y. Minekawa. 1995. Nucleotide sequence analysis of recent epidemic strains of enterovirus 70. Microbiol. Immunol. **39**:429-432.

Yamazaki, S., and K. Miyamura. 1989. General Characteristics of Enterovirus 70. In *Acute Hemorrhagic Conjunctivitis. Etiology, Epidemiology and Clinical Manifestations*. Edited by Y. Uchida, K. Ishii, K. Miyamura, and S. Yamazaki. S. Karger, AG, New York. pp. 345-357.

Yamazaki, S., K. Natori, and R. Kono. 1974. Purification and biophysical properties of acute hemorrhagic conjunctivitis virus. J. Virol. **14**:1357-1360.

Yin-Murphy, M. 1984. Acute hemmorrhagic conjunctivitis. Prog. Med. Viro. **29**:23-44.

Yin- Murphy, M., and K.H. Lim. 1972. Picornavirus epidemic conjunctivitis in Singapore. Lancet 857-858.

Yoshii, T., K. Natori, and R. Kono. 1977. Replication of enterovirus 70 in non-primate cell cultures. J. Gen. Virol. **36**:377-384.

Zajac, A.J., E.M. Amphlett, D.J. Rowlands, and D.V. Sangar. 1991. Parameters influencing the attachment of hepatitis A virus to a variety of continuous cell lines. J. Gen. Virol. **72**:1667-1675.

Zajac I., and R.L. Crowell. 1965a. Effects of enzymes on the interaction of enteroviruses with living HeLa cells. J. Bacteriol. **89**:574-577.

Zajac I., and R.L. Crowell. 1965b. Location and regeneration of enterovirus receptors of HeLa cells. J. Bacteriol. **89**:1097-1100.

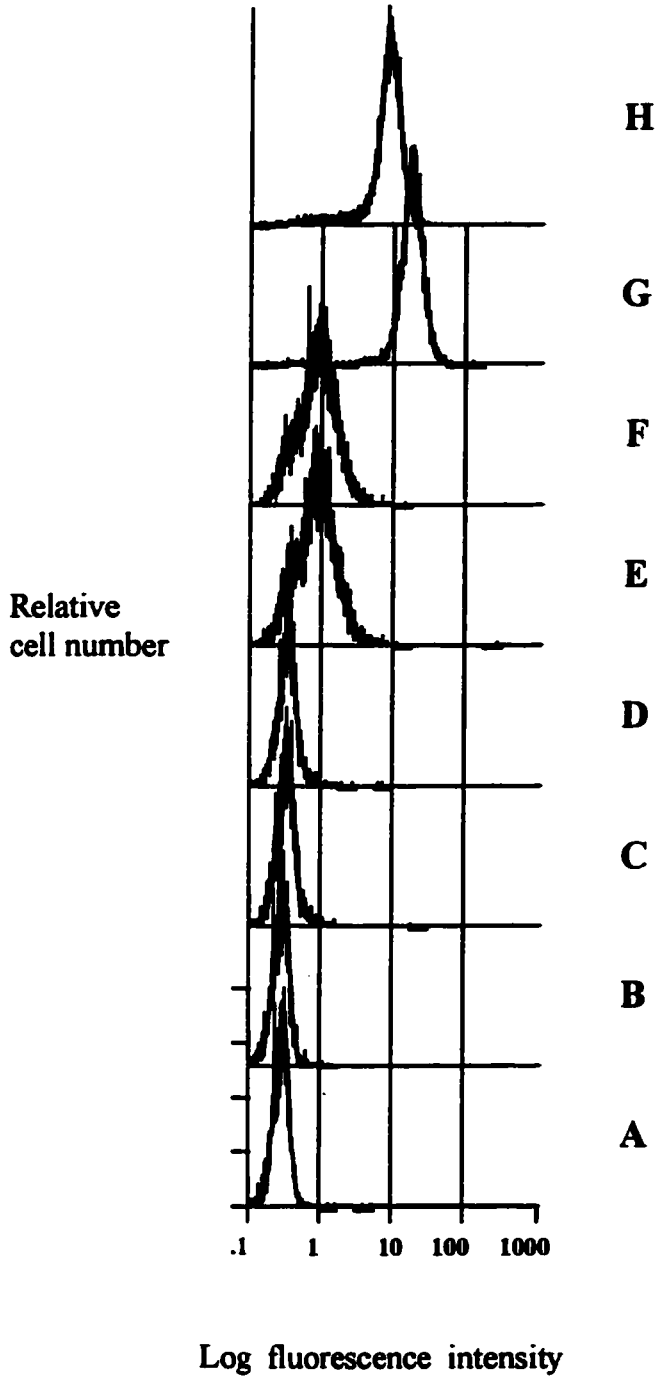
Zibert, A., and E. Wimmer. 1992. N glycosylation of the virus binding domain is not essential for function of the human poliovirus receptor. J. Virol. **66**:7368-7373.

