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Role of annexins in cytomegalovirus infection.

I. Annexin 2 tetramer enhances infection.

II. Annexins 5 and 1 inhibit infection.

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ABSTRACT

Cytomegalovirus (CMV), a widespread pathogen, causes significant pathology in immunocompromised individuals, and chronic infection has been linked to heart disease. Since CMV infection can be controlled, but not prevented, therapeutics aimed at the virus-cell interaction would be beneficial, but CMV-host cell interactions are not well understood. Cell-free experiments have suggested a function for annexin 2 (AnxA2), which exists in heterotetrameric ($p36_2p11_2$, AnxA2t) or monomeric (p36) form, but a role for AnxA2 in productive infection of cells is controversial. In the first part of this study we followed the effect on various stages of infection of endogenous AnxA2, using inhibitory antibodies, and purified AnxA2. Anti-p11 or anti-p36 antibodies inhibited CMV production by up to 95%, immediate-early (IE72) expression by 90% and plaque formation by 50%, but ^{35}S-CMV-cell binding was unaffected. Confirming the role of endogenous AnxA2, AnxA2t and p11, but not p36, enhanced virus production 7-fold and IE72 expression 3-fold. Interestingly, purified proteins did not alter plaque formation, whereas AnxA2t, but not p36, enhanced ^{35}S-CMV-cell binding 3-fold, suggesting biochemical differences between endogenous and purified AnxA2. IE72 expression was enhanced 3-fold in the p36-null cell line HepG2 following p36 transfection. Co-immunodepletion, chemical crosslinking, and ligand blots suggested an interaction between p11 and the viral fusogen glycoprotein B. In the second part of this study, we investigated whether purified AnxA5 and AnxA1, which interact with AnxA2, affect CMV infection by altering AnxA2 function. AnxA1 or AnxA5 inhibited CMV production by up to 80%, IE72 expression by 60% and plaque formation by 50%. However, ^{35}S-CMV-cell binding was unaffected. Combined AnxA5 and AnxA1 were not

additive at saturating concentrations, indicating that they inhibit by a similar pathway. Inhibition was reversed by preincubation with purified AnxA2t, and by anti-AnxA2 mAbs that alone had no effect on infection. Attenuation by AnxA1 or AnxA5 was not additive with inhibitory anti-AnxA2 antibodies, implying that a common pathway, i.e. AnxA2t, was impeded. AnxA1 or AnxA5 inhibited IE72 gene expression by 80% in p36-transfected but not native HepG2. These data demonstrate that 1) AnxA2t enhances CMV infection, and 2) purified AnxA5 and AnxA1 inhibit CMV infection by disrupting AnxA2t activity.

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LIST OF ABBREVIATIONS

^{o}C	degrees Celcius
AIDS	Acquired immuno-deficiency syndrome
Ala	alanine
AnxA1	Annexin 1
AnxA2	Annexin 2
AnxA2t	Annexin 2 tetramer
AnxA4	Annexin 4
AnxA5	Annexin 5
AnxA6	Annexin 6
AnxA7	Annexin 7
AnxA12	Annexin 12
ATCC	American Tissue Culture Collection
ATP	adenosine triphosphate
BME	Basal Medium Eagle
BSA	Bovine serum albumin
Ca	calcium
cAMP	cyclic adenosine monophosphate
CD13	cell differentiation cluster 13
CD44	cell differentiation cluster 44
CMV	cytomegalovirus
CMVFR	cytomegalovirus fusion receptor
COX-2	cyclooxygenase-2

cpm	counts per minute
DNA	deoxyribonucleic acid
DEAE	diethylaminoethyl
DSP	dithiobis(succinimidylpropionate)
εACA	ε-aminocaproic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGTA	ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ERK	extracellularly regulated kinase
g	gravity
gB	glycoprotein B
gB_H	glycoprotein B heavy chain
gB_L	glycoprotein B light chain
gCII	glycoprotein CII
gCIII	glycoprotein CIII
gH	glycoprotein H
gL	glycoprotein L
Glu	glutamic acid
GM-CSF	granulocyte/macrophage colony-stimulating factor
gO	glycoprotein O
GTP	guanosine triphosphate
HBS	HEPES-buffered saline

HBST	HEPES-buffered saline with Tween
HBV	hepatitis B virus
HFF	human foreskin fibroblasts
His	histidine
HLA	human leukocyte antigen
HSP	heparan sulfate proteoglycan
IC50	concentration at which half-maximal inhibition occurs
IE	immediate-early
IgG	immunoglobulin G
II	prothrombin
IIa	thrombin
IIase	thrombinase
IFN	interferon
IL	interleukin
I_{max}	maximum inhibition
iNOS	inducible nitric oxide synthase
kB	kilobases
kD	binding affinity constant
kDa	kilodaltons
Leu	leucine
Lys	lysine
µCi	microCurie
µg	microgram

µl	microlitre
µM	micromolar
mAb	monoclonal antibody
MEM	Minimum Essential Medium
mA	milliamperes
mg	milligram
ml	millilitre
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
ND10	nuclear domain 10
nm	nanometre
nM	nanomolar
PAR1	protease-activated receptor 1
PBS	phosphate-buffered saline
pfu	plaque-forming units
Phe	phenylalanine
PI3-K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PL	phospholipid
PLA2	phospholipase A2
pp	phosphoprotein
PVDF	polyvinylidinedifluoride

RNA	ribonucleic acid
RU	response unit
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
sHBsAg	small hepatitis B virus surface antigen
SPR	surface plasmon resonance
sulfo-BSOCOES	bis[2-(sulfosuccinimidooxycarbonyloxy) ethyl]sulfone
TBST	Tris-buffered saline with Tween
Thr	threonine
tPA	tissue plasminogen activator
Trp	tryptophan
Tyr	tyrosine
TNFα	tumour necrosis factor α
U	unit of enzymatic activity
UL	unique long open reading frame
US	unique short open reading frame
vp	virus particles
v/v	volume per volume
w/v	weight per volume

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1. INTRODUCTION.

1.1. OBJECTIVES AND HYPOTHESES.

The objective of the current study was to investigate the roles of annexins in cytomegalovirus (CMV) infection. Based on reports in the literature implicating annexin 2 (AnxA2) as a potential CMV receptor, we hypothesized that AnxA2 could enhance CMV infection of host cells by augmenting CMV-cell entry. Since AnxA2 has been reported to bind to annexins 5 (AnxA5) and 1 (AnxA1), we further postulated that AnxA5 and AnxA1 could inhibit CMV infection by interfering with AnxA2-mediated infection events.

1.2. CYTOMEGALOVIRUS.

1.2.1. General information. CMV is present in approximately 60% of the North American population (581). Infection occurs through exposure of mucosal tissues to the virus, and is disseminated in the body in the vascular endothelium and the monocyte population. Following infection, the virus normally enters a period of latency, and remains subclinical for most individuals. However, in immunocompromised individuals, such as AIDS patients or transplant recipients, the virus may become reactivated, spreading to various sites in the body, replicating in a manner destructive to the host cell and causing a range of potentially life-threatening diseases. In newborns who acquire the infection congenitally, the most common consequence is hearing loss and mental retardation, but may also include growth retardation, spleen and liver dysfunction, and

immune disorders. In immuno-deficient adults, in whom the virus in usually reactivated from latency rather than acquired in a primary infection, the most common manifestations are pneumonia, retinitis, and gastrointestinal disease, which may be fatal to the patient. Seropositivity for CMV has also been linked with the development of several pathologies including Sezary syndrome and mycosis fungoides (221). Furthermore, even in apparently healthy patients, infection by CMV and other Herpesviruses is linked to the development of atherosclerosis, since Herpesvirus stimulation has been reported to alter cytokine production, recruitment of inflammatory cells, lipid and cholesterol metabolism, and thrombosis on the surface of infected cells (395). Since the only clinically available course of treatment for combating CMV infection is anti-viral drugs, which can cause serious side effects and can lead to viral resistance (581), the development of therapeutics targeted at CMV-cell interactions would be beneficial.

CMV is a member of the viral family Herpesviridae (11). Also known as Human Herpes Virus-5, CMV possesses the structural features typical of Herpesviruses, consisting of large spherical particles of approximately 150-200 nm consisting of a nucleocapsid, a surrounding tegument, and a phospholipid envelope (figure 1). The nucleocapsid consists of the viral genome, a linear double strand of DNA of approximately 230 kB, anchored to various capsid proteins. The capsid is surrounded by the tegument, a structure consisting of as many as twenty CMV-encoded proteins (598). While the role of most tegument proteins is still unclear, some have been implicated in gene transactivation, control of host cell growth and apoptosis, and virus maturation. The tegument is contained by the envelope, consisting of a phospholipid bilayer. The


A B C

Figure 1. Structural features of human cytomegalovirus. CMV possesses a structure typical of Herpesviruses, containing a nucleocapsid (A) consisting of the viral genome and the nucleocapsid proteins. The nucleocapsid is surrounded by the tegument (B), a poorly defined structure consisting of proteins involved in viral gene activation, control of host cell growth, and viral assembly. The virus is contained within the envelope (C), a bilayer of phospholipid that also contains both viral and host-derived proteins involved in virus entry and immune evasion.

envelope is derived from host membranes from a cytosolic post-Golgi compartment, possibly the early endosome (555), and contains anionic phospholipid (442) as well as virus protein spikes, the function of which is to aid in virus-cell entry and intracellular signalling (64, 505, 616, 617). The envelope also contains host-derived proteins (359, 430, 458, 602), which are thought to aid in entry and immune evasion. It is unclear whether there is a process for the virus to select envelope constituents.