Appendix I.

Flow cytometric analysis of DAF expression in 3T3-RDAF and 3T3-DAF cell lines using mAbs 1H4 and EVR1.

Cells were prepared and processed as described in Materials and Methods. Primary antibody dilutions were 1:5 (1H4) and 1:100 (EVR1),

A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) 3T3-RDAF cells; EVR1
D) 3T3-RDAF cells; mAb 1H4
E) 3T3-DAF cells; EVR1
F) 3T3-DAF cells; mAb 1H4
G) HeLa cells; EVR1
H) HeLa cells; mAb 1H4


H
G
F
E
D
C
B
A
Relative
cell number
.1
1
10
100
1000
Log fluorescence intensity

Appendix II.

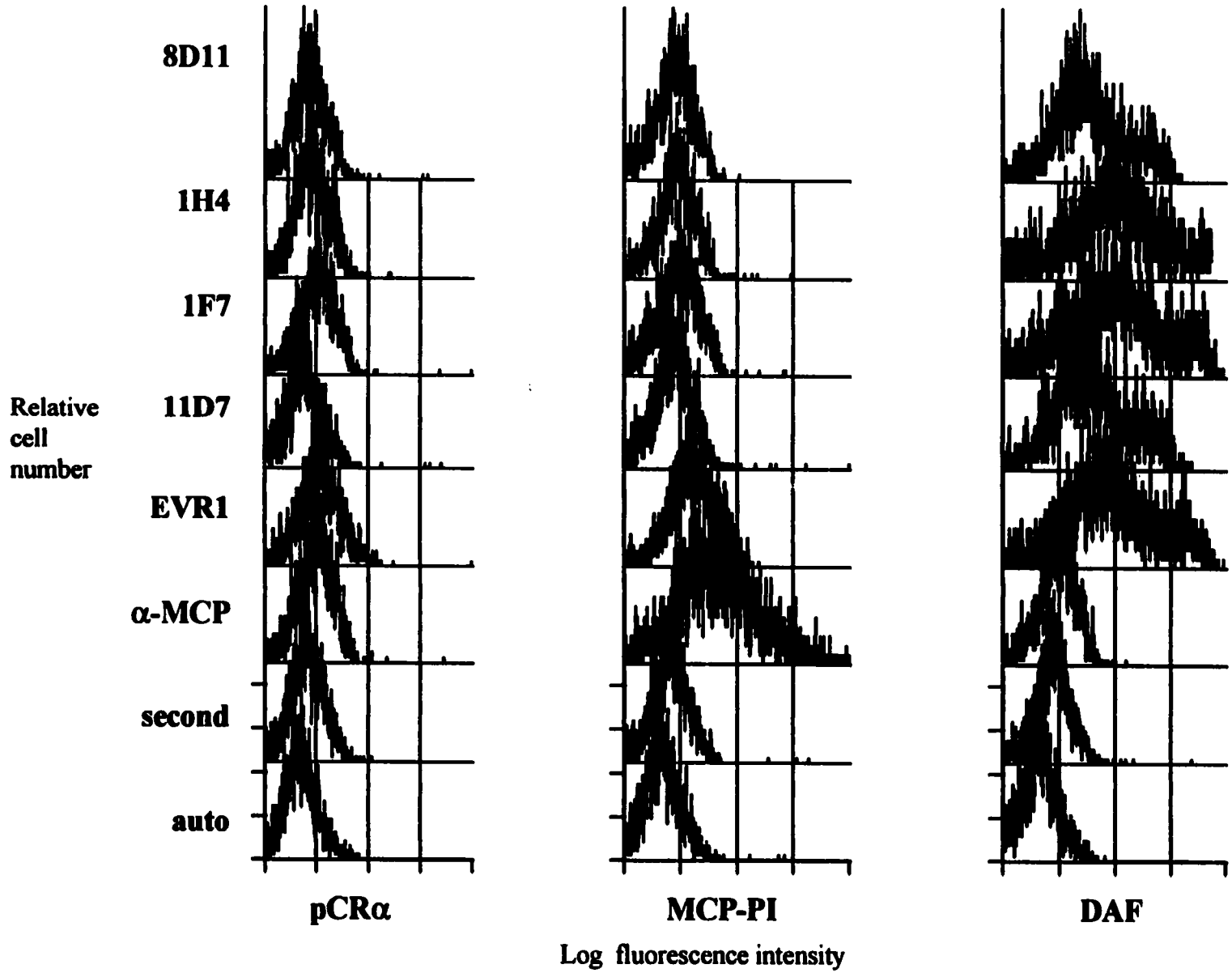
Flow cytometric analysis of DAF-specific and MCP-specific antibody binding to control samples used in DAF/MCP chimera expression studies.

Cells were prepared and processed as described in Materials and Methods. Primary antibody dilutions are listed in Table 5.

pCRα: 3T3 cells transfected with vector pCRα
MCP-PI: 3T3 cells transfected with pSRαEN-MCP-PI
DAF: 3T3 cells transfected with pCRαDAF

auto: autofluorescence control
second: non-specific secondary antibody binding control

α-MCP: mAb vs. MCP
11D7: mAb vs. DAF SCR1
1F7: mAb vs. DAF SCR2
1H4: mAb vs. DAF SCR3
8D11: mAb vs. DAF SCR4


8D11
1H4
1F7
11D7
EVR1
α-MCP
second
auto
Relative cell number
pCRα
MCP-PI
DAF
Log fluorescence intensity

Appendix III.

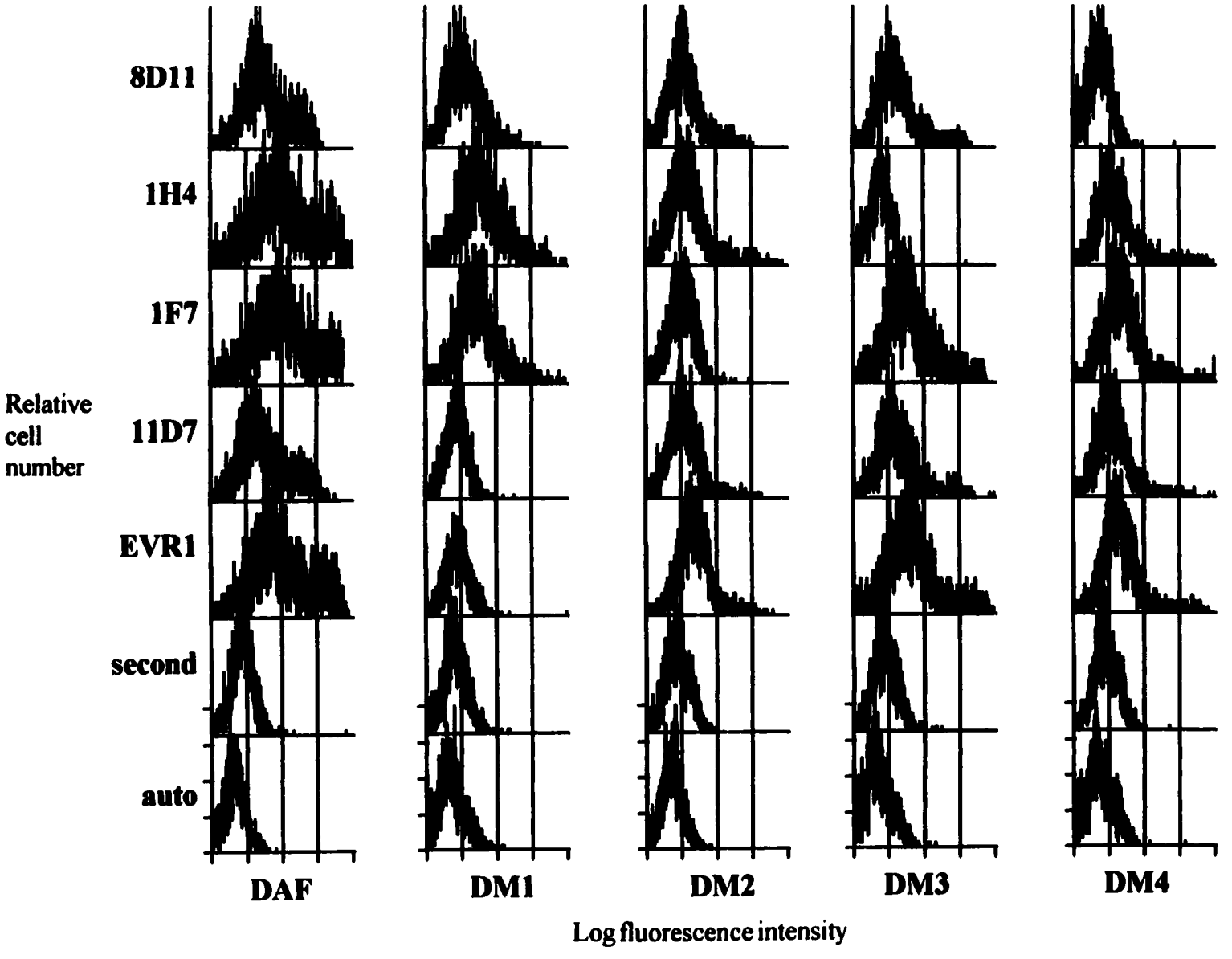
Summary of DAF/MCP chimera expression studies.

Flow cytometric analysis of DAF-specific and MCP-specific antibody binding to DAF/MCP chimeras. Cells were prepared and processed as described in Materials and Methods. Primary antibody dilutions are listed in Table 5.

DAF: 3T3 cells transfected with pCRαDAF
DM1: 3T3 cells transfected with pCRα-DM1
DM2: 3T3 cells transfected with pSRαEN-DM2
DM3: 3T3 cells transfected with pCRα-DM3
DM4: 3T3 cells transfected with pCRα-DM4

auto: autofluorescence control
second: non-specific secondary antibody binding control

11D7: mAb vs. DAF SCR1
1F7: mAb vs. DAF SCR2
1H4: mAb vs. DAF SCR3
8D11: mAb vs. DAF SCR4


8D11
1H4
1F7
11D7
EVR1
second
auto
Relative cell number
DAF
DM1
DM2
DM3
DM4
Log fluorescence intensity

Appendix IV.

Comparison of the transient expression levels of pcDNA3-DAF and pCRα-DAF in 3T3 cells.

Cells were prepared and processed as described in Materials and Methods. Primary antibody (mAb 1H4) dilution was 1:5.

A) Autofluorescence control; unstained cells
B) Secondary antibody control; cells stained with secondary antibody only
C) pCRα; mAb 1H4
D) pcDNA3; mAb 1H4
E) pCRα-DAF; mAb 1H4
F) pcDNA3-DAF; mAb 1H4