1.2.2. Life cycle (11). CMV begins its infection cycle by attaching to the host cell surface receptors. Attachment is followed by fusion of the envelope with the cell membrane. Although there is some controversy as to whether the virus retains its envelope or proceeds denuded through the cell (596), the virus makes its way to the cell nucleus, where viral gene replication and expression begin. CMV DNA transcription begins immediately upon entry into the nucleus, and peaks late in infection. Gene expression is temporally divided into the following categories: immediate-early genes, which are involved in gene transactivation and stimulation; delayed-early genes, which are involved in DNA replication and nucleotide metabolism, and late genes, which are involved in structural or maturation functions. Although little is known of CMV maturation events, virions are believed to assemble as nucleocapsids in the nucleus, from which they egress into the cytosol and proceed through the endoplasmic reticulum and Golgi apparatus before budding out of the cell membrane, possibly through the endocytic pathway (555). In cell culture, most of the virions are released into the extracellular space, but a significant amount spreads directly to adjacent cells.

1.2.2.1 Pre-entry infection events. AnxA2 has been implicated as a receptor for attachment and fusion of CMV to host cell, stages in infection that occur prior to entry. Pre-entry events comprise both extracellular and intracellular phases, mediated by CMV to optimize infection of the host cell.

1.2.2.1.1. Extracellular events. Extracellular events comprise two distinct steps: attachment and fusion. The virus commences infection by attaching to the cell surface through a well-characterized interaction with cellular heparan sulfate proteoglycans (HSP) (112), a strategy employed by several other Herpesviruses including herpes simplex viruses 1 and 2 (503, 522, 607), Kaposi's sarcoma-associated virus (8, 9, 588), and pseudorabies virus (487). Cellular HSP binds CMV in a weak, heparin-inhibited interaction, using glycoprotein CII complex (gCII) (270, 271) or glycoprotein B (gB) (399).

The initial interaction is followed by a second attachment step, a higher-affinity, heparin-insensitive interaction (112). Though it is not currently known which viral or cellular components mediate the second attachment interaction, one proposed candidate is gB, shown to bind two separate receptors on the host cell surface, one of which was heparin-insensitive (63). Several heparin-independent cellular species have been identified as candidates for mediating CMV-cell attachment, including CD13 on T cells (190, 509) and AnxA2 on endothelial cells and fibroblasts (399, 602, 603).

Attachment is followed by fusion of the virus envelope with the cell membrane. Fusion is mediated by two viral glycoprotein clusters, the first consisting of a dimer of gB, which is indispensable for growth of CMV in cultured cells (226), and which

mediates not only virus-cell fusion but also cell-cell spread and formation of multinucleated giant cells called syncytia (391, 561, 562). The gB ligand on the host cell has not been definitively identified. Though gB is known to bind heparin and therefore can probably bind cellular HSP (399), a heparin-insensitive cellular ligand exists (63). Several candidates have been proposed, including epithelial growth factor receptor (EGFR) (592), CD13 (190, 509), and AnxA2 (61, 430).

Following fusion initiation by gB, fusion is further augmented by a second viral glycoprotein cluster, glycoprotein CIII (gCIII), composed of glycoprotein H (gH), glycoprotein L (gL), and glycoprotein O (gO) (235, 236, 326). Though gCIII cannot initiate CMV-cell fusion on its own, fusion is greatly enhanced by gCIII (280, 375, 412). gH and gL are indispensable for growth of CMV in cell culture, and loss of gO results in a severe growth deficit (226). The cell-surface ligand for gCIII has been identified as a phosphorylated glycoprotein (281) called p92.5 or CMV fusion receptor (CMVFR), which binds the gH component of gCIII (280, 283, 453). CMVFR, like gCIII, is required for CMV-cell fusion (35, 36).

1.2.2.1.2. Intracellular events. In addition to attachment and fusion on the cell surface, there is evidence that the CMV-cell interaction triggers host cell intracellular events associated with cellular proliferation and transcription, resulting in cellular conditions beneficial for viral replication. It has been proposed that the primary HSP-dependent interaction results in, and may be necessary for, the priming of the cell to favor downstream events (112). In agreement with this hypothesis, results from our laboratory (M. Sutherland and E. Pryzdial, unpublished) have shown that stimulation of the cells

with thrombin, which can be produced by the virus (535), results in increased infection by Herpesviruses. Providing insight into the possible mechanism of priming, stimulation of the cell by thrombin (426) or by ultraviolet-inactivated (i.e., transcriptionally inert) CMV (M. Sutherland and E. Pryzdial, unpublished) resulted in increased cell-surface expression of AnxA2, a candidate receptor for CMV. Other CMV-induced signalling events include increased intracellular calcium levels (173, 282, 396), and activation of cytosolic phospholipase A2 (PLA2), resulting in augmented release of arachidonic acid and its derivatives (3, 397, 502) and subsequent elevation of intracellular cyclic adenosine monophosphate (cAMP) (293, 397). The promoter of the major immediate-early gene 1, also called immediate-early 72 (IE72), contains response elements to cAMP (242, 475), suggesting that elevated cAMP levels are beneficial for infection. Infection has also been shown to activate the phosphatidylinositol-3-kinase (PI3-K) pathway, resulting in the activation of the cellular kinases Akt and p70S5K and the transcription factor NF-κB (247). These factors, when activated, promote cellular proliferation, a process favourable for CMV since it relies on the cellular machinery for replication. CMV has also been shown to inhibit anti-viral host cell signalling by receptors for tumour necrosis factor α (TNFα) (33, 437), a potent pro-inflammatory cytokine.

Two CMV proteins, gB and the gH component of gCIII, have been reported to be responsible for at least some of the observed CMV-dependent activation of signal transduction pathways. Both gB and gH have been implicated in induction and activation of Sp-1, A20, NF-κB, and IκBα, transcription factors associated with cellular proliferation and activation (616, 617). gB and gH have also been shown to mediate induction of p38 kinase, a hallmark of monocyte activation, and of the pro-inflammatory

cytokine interleukin-1β (IL-1β) (616). gB has also been reported to activate the interferon (IFN)-responsive pathway, in a mechanism involving the extracellularly regulated kinases (ERK)-1 and -2 (64), and in a second, non-traditional IFN-dependent pathway (505). Ultraviolet-inactivated CMV has been shown to upregulate genes in the human leukocyte antigen (HLA) class I region, an induction inhibited by the addition of heparin or by the prior treatment of the cell with heparinase I, which implicates the HSP-gB or HSP-gCII interaction (516). The cell proteins through which CMV mediates its signalling is currently unknown, but gH is known to bind CMVFR (280, 283, 453), and gB has been reported to bind to several cellular proteins, including HSP (399), EGFR (592), and AnxA2 (61, 430), all of which have been implicated in signal transduction.

1.2.2.2. Post-entry infection events. Following penetration into the host cell, the virus proceeds to the nucleus to begin gene expression and replication. The site of viral disassembly remains controversial, since there is evidence that the virion remains intact with its envelope until entry into the nucleus of the host cell (596). However, disassembly of the virion and release of the genome from the particle appears to require the products of genes UL47, UL48 and UL69, all proteins that exist in the viral tegument (44).

Upon disassembly of the virus particle, the tegument protein pp71 is released. Required for CMV infection (34), pp71 is a potent transactivator of cellular and viral genes containing a variety of promoters (230, 308, 331), including CMV immediate-early (IE) gene products IE72 and IE86 (66). In addition to its transactivation properties, pp71 has been suggested to control cell cycle progression (260, 261, 262).

The first viral genes to be expressed consist of the immediate-early (IE) genes, the function of which are to regulate viral and cellular gene expression during the course of infection. The most important of these are two products of the major IE gene, IE72 and IE86, which are required for CMV infection (6, 175, 595). IE72 and IE86 are transactivators of both viral and cellular genes (96, 171, 177, 178, 179, 257, 302, 525). IE86 has been shown to both activate and repress gene expression (96, 435). In particular, IE86 acts to modulate the expression of genes that regulate the cell cycle, p53 and pRb (386, 398). Other IE proteins have been identified that effect gene transactivation, including four minor products from the alternatively spliced RNA of the major IE gene (525), and products from the viral genes US3 (553) and UL36-38 (109).

Viral DNA synthesis begins following gene transactivation. Although this process is incompletely understood, replication has been shown to require pp65 (129). Viral DNA synthesis occurs at nuclear domain 10 (ND10), a cellular structure involved in DNA replication. This process has been suggested to involve pp71 since it has also localized to ND10 (355). In addition, IE72 has also been localized to ND10, and can furthermore disrupt its structure (300), an event which is believed to diminish replication of cellular DNA while enhancing that of viral DNA.

When replication is complete, the viral DNA is then packaged into capsids containing the small capsid protein (75). Non-structural proteins involved in packaging include the UL80-encoded protease which cleaves the small capsid protein into the mature protein, a process essential to genome encapsidation and capsid maturation (75, 484), and the UL97-encoded kinase, which enhances DNA packaging (309, 599). Following packaging of the viral genome, the mature capsid undergoes nuclear egress

into the cytoplasm, a process requiring the UL97 kinase (309), the UL32 gene product pp150 (220, 373), and the UL53 gene product (130). Final virus maturation occurs at a cytosolic vacuole-like structure derived from Golgi membranes or the early endosome (151, 272), where the virus acquires its final tegument proteins, envelope, and envelope proteins (548, 555), a process requiring the UL99 gene product pp28 (253). The mature virion then exits the cell either into the extracellular space or into neighbouring cells.

1.2.3. Annexins in CMV infection. Given its known *in vitro* properties of mediating phospholipids membrane aggregation and fusion, cytoskeletal rearrangement, and intracellular signalling, AnxA2 is an attractive candidate for CMV to exploit during infection. AnxA2 was first identified as a potential CMV receptor when it was identified as a cell-surface, CMV-binding protein on endothelial cells (603), correlating with earlier reports of a 32-kDa cellular protein that bound CMV particles and that coincided with CMV tropism (115, 399). AnxA2 has been detected on the surface of cells known to support productive CMV infection (207, 426), and is also present on the viral envelope (430, 458, 602), indicating that it is available to CMV during infection. The interaction between CMV and AnxA2 has been suggested to be mediated at least in part by gB, following the observation of a stable AnxA2- and gB-immunoreactive complex not disrupted by detergent-mediated denaturation or bond reduction (430), and of the inhibition of cell-cell fusion in gB-transfected cells by an anti-AnxA2 antibody (61). AnxA2 may also bind the anionic phospholipid that is present in the CMV envelope (602). In addition to the considerable indirect support for a role for AnxA2 in CMV infection, our laboratory has shown directly that AnxA2 enhanced both binding and

fusion of CMV to well-characterized model phospholipid membranes (458), and that a polyclonal anti-AnxA2 antibody inhibited CMV plaque formation (602).

Reports exist in the literature of involvement of annexins in other virus infections. An interaction has been reported between p11, an important regulatory ligand of AnxA2, and hepatitis B virus (HBV) polymerase (98), resulting in inhibition of its ability to polymerize DNA. An interaction between p11 and bluetongue virus protein NS3 has been demonstrated, with p11 proposed to mediate viral release (43). AnxA2 was reported to bind respiratory syncytial virus G protein (352), mediating virus-cell attachment. These reports suggest that AnxA2 may act as a bridge between virus and host cell membranes.

Annexin 5 (AnxA5), a related protein, has been reported to influence infection of various viruses. Our laboratory has demonstrated that AnxA5 can inhibit AnxA2-dependent fusion between CMV and phospholipid vesicles (458). AnxA5 has been identified as a cellular ligand of influenza A and B (409), an interaction that may involve envelope phospholipid since the addition of external phospholipids reduced infection (233). Perhaps the most characterized viral activity of AnxA5 is as a specific receptor for hepatitis B virus (HBV). Human, but not rat, AnxA5 is a documented ligand of the small HBV surface antigen (sHBsAg) (133, 137, 139, 222, 223). AnxA5 has also been shown to be responsible for mediating both sHBsAg internalization and HBV infection of cells (132, 140, 197). The receptor mechanism has been proposed to consist of bridging sHBsAg, and thus HBV, to phospholipid in the host cell membrane (138, 394), a mechanism that may also be generally applicable to AnxA5 in other virus infections, and to AnxA2 in CMV infection.

Despite significant biochemical evidence implicating AnxA2 in CMV infection, its role in CMV infection of host cells remains controversial. While our laboratory reported that an anti-AnxA2 serum was inhibitory to CMV plaque formation (602), other groups have concluded that AnxA2 was dispensable for infection of Caco-2 cells (154), and that AnxA2 had no discernible effect in CMV-cell entry or infection in fibroblasts (429). However, it is difficult to definitively assert that AnxA2 is not involved, since AnxA2 is present both on the cell and on the virus.

1.3. ANNEXINS.

AnxA2, a candidate CMV receptor, belongs to the annexin family, consisting of a large multigene family of highly conserved, widely expressed proteins that are characterized functionally by the ability to bind phospholipid in a calcium-dependent manner. Annexins gene products are found in nearly every eukaryotic cell type (381), ten of which are expressed in mammals (208), and may constitute up to 2% of total protein (457). Within the cell, annexins are found mainly in the cytosol, at the plasma membrane, and near calcium-segregating structures, and the nucleus and nuclear envelope. Annexins have also been reported on the extracellular face of the plasma membrane and in the extracellular fluid, though no signal peptide exists in the gene. Several externalization mechanisms have been proposed, including interactions with secreted proteins (189), as well as Golgi-independent pathways (85, 428) including multiporic membrane disruption during exocytosis resulting in annexin release (158, 419) and concurrent translocation of annexins with anionic phospholipid from the inner to the outer leaflet of the plasma membrane through the calcium-regulated phospholipid scramblase pathway (463).

1.3.1. Structure. Annexins are characterized by the presence of a common C-terminal domain consisting of approximately 34 kDa, termed the tetrad core, plus a unique N-terminal domain (figure 2). Early three-dimensional models of annexins of the C-terminal domain consisted of four repeats with internal 2-fold homology, forming a core containing putative calcium-binding loops (546). Crystal structures of AnxA5 confirmed these models, revealing four domains of similar structure, denoted I-IV, each of which contains five α-helices, denoted A-E. The four domains are arranged in an almost planar cyclic array, with further hydrophobic packing between domains I/IV and domains II/III. The repeats form a central hydrophilic pore, the surface residues of which are polar charged residues conserved between domains and among annexins (237, 238, 239). AnxA5 was proposed to bind to membranes by its convex face, which contains the calcium binding sites (237, 239, 324, 364). The tetrad core is conserved both in primary structure, with 23-35% sequence identity among repeats and 45-55% identity among annexins, and in secondary structure in phospholipid-bound annexins. The exception to this structure is annexin 6 (AnxA6), which contains eight repeats, consisting of two tetrad cores in anti-parallel orientation to each other. The tetrad core has been considered to be responsible for functional properties common to all annexins, but there is evidence that unique functions may be conferred by the core domain (152, 153, 164, 252, 323, 618).

In contrast to the conserved C-terminal tetrad core, the N-termini of annexins possess little similarity from one annexin to the next. They range in length from 11-196 amino acids and are found on the aqueous side of the molecule, opposite from the membrane, normally interacting with the tetrad core through hydrophobic interactions

A

B

C

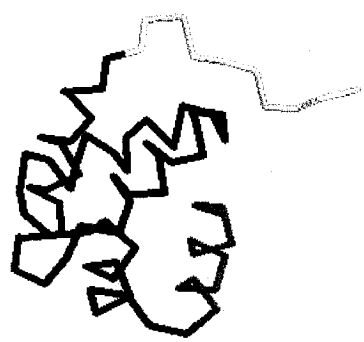


Figure 2. Structure of annexins. The crystal structure of AnxA1 (594) displays the typical structural features common to annexins. The side view (A) and top view (B) show a unique N-terminal tail (yellow) and a conserved C-terminal tetrad core containing four annexin repeats, domain I (green), domain II (blue), domain III (purple), and domain IV (red). The domains are arranged into two modules consisting of domains I and IV and domains II and III. Annexins concomitantly bind calcium (gray) and phospholipid (not shown). C. Each repeat contains five α-helices, exemplified by domain I. The helices are denoted A (green), B (blue), C (purple), D (red), and E (orange). The N-terminus is indicated in yellow.

(181). The N-termini are subject to various post-translational modifications, including phosphorylation (192, 199, 232, 574), N-acetylation (210), and other covalent linkages (208, 440). The N-terminus is generally considered to confer the specific functional properties of individual annexins.

1.3.2. Functions. Though the biological roles of annexins have not yet been definitively proven, their *in vitro* properties have implicated them in cellular trafficking, inflammation, ion transport, signalling, lipid metabolism, proliferation and differentiation, coagulation and fibrinolysis, chemotaxis, and gene regulation. To perform their biological roles, annexins may cycle between soluble and membrane-bound states in order to respond to changes in intracellular calcium concentration. Almost all the proposed biological functions are dependent on and regulated by the ability of annexins to bind calcium and phospholipid.