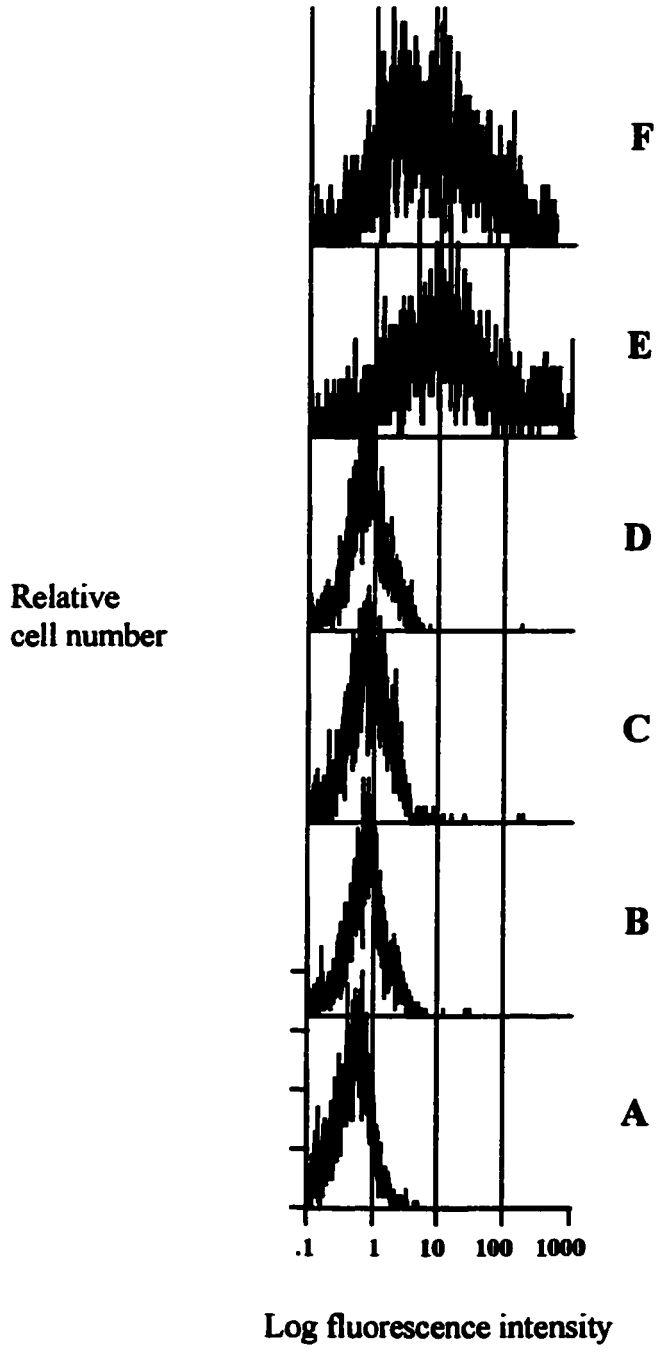
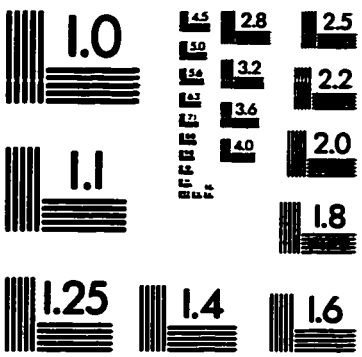
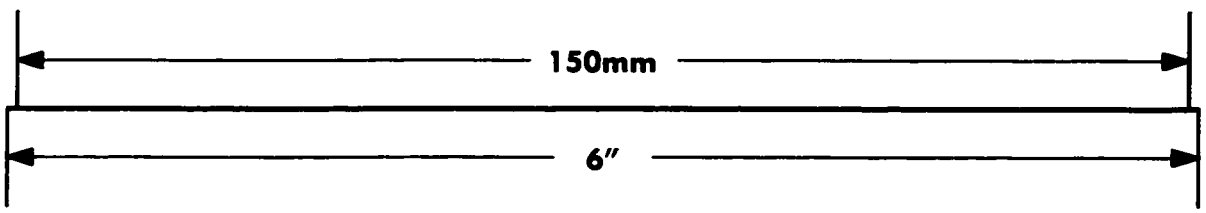

F
E
D
C
B
A
Relative
cell number
.1
1
10
100
1000
Log fluorescence intensity

IMAGE EVALUATION TEST TARGET (QA-3)





APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989