1.3.2.1. Calcium binding. Two types of calcium binding sites have been identified as a general structural feature of annexins: type II and type III (594). Type II sites are contained in the so-called endonexin fold, consisting of a loop formed by helices A and B at the consensus sequence GXGT-(X_{38})-D/E (176, 181). Calcium occupation of the high-affinity (micromolar range) type II sites results in a conformational change, allowing occupation of the low-affinity (millimolar range) type III calcium-binding site (78, 79, 583). In AnxA5, the conformational change induced in domain III following calcium occupation results in a red shift in the emission of the unique tryptophan at position 187 correlating with its exposure to a hydrophilic environment. The emission shift has been

used to study the calcium- and phospholipid-binding properties of AnxA5 (364, 517, 520).

In addition to the known type II and type III sites, crystal structures have shown that several binding sites for calcium exist that were not anticipated by analysis of primary structure. In AnxA5 crystal structures, two calcium ions were observed in domains I and IV (324). Another structure was reported containing five calcium ions, with three high-affinity sites in calcium-binding loops coordinated with asparagine and glutamic acid residues in homologous positions in domains I, II, and IV, and two weaker-affinity sites in domain I (238, 240). A crystal structure of AnxA1 was shown to bind 8 calcium ions, including two in the N-terminus (471). An AnxA2 mutant having lost all known calcium-binding sites still exhibited calcium-dependent properties, indicating that it contains an unidentified site (164). These reports show that calcium binding by annexins is not yet completely understood, and more study is required to understand how annexins bind calcium and binding regulates function.

1.3.2.2. Phospholipid binding. Calcium occupation of annexins correlates with their binding to phospholipid membranes, with maximal binding occurring in the millimolar range (363, 526). Indeed, membrane binding has been shown to be cooperative with calcium binding (492). While crystals of soluble and phospholipid-complexed AnxA5 were very similar in overall structure (67, 237), indicating that membrane binding by annexins does not induce a large-scale conformational change, calcium and phospholipid binding prevent thermal denaturation, suggesting that the secondary structure of annexins is stabilized upon membrane binding (605).

It is known that the characteristic phospholipid binding by annexins involves a negative charge on the target membrane (362, 363). Consequently, annexins bind anionic phospholipids, with a preference for phosphatidylserine. Membrane binding, which is reversible by EDTA chelation (363), has been proposed to be mediated by direct binding of phosphatidylserine headgroups and calcium in the loops (67). It has been hypothesized that annexins bind to pre-formed phospholipid-calcium complexes (538), but this hypothesis remains unproven. The classical model of phospholipid binding has been proposed to occur at type III sites, containing the unique tryptophan residue (78, 79, 583). In AnxA5, the annexin-phospholipid contact domain contains essential serine, threonine and arginine residues, and a unique tryptophan in the molecule, which becomes accessible after calcium occupation of type II binding sites (114, 518). AnxA5 crystal structures have revealed that two calcium ions bridge the contact between the phosphatidylserine headgroup and the tryptophan residue (536).

More recent reports contradict the notion that only one domain is responsible for phospholipid binding. AnxA5 has been shown to bind to at least three phospholipids (240). Photoreactive phospholipid crosslinking led to the observation of four to five phospholipids bound per AnxA5 (363). Domain III, containing the putative phospholipid-binding site, was suggested not to interact strongly with phospholipid (520). More recently, using site-directed mutagenesis and molecular simulation, a consensus sequence for phospholipid binding was identified, and localized to domains I or II or both, but not domains III or IV (382). Site-directed mutagenesis and crystallization of AnxA5 have shown that mutations in residues on the convex side of the molecule, including Thr-72-Ser and Thr-72-Lys, but not Thr-72-Ala, and lysine replacements at Ser-144, Ser-228,

and Ser-303, reduced phospholipid interactions, indicating first that amino acids in all domains may affect binding, and second that side chains may participate in intermolecular contacts with phospholipid (78). Like calcium binding, phospholipid binding by annexins requires more study to fully understand its mechanism and regulation.

In addition to the classical phospholipid binding, several annexins have been reported to interact with membranes in a calcium-independent manner. Though some of these interactions are believed to occur between the annexins and a proteinaceous component of the membrane or cholesterol, AnxA5 has been reported at low pH to bind to and insert in phospholipid vesicles (296), probably due to acid-induced unfolding resulting in exposure of the tryptophan (46, 181). At neutral pH, the N-terminus of AnxA1 has been proposed to bind phosphatidylcholine membranes following tetrad core binding (56, 57, 227). Thus, membrane binding by annexins may be regulated by the physiological environment or by the makeup of the available membranes.

1.3.2.3. Ion transport. Based on structural observations, ion channel activity has been proposed as a feature of all mammalian annexins (49), and many annexins have been shown to mediate calcium transport across synthetic phospholipid membranes (76, 108, 357). The putative ion channel is contained in the tetrad core, formed by the central hydrophilic pore of the annexin molecule around which are arranged the four annexin repeats. The hydrophilic pore is coated with highly conserved acidic amino acid residues (95, 303). Crystallographic and molecular dynamics simulation studies of AnxA5 and AnxA12 have suggested that the accessibility of the pore is due to intramolecular

flexibility, the so-called hinge movement, between two modules of the core formed by domains I/IV and II/III, respectively (118, 265). The exact mechanism of transport is unknown, but two different models have been proposed: by peripheral membrane binding, leading to electrostatic disturbance of membrane and altered ion conductance, or a change in conformation resulting in insertion into the membrane and deeper penetration of the membrane (303).

1.3.2.4. Annexin-annexin interactions. Various annexin-annexin interactions have been reported, including self-association of AnxA2 (333), AnxA1 (7, 528), and AnxA5 (113, 383, 518, 582), as well as other annexins including AnxA4 (259, 618), AnxA6 (37, 48, 619), AnxA7 (119, 329, 619), and AnxA12 (84, 319, 341, 351). Associations between different annexins have also been reported, including AnxA4-AnxA7 and AnxA6-AnxA7 (618), and AnxA2-AnxA4 and AnxA2-AnxA6 (333). Since annexin-annexin interactions have been demonstrated to alter biochemical properties (119, 333, 369, 458, 528, 618, 619), these interactions may therefore constitute a general mechanism of functional regulation.

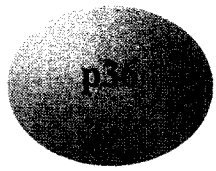
1.3.3. Annexin 2. AnxA2 has been implicated by several studies as a potential CMV receptor (61, 430, 458, 602, 603), but the involvement of AnxA2 in infection of cells is controversial (154, 429).

1.3.3.1. Structure. AnxA2 is a protein of 36 kDa, containing 336 amino acids. Though most cells express a single isoform, two isoforms of AnxA2 have been identified in

human cultured skin keratinocytes (342), with the minor isoform containing two extra amino acids in the N-terminus (570). The significance of the second isoform is unknown. The C-terminal tetrad core contains one type II calcium-binding site in each of domains II, III and IV, and two type III calcium-binding sites in domain I (255, 551). In addition, the presence of at least one additional calcium binding site was suggested by the observation that AnxA2 still displayed calcium-dependent behaviour even when all the known sites were mutated (164), in agreement with the report that p36 could bind 10-11 calcium atoms (156). A unique tryptophan residue occurs at position 212 in domain I, which is postulated to become accessible upon calcium occupation of other sites, and where phospholipid binding is proposed to occur (255), though, like several other annexins, AnxA2 contains a phospholipid-binding consensus site in domain II (382). The N-terminus of AnxA2 is comprised of 36 amino acids, containing binding sites for protein-protein interactions, as well as target sites for post-translational modifications including serine and tyrosine phosphorylation and N-terminal acetylation.

AnxA2 exists in two known forms: the monomeric form, typical of annexins, consisting of one p36 unit, and the heterotetrameric form, called AnxA2t and consisting of two p36 subunits bridged by a dimer of p11 (figure 3). Also called S100A10, p11 is a member of the S100 family of low-molecular weight acidic proteins, characterized by their helix-loop-helix calcium-binding domain, the EF-hand. S100 proteins have been implicated in regulating cytoskeleton assembly, cytosolic enzyme activity, and membrane dynamics (488). While the tetrameric form of AnxA2 is considered to be atypical among annexins, there are two other known instances of S100-annexin interactions: calcyclin (S100A6) with annexin 11 (AnxA11) (530, 531), and S100C (also called S100A11 or

A



B

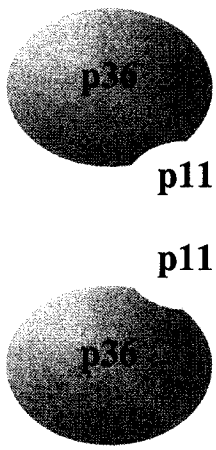


Figure 3. Monomeric and heterotetrameric forms of AnxA2. AnxA2 exists in two known forms: the monomeric form (A) consisting of a single 36-kDa unit, and the heterotetramic form (B) consisting of two p36 subunits, bridged by a dimer of the S100 protein p11.

calgizzarin) with annexin 1 (AnxA1) (494).

1.3.3.2. Functions. The biological role of AnxA2 has not yet been definitely proven, but AnxA2 has been implicated in cell trafficking, fibrinolysis, and cellular signalling, all properties that CMV could exploit to enhance infection. These functions require AnxA2 to be available on the cell surface as well as in the cell. Like all annexins, AnxA2 does not possess a signal sequence, but has been shown to be externalized within 16 hours of synthesis (206) and has been reported to be present on the surface of many cell types (39, 70, 86, 105, 106, 157, 207, 307, 342, 344, 426). In addition to being on the cell surface, AnxA2 has also been reported to be secreted into the extracellular fluid by cell lines (131, 158, 273) and by primary cultures of isolated human tissue (1), and has also been detected in broncheoalveolar lavages (131).

1.3.3.2.1 Cell trafficking. A wealth of evidence implicates AnxA2 in intracellular trafficking, which could promote CMV infection for processes such as cell entry and egress. AnxA2 has been localized within cells to relevant regions such as secretory granules and the plasma membrane (147, 497, 563), as well as early endosomes in the cortical region, the junction in strand structures between aggregated secretory vesicles, between vesicles and the plasma membrane (214, 318, 390, 498), and zymogen granules in pancreatic acinar cells (356). Implicating AnxA2 specifically in endocytosis, AnxA2 has been localized to membrane microdomains (i.e. lipid rafts) in clathrin-coated pits (564), and has co-immunoprecipitated with caveolin (569), implicating it in caveola-mediated transport. AnxA2 has also colocalized in receptor-recycling compartments with

actin, α-actinin, dynein, tubulin, and kinesin (436), cytoskeletal species that undergo rearrangement during trafficking.

In addition to cellular localization, AnxA2 possesses biochemical properties consistent with a role in cellular trafficking. AnxA2 mediates the aggregation and fusion of phospholipid-containing liposomes (59, 336). In cells, AnxA2 was shown to promote aggregation and fusion of chromaffin granules at micromolar calcium concentrations (13, 147), and translocation of AnxA2 to peri-membranous regions of the cell has been found to be required for secretion (92). The specificity of AnxA2 was demonstrated when only AnxA2, but not AnxA1, AnxA5, or AnxA6, restored secretion in chromaffin cells (483). Several studies have implicated AnxA2t, rather than p36, as the species responsible for mediating trafficking, since disruption of AnxA2t complex reduced endocytosis (93, 214, 298), and only AnxA2t, but not p36 alone, could mediate exocytosis (93). The domain responsible for intracellular trafficking appears not to be discrete, but to require several areas in the molecule, since both the N-terminus (92) and the tetrad core (506) are required, but neither alone is sufficient to mediate aggregation and fusion of secretory granules (147) or secretion (12). These observations imply that several domains and activities of AnxA2 are required to mediate intracellular trafficking.

Another important factor implicating AnxA2 in intracellular trafficking is its ability to modulate cytoskeletal elements. AnxA2 has been colocalized with several cytoskeletal elements involved in trafficking (342, 436). AnxA2-mediated exocytosis in chromaffin cells results in cortical actin network disruption, implicating actin in AnxA2-dependent trafficking (94). *In vitro*, AnxA2 was shown to interact directly with actin in a calcium-dependent manner (459), with amino acids 308-316 essential for actin binding (164) and

amino acids 286-294 implicated in actin bundling (252). The importance of the AnxA2-actin interaction was confirmed when AnxA2 was observed to cause the formation of actin bundles in cells, in the presence of calcium (343).

In addition to the typical annexin property of calcium-dependent phospholipid binding, AnxA2 has also been reported to interact with cellular membranes in a calcium-independent manner, likely through specific membrane domains (337, 559, 564). The proportion of calcium-independently bound AnxA2 to cellular membranes has been reported to be as high as 50% (216). The calcium-independent interaction of AnxA2 with membranes may be dependent on associations with proteins, including cytoskeletal proteins, membrane proteins, and caveolin (215, 337), or with lipids, including glycolipids and cholesterol (215, 216). AnxA2 has been proposed to act as an interface between elements of the membrane and of the cytoskeleton (216). The domain of AnxA2 that regulates calcium-independent binding appears to reside in the N-terminus, in the region spanning amino acids 15-24, confirming the observation that AnxA2t complex formation was unnecessary for binding (256, 297), though the formation of AnxA2t may stabilize such an association. It has been suggested that the various mechanisms by which AnxA2 associates with membranes may constitute a form of regulation of association with different membranes.

Interestingly, there are reports in the literature that AnxA2 had no effect in intracellular trafficking (201). Exocytosis occurred in absence of AnxA2 in permeabilized chromaffin cells, though addition of AnxA2t increased rates of secretion (338). These reports, combined with the literature supporting a role for AnxA2 in intracellular trafficking, suggest that AnxA2-dependent and -independent secretion

pathways exist in cells, and perhaps that the importance of AnxA2 in trafficking may vary according to cell type or other conditions.

1.3.3.2.2. Signalling. Though most studies have focused on AnxA2 as a mediator of intracellular trafficking, reports are emerging in the literature further implicating AnxA2 in several different signalling pathways in cells. Since CMV has been shown to trigger signalling cascades in the host cell (64, 505, 616, 617), and since such cell "priming" has been proposed to favour downstream stages in infection (112), the ability of AnxA2 to mediate signalling may be beneficial to CMV. On the cell surface, AnxA2 has been implicated in mediating cell binding and signalling of 1,25-dihydroxyvitamin D3 (38, 39, 371) and tenascin-C (106). AnxA2 has also been shown to colocalize with CD44 in lipid rafts, with extracellular CD44 localization mirroring that of AnxA2 and the cortical actin network on the inner face of the membrane (404). Inside the cell, AnxA2 has been reported to inhibit GTP-γS-stimulated inositol phosphate-3 formation, a process regulated by G-proteins (489), and to inhibit proliferation and DNA synthesis (2, 97). In contrast, AnxA2 induced T cell secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), a stimulator of proliferation in certain bone marrow cells and immune cell precursors (371). AnxA2 has also been shown to bind nucleic acids, raising the possibility that AnxA2 might regulate gene expression. AnxA2 has been reported to bind to DNA (62) and cytoskeleton-associated polysomes containing mRNA (575), and to enhance replication of exogenous DNA in Xenopus (580). AnxA2 may therefore be involved in regulation of signalling by interacting with, and modulating the function of, various species in the cell, including proteins, lipids, and nucleic acids. These multiple

effects suggest that AnxA2 may have different roles on a variety of cell types and molecules.

1.3.3.2.3. Fibrinolysis. Another important physiological process in which AnxA2 has been implicated is fibrinolysis, the process in which the blood enzyme plasmin degrades the fibrin clot following coagulation. Although no link has been shown between fibrinolysis and CMV infection, Herpesviruses are known to produce thrombin on their envelope (535), a process proposed to enhance infection (M. Sutherland and E. Pryzdial, unpublished). Furthermore, plasmin has been implicated in various physiological processes, including proliferation, signalling, and cell trafficking (245, 327, 411, 445). Since these processes may modulate infection, control of the fibrinolytic activity of AnxA2 may therefore confer an advantage to CMV. AnxA2 is believed to be a cofactor for tissue plasminogen activator (tPA), an enzyme that activates plasmin from its precursor plasminogen. AnxA2 was shown to bind tPA, plasmin and plasminogen (86, 207). C-terminal lysine residues are important to these interactions, since they are sensitive to carboxypeptidase B degradation (207). Purified AnxA2 was also shown to accelerate tPA-dependent conversion of plasminogen to plasmin, a process that was inhibited by both carboxypeptidase B and ε-aminocaproic acid (εACA), confirming the importance of the C-terminal lysine (86). AnxA2t has been detected in a blood clot, demonstrating that it is present at a site where fibrinolytic activity would occur (99). Implicating AnxA2 in fibrinolysis in a physiological setting, extracellular matrix remodelling by macrophages, a process mediated by plasmin, was augmented by AnxA2 (70, 71, 157). Interestingly, AnxA2 was shown to inhibit plasmin-dependent lysis of clot

and fibrin polymer, by two mechanisms: stimulation of plasmin autoproteolysis, an εACA-sensitive process (166), and non-competitive fibrin binding, an εACA-insensitive process (99, 100). While at first glance these observations seem to contradict a role for AnxA2 in fibrinolysis, it has been suggested that AnxA2t provides a quick pulse of plasmin activity by first enhancing tPA-dependent plasminogen conversion, followed by quick inactivation of plasmin.

1.3.3.3. Regulation.

1.3.3.3.1. Calcium. Like all annexins, AnxA2 function is regulated by calcium concentration. Calcium is required for AnxA2 binding to membranes, as well as the cytoskeletal elements actin and fodrin (406, 496). The calcium requirement for membrane binding by annexins is in the millimolar range (30, 234, 560, 584), which is not achieved in resting cells and which provides a manner for cells to regulate AnxA2 activity. Like other annexins, calcium occupation and membrane binding of AnxA2 results in a conformational change in both AnxA2t and p36, implying functional changes as well (30, 169, 432). In addition to membrane binding, calcium may be required for intracellular trafficking, including aggregation of phospholipid vesicles (59), binding to actin (459), and exocytosis in chromaffin cells (94). The addition of calcium to cells resulted in the disintegration of cortical cytoskeleton (214) and in translocation of AnxA2 from cytosol to plasma membrane (40, 60), further implicating calcium in regulation of trafficking. The calcium requirement for exocytosis may be a consequence of the calcium requirement for membrane association rather than a direct requirement for trafficking.

Calcium has also been shown to be required for AnxA2 to interact with heparin and heparan sulfate proteoglycans (276) as well as with AnxA5 (69) and AnxA1 (323), the properties and functioning of which may be regulated by their interaction with AnxA2.

1.3.3.3.2. p11. A second very important regulatory factor for AnxA2 is p11, a member of the S100 family of EF-hand type proteins, a group of calcium-binding proteins that have been implicated in calcium signalling (488). Also called S100A10, p11 was first identified as an S100 protein by sequence analysis of a p36-binding protein (182). The binding site for p11 on p36 resides in amino acids 1-9, a region that contains a hydrophobic cluster (45, 192, 248). On p11, the p36 binding site has been localized to amino acids Tyr-85 and Phe-86, located near the C-terminus in a sequence unique to p11 among S100 proteins (310, 311). Other S100 family proteins have been reported as p36 binding partners, notably calcyclin, also known as S100A6 (162, 163, 620) and an unnamed S-100, which can compete with p11 for p36 binding (55), but the significance of these interactions is unclear.

p11 is an important regulator of AnxA2 function. It has been reported that p11 induces conformation change in p36 (30, 169). Though p11 does not itself bind calcium, its interaction with p36 alters the calcium-binding properties of p36 subunit, decreasing its number of calcium binding sites (156). Although p11 has been reported to be unable to bind intracellular membranes on its own, requiring the presence of p36 (92, 406, 621, 622), the binding of p11 to p36, resulting in the formation of AnxA2t, has been shown to diminish the calcium requirement for phospholipid binding (156) and aggregation (334, 338), compared to p36 alone. A potential consequence of the diminished calcium

requirement is that AnxA2t may be better equipped than p36 to mediate trafficking processes at intracellular calcium levels. In addition to lowering the calcium requirement, AnxA2t, but not p36, was shown to mediate phospholipid fusion (334), and has been reported as essential for regulated exocytosis (298). However, whether AnxA2t only, but not p36, mediates fusion of membranes is controversial since p36 has been reported as a mediator of fusion of chromaffin granules (12, 13), though it is possible that p11 existed in the AnxA2 preparation as a contaminant. In addition to calcium and membrane binding, intact p11 binding sites in p36 were shown to be required for association of AnxA2 with submembranous skeleton in cells (550), a potentially important property for trafficking.

Several studies implicate the tetrameric form of AnxA2 in tPA-dependent plasminogen conversion over the monomeric form. AnxA2t and p11 greatly enhanced formation of plasmin, compared to p36 (275). Enhancement of plasmin formation by AnxA2t or p11 was attenuated by inhibition of p11 activity, either by addition of a competing peptide corresponding to amino acids 85-96 of p11, or by mutation of the last 2 amino acids (275). In inhibition of plasmin-dependent fibrin clot lysis, AnxA2t, but not p36, was shown to stimulate autoproteolysis of plasmin (166). It has been argued that p36 alone could bind and accelerate tPA. Homocysteine, which binds to amino acids 7-12 of p36, competitively inhibited tPA-AnxA2 binding (209). It was concluded that a direct tPA-p36 interaction existed, but given that a later study reported an interaction between tPA and p11 only, but not p36 (346), it is more likely that homocysteine inhibits tPA-AnxA2 binding by disrupting AnxA2t formation. Furthermore, AnxA2-dependent enhancement of tPA-mediated conversion of plasminogen was dependent on the presence

of a C-terminal lysine (207), but p36 does not posses a C-terminal lysine. Though p36 has been suggested to undergo proteolysis to expose a C-terminal lysine (207), there is no evidence that such processing occurs. However, p11 does possess a C-terminal lysine, and C-terminal lysine-dependent binding sites for both tPA and plasminogen were identified in p11 (346). Therefore, these studies all point to p11 as an important regulator of the fibrinolytic activity of AnxA2.

1.3.3.3.3. Post-translational modifications. The best-characterized post-translational modifications undergone by AnxA2 are phosphorylations at Tyr-23 and Ser-25, in the N-terminus. p36, first identified as a substrate of the viral kinase pp60v-src (192), is also a substrate of platelet-derived growth factor kinase, though perhaps not directly (65), and the insulin receptor kinase (269). Tyrosine phosphorylation of p36 appears to be a negative modulator of function. Phosphorylation by pp60-vsrc, while having no effect on p11 binding or phospholipid binding, was reported to inhibit actin binding and bundling, chromaffin granule aggregation, cortical granule bridging to the plasma membrane, and heparin binding (234, 439).

p36 has also been identified as a substrate of the serine-threonine kinases, notably protein kinase C (PKC), and also cAMP- and calmodulin-dependent kinases (249). The main phosphorylation site has been identified as Ser-25 (199), though other serines exist in the N-terminus, and different populations of serine-phosphorylated AnxA2 have been identified based on functional differences (249). Though the phosphorylation sites for Tyr-23 and Ser-25 are very close together, the two residues are on opposite sides of the molecule, implying that both sites may be phosphorylated at once (552). Indeed, though

p36 may be either serine- or tyrosine-phosphorylated, but not both, AnxA2t has been reported as being phosphorylated at both sites (459). Accounts of the effects of serine phosphorylation on AnxA2 function vary, possibly due to the multiple phosphorylation sites available, which could affect functional properties in different ways. Serine phosphorylation of AnxA2t had no discernible effect on binding of F-actin (459) or phospholipid (29, 250, 459). PKC phosphorylation of AnxA2t, presumably at Ser-25, was reported to enhance exocytosis (120, 141, 483). However, PKC reportedly decreased the rate and extent of aggregation of liposomes and to increase the requirement for calcium (29, 250). This apparent contradiction may be explained by the observation that serine phosphorylation by either cAMP- or calmodulin-dependent kinases, but not PKC, prevents AnxA2t formation (249), which may be explained by phosphorylation at or near the p11-binding site, i.e. not at Ser-25. This notion was confirmed by the disruption of AnxA2t formation following Ser-2 phosphorylation (254), and by a Ser-11-Glu mutant, which mimics the structure of serine phosphorylation, which had decreased affinity for p11 (29). Since p11 regulates calcium and membrane binding, impaired p11 association could explain the observed decrease in lipid aggregation and increased calcium requirement upon phosphorylation (29, 250). The cell type and the kinase could play an important role in the phosphorylation site, thus differentially regulating AnxA2 function.

AnxA2 is also known to undergo other post-translational modifications. The N-terminal amino acid is acetylated, which has been found to be important in p11 association to short peptides corresponding to the extreme N-terminus (45, 248). A full-length recombinant p36 expressed in *E. coli* was not N-terminally acetylated, but no difference was noted in p11 binding, phospholipid binding, aggregation, or actin binding

(47). It is possible that N-terminal acetylation is a contributing factor to stabilizing the AnxA2t complex, but it may not be essential. However, acylation of other proteins has been shown to influence their localization in the cell to certain membrane microdomains, including sphingolipid-enriched domains and caveolae (332, 465, 499). Since AnxA2 has been reported to associate with certain membrane domains including lipid rafts and caveolae (31, 215, 564), acetylation of AnxA2 could play a role in its intracellular localization.

1.3.4. Annexin 5. AnxA5 interacts directly with AnxA2 (69), and furthermore has been reported to inhibit AnxA2-mediated fusion of CMV to liposomes (458). We hypothesized that the inhibition of fusion was due to the direct interaction between AnxA2 and AnxA5, that that this binding could inhibit CMV infection by interfering with AnxA2-mediated events.

1.3.4.1. Structure. AnxA5 is a 316-amino acid protein, consisting of a short N-terminus of 18 amino acids, plus the tetrad core. Two isoforms have been identified on the basis of their apparent size, 33 kDa and 37 kDa, by SDS-PAGE. These two species differ by two amino acid substitutions at positions 36 and 125 in the tetrad core, which is proposed to result in an alteration in the compactness of the molecule (79, 322). The tetrad core of AnxA5 has been revealed by several crystal structures to contain calcium-binding sites in all four repeats, with type II sites in the classical endonexin folds in domains I, II, and IV, and two weaker-affinity sites in domain I (52, 238, 239, 324). Domain III contains a unique tryptophan residue which is exposed upon calcium binding, and which contains

the classical phospholipid binding site (78, 79, 114, 521). Calcium binding to AnxA5 is cooperative (492). Crystal structures have revealed that two calcium ions bridge the contact between the phosphatidylserine headgroup and the tryptophan residue in domain III (536). However, other domains have also been proposed to possibly mediate phospholipid binding (78, 240, 363), including a consensus sequence in domain I (79, 382). These phospholipid- and calcium-binding sites are thought to confer the phospholipid specificity upon AnxA5, which interacts with several different anionic phospholipids in addition to phosphatidylserine, including blends containing oleic acid and phosphatidylcholine (363), phosphatidylethanolamine (59, 526), and cholesterol (28), but not phosphatidylcholine alone (59, 363, 538), sphingomyelin (526), or cholesterol alone (526). AnxA5 has a much shorter N-terminus than either AnxA1 or AnxA2, of only 18 amino acids. While the function of the N-terminus in AnxA5 has not been defined, a possible role in stabilization of the conformation of the tetrad core has been suggested (26).

In addition to its monomeric form, AnxA5 has also been shown to self-associate into trimers, higher-order oligomers, and large two-dimensional crystals on the surface of lipids that cover the monolayer surface (87, 168, 383, 433, 454, 464, 582). Self-association occurs only upon membrane binding (113, 379) and coincides with a conformational change (405, 582). The structure of the trimer has been shown to involve a basic residue cluster, located mainly in domain I, which is also involved in membrane binding (379).

1.3.4.2. Functions. Like all annexins, no biological function for AnxA5 has been definitively identified, though the *in vitro* properties of AnxA5 suggest involvement in regulation of coagulation, ion transport, and signalling, all of which could affect CMV infection. AnxA5 is most abundantly expressed in the cytosol of cells, but has also been found on the inner face of the plasma membrane (185, 196, 266, 301, 589), and in the nucleus (41, 301, 534). Within the cytosol, AnxA5 has been detected near intracellular membranes such as the endoplasmic reticulum (185), calcium-segregating organelles (144, 145, 184, 524, 589), and mitochondrial membranes (367, 368, 533). AnxA5 has also been reported to be present to the cell surface (305, 413, 451) and to be secreted into the medium (301, 510).

1.3.4.2.1. Anti-coagulation. AnxA5 has been implicated as an anti-coagulant. Herpesviruses are known to produce thrombin on the envelope (535), which has been proposed to enhance infection (M. Sutherland and E. Pryzdial, unpublished). Therefore, the anti-coagulant property of AnxA5 could counteract CMV infection. Purified AnxA5 has been shown to inhibit the generation of thrombin (89, 452, 572) and factor Xa (340, 572), to prolong blood clotting time (467), and to neutralize the pro-coagulatory effect of activated platelets (467). In animal models, AnxA5 has also been reported to inhibit fibrin generation (101, 573), fibrin accumulation (549, 572), platelet adhesion (549, 572), and thrombosis (468, 549). The basis for the anti-coagulatory effect is believed to be the formation of a shield or lattice over the membrane, resulting in the protection of anionic phospholipids from binding by coagulation factors. This hypothesis is consistent with the observations that AnxA5 anti-coagulatory effects are dependent upon calcium- and

phospholipid-binding (24, 79, 467) and that AnxA5 reduces the lateral mobility of prothrombin and factor Xa on the membrane (24).

Evidence for the biological significance of the anti-coagulant activity of AnxA5 is provided by pathological states in patients with low AnxA5. Decreased AnxA5 binding has been associated with recurrent pregnancy loss and preeclampsia (79, 448, 451, 504), and has also been suggested to be the mechanism behind pro-coagulant phenotypes observed in systemic lupus erythematosus, due to anti-phospholipid or anti-AnxA5 antibodies (79, 258, 446, 447, 449, 450, 451, 486, 532). These studies suggest that AnxA5 acts to preserve blood fluidity by inhibiting thrombosis, implicating AnxA5 in the maintenance of blood hemostasis.

1.3.4.2.2. Ion transport. CMV infection has been shown to coincide with influx of calcium into the cell (173, 282, 396). This process may be regulated by AnxA5, which has been suggested to play a role in ion transport. AnxA5 has been localized to calcium-segregating organelles such as the sarcolemma, sarcoplasmic reticulum, transverse tubules, and intercalated disks in heart tissue (145, 184, 444, 524, 589), mitochondrial membranes (367, 368, 533), synaptic vesicles in axons (198), atrial specific secretory granules (144), and matrix vesicles in chondrocytes (27, 180, 229, 444). Localization to these structures, which regulate ion release, suggests that AnxA5 may play a role in modulating ion transport across membranes.

AnxA5 has been reported to mediate transport of calcium across synthetic membranes (50, 51, 243, 357). However, although ion transport in liposomes has been observed or proposed for all annexins, AnxA5 is the only annexin for which ion channel

activity has been observed in living cell membranes. AnxA5 has been shown to mediate calcium influx into the calcium-accumulating matrix vesicles in chondrocytes (27), and into cells during apoptosis (312). Interestingly, uptake of calcium by chromaffin granules in adrenal medulla cells was inhibited by the addition of AnxA5 (165). These results indicate that AnxA5 may modulate calcium transport into living cells as well as into liposomes.

Ion transport is believed to occur through the central hydrophilic pore of the protein, formed by the tetrad core (466). Residues within the hydrophilic pore regulate voltage dependence of channel gating by charge alteration (142, 328), and ion selectivity (51, 77, 328). AnxA5 is most selective for calcium, but also regulates the passage of other divalent cations such as barium, strontium and magnesium, and monovalent cations such as lithium, scandium, sodium, and potassium (466). Ion transport activity is also altered by lipid composition of the membrane, with high phosphatidylserine concentrations causing high calcium influx (291).

Structures have provided clues to the mechanism of ion transport. The current model consists of the so-called hinge movement between the relatively stable domain I/IV module and the more flexible domain II/III module, with calcium occupation favouring the open conformation (77, 118, 405). A crystal structure of AnxA5 complexed with K201, a calcium channel blocker, has revealed that K201 binds in the putative hinge cavity region (265), further supporting the notion that ion transport occurs through the central pore. Hinge movement may correlate with the proposed local disordering of the membrane following AnxA5 binding, allowing the passage of ions (67, 238, 240).

AnxA5 has been proposed to form two types of channels: the first occurs at low concentration, consists of membrane adsorption of monomeric AnxA5, and is regulated by the voltage across the membrane, since ion permeation occurs in discrete conductance states (228, 237, 238, 393). The result of voltage regulation is that ion transport occurs in bursts due to the quick opening and closing of the channel (228, 393, 466). The second type of channel postulated is more controversial. This channel is proposed to occur at higher protein concentrations, likely forming a dimer of trimers that penetrates the membrane completely to the other side (243, 291, 393). Though formation of ion channels is dependent on membrane binding, AnxA5 has been reported to form tight associations with membranes in the presence of calcium that are subsequently resistant to EDTA chelation or Triton X-100 extraction in several different tissues (54, 304, 444), further supporting the notion of insertion into the membrane. However, membrane electroporation has been observed to be decreased upon increasing protein concentration (228, 554). More investigation is needed to further characterize the ion transport properties of AnxA5.

1.3.4.2.3. Signalling. AnxA5 has been implicated in regulation of intracellular signalling, a property that could affect CMV infection events. AnxA5 has been suggested to regulate intracellular trafficking due to localization to structures such as atrial specific secretory granules (144) and synaptic vesicles in neuronal cells (198). AnxA5 has been colocalized with the cytoskeleton in cells, specifically with γ-actin, which it binds in a calcium-dependent manner (294, 566, 567, 568). AnxA5 has also been found to interact with lipid microdomains in the membrane, specifically with glycerophospholipid-containing

microcompartments, at which AnxA2 was not co-detected (31). Importantly, in addition to intracellular localization, AnxA5 has been shown to be inhibitory to trafficking events, inhibiting liposome aggregation, possibly by steric hindrance (296) or by competing for binding sites with other annexins (25). AnxA5 was also observed to inhibit fusion of liposomes with isolated cell membranes (408) and AnxA2-mediated fusion of CMV (458), and was shown to attenuate secretion in neutrophils (469).

In addition to regulating intracellular trafficking, AnxA5 has been proposed as an inhibitor of apoptosis (187, 539), a property that may be dependent upon ion transport or phospholipid binding (188). AnxA5 has been proposed to be a negative modulator of proliferation, since AnxA5 levels have been reported to be elevated in non-proliferating cells (145, 184, 185, 444, 524, 589), and in cells treated with anti-proliferative stimuli (278, 279, 501, 609). Several mechanisms have been proposed. AnxA5 has been reported to suppress the pro-mitotic Ras/ERK signalling pathway (485). AnxA5 has also been reported to interact with vascular endothelial growth factor receptor (593), heparin and HSPs (82, 83, 244), and the cytoplasmic tail of the β5 subunit of αvβ5-integrin (20), all of which are involved in mitotic signalling.

Another potential mechanism of signalling regulation consists of inhibition of PKC. Though AnxA5 has been reported to be phosphorylated *in vitro* by PKC (322), no PKC phosphorylation has ever been detected in cells (110, 455). AnxA5 has been shown to inhibit PKC-mediated phosphorylation of several substrates (246, 455, 474, 491, 500), including AnxA1 (455, 474, 491) and AnxA2 (149), but AnxA5 has no effect on PKC auto-phosphorylation (455), or on kinase activity of calcium-independent PKC, EGFR, or cAMP-dependent kinase (474, 491), indicating specificity of inhibition. AnxA5-mediated

inhibition of PKC activity has been shown to be dependent on calcium (148, 500) and membrane binding (148, 455, 491). However, phospholipid sequestration is unlikely to be the mechanism of inhibition, since it could be only partly reversed by the addition of more phospholipid (149, 474, 491, 500). Inhibition probably occurs by a direct and specific interaction between AnxA5 and PKC (491, 560).

1.3.4.3. Regulation.

1.3.4.3.1. Calcium. Like all annexins, membrane binding by AnxA5 requires calcium. Calcium has also shown to regulate the phospholipid specificity of AnxA5, with increasing calcium associated with a preference for anionic phospholipids (23, 347), and with tighter binding affinity (228). Calcium levels may also influence intracellular distribution, with an elevation in concentration associated with a translocation from a soluble or protein-bound phase to a phospholipid-bound phase, including at the inner face of the plasma membrane, the nuclear membrane, and the cytoskeleton (40, 60, 294, 380, 456, 560, 565, 567, 568). Calcium has also been shown to induce a tight association with phospholipid membranes that is resistant to calcium chelation (23, 60), suggesting the interesting notion that calcium occupation induces calcium-independent binding.

1.3.4.3.2. Self-association. AnxA5 has been reported to self-associate into trimers, higher-order oligomers, and two-dimensional lattices upon membrane binding that forms a shield over the monolayer (87, 113, 379, 383, 433, 454, 464, 518, 582) and results in a conformational change (392, 431, 582). In addition to its effect on its own conformation,

self-association of AnxA5 has been proposed to impact membrane fluidity and permeability (113). AnxA5 two-dimensional lattice formation, induced at high AnxA5 concentrations, caused complete coverage of bilayers and concurrent near-immobilization of both the phospholipid and the AnxA5 (87). This observation confirmed and clarified earlier reports of decreased lateral mobility of phospholipids upon AnxA5, possibly by increasing the rigidity of the phospholipids and altering the organization of the vesicle (362, 369, 537). The observation that AnxA5 may reduce fluidity of model membranes upon self-association was confirmed in isolated mitochondrial membranes, with increased rigidity of the inner leaflet observed. Rigidity of cardiolipin was observed to be more strongly affected than other lipids in the mitochondrial membranes, while in vesicles cardiolipin was affected to the same degree, suggesting that AnxA5 exerts a specific effect on mitochondrial function and that other factors may regulate membrane fluidity (367).

The shielding effect exerted by AnxA5 upon self-association, and subsequent decrease in membrane fluidity, is believed to be the basis for the anti-coagulant property of AnxA5, as well as its observed inhibition of PLA2 activity (146, 159, 418, 571). The latter activity is not due solely to phospholipid sequestration since partial AnxA5 coverage of liposomes results in almost complete inhibition of PLA2-mediated lipid hydrolysis (523), and since more calcium is required for inhibition than can be accounted for by binding requirements (73). The effect of AnxA5 on membrane fluidity and the high calcium requirement for self-association (518) may reconcile these observations.

1.3.4.3.3. pH. Under mildly acidic conditions, AnxA5 has been reported to bind liposomes in the absence of calcium, possibly due to a charge difference in the calcium-binding pockets (296). Furthermore, in contrast to its inhibitory effect on aggregation at neutral pH, AnxA5 was reported to promote aggregation of liposomes in a calcium-dependent manner (227, 296). AnxA5 was also reported to self-associate more readily (519) and to insert into the membrane, forming ion channels (243). These altered properties of AnxA5 under acidic conditions are proposed to reflect a difference in charge in the calcium-binding pocket in domain III (296, 519), and could be an important form of regulation of the properties of AnxA5 in structures such as mitochondria and endosomes.

1.3.5. Annexin 1. Annexin 1 (AnxA1), like AnxA5, has been reported to interact with AnxA2 in a calcium-dependent manner (323), though no link has been reported between this interaction and CMV infection. Since annexin-annexin interactions have been proposed as a mechanism of functional regulation (618), we hypothesized that the binding of AnxA2 by AnxA1 might inhibit CMV infection, by interfering with AnxA2-mediated events.

1.3.5.1. Structure. AnxA1 is a protein of 37 kDa, containing 349 amino acids. The tetrad core contains at least six calcium-binding sites (471), with the phospholipid binding site proposed to reside in domain I (152, 153), though no tryptophan residue is contained in this domain. The N-terminus consists of 46 amino acids and contains several unique features, including sites for protein-protein interaction, phosphorylation, and proteolytic

cleavage. Crystal structures of calcium-bound and calcium-free AnxA1 have revealed that, in the absence of calcium, the N-terminus interacts with the tetrad core, with the helical domain encompassing amino acids 2-12 inserting into the core and displacing helix D on domain III. Helix D is unwound and covers the exposed part of the N-terminus (470). Upon calcium-binding, however, helix D is back in place in the core, with the N-terminal helix expelled and disordered (471). The N-terminus also contains binding sites for two calcium ions (471) and an additional binding site for phospholipids which is formed by a conformational change upon phospholipid binding by the core domain (135).

In addition to the monomeric form, AnxA1 exists in several additional forms. AnxA1 exists in at least three isoforms in esophageal epithelia, likely due to proteolytic cleavage (608). AnxA1 has also been shown to exist in covalent dimers, formed enzymatically by transglutaminase (440). There is also evidence that AnxA1 may exist in a heterotetrameric form similar to AnxA2t, interacting in a calcium-dependent manner with S100C, also called S100A11 or calgizzerin (350, 462). Binding, which is maintained by a hydrophobic interaction (143), has been localized to amino acids 1-12 on AnxA1 (350, 462, 494) and to amino acids 91-94 of S100C (143, 494).

1.3.5.2. Functions. While no biological function has yet been identified for AnxA1, it has been implicated in several processes, notably in cell trafficking, anti-inflammatory actions, and proliferation and apoptosis. AnxA1, first identified as a substrate of EGFR (232), is widely expressed, generally in the cytoplasm of cells, but has been reported in the nucleus (41, 277, 402, 403) and in mitochondria (615). AnxA1 has also been detected

on the cell surface (102, 292, 427, 543) and has been reported to be secreted (85, 90, 91, 111, 268, 358, 529, 576, 579), though the secretion pathway of AnxA1 is unknown since there is no signal sequence in any annexin. AnxA1 secretion in some cell lines has been shown to be insensitive to inhibitors of the classical endoplasmic reticulum-Golgi pathway (111, 428), but alternatively may utilize ATP-binding cassette transporters for export (91). Another report has shown that AnxA1 localized inside secretory granules was released upon fusion of the granules with the plasma membrane (419). There may therefore be multiple secretion pathways for AnxA1, depending on cell type or stimulation.

1.3.5.2.1. Cell trafficking. Like AnxA2, AnxA1 has also been implicated in intracellular trafficking, a property that could affect CMV infection. AnxA1 has been localized to various locations in the cell suggestive of a role in trafficking, although not identical to AnxA2, which suggests different roles. In the cell, AnxA1 has been localized to secretory granules (195, 401, 402, 403, 469), and to the inner leaflet of the plasma membrane in cells of the anterior pituitary (160, 191, 403, 563). AnxA1 has also been reported to be associated with the surfactant layer in lung alveolar cells (358).

Like AnxA2, purified AnxA1 has been reported to aggregate liposomes (191, 365) as well as biological membranes in cells (108, 366, 388). Whether AnxA1 can mediate fusion in addition to aggregation is controversial. Unlike AnxA2, AnxA1 has failed to enhance fusion proper between phospholipid vesicles (59, 170, 365). However, AnxA1 has been shown to accelerate the fusion of vesicles containing lipid components of lung surfactant (299) and of liposomes to isolated biological membranes (170, 366,

408), and was reported to enhance calcium-stimulated secretion in permeabilized neutrophils (469). AnxA1 may participate in a multiprotein system to mediate membrane-membrane contact, regulating aggregation and fusion (366).

While the AnxA1 aggregation and cell trafficking are dependent on the tetrad core-mediated binding to membranes (152), the N-terminus has also been implicated in aggregation (25, 57, 227). A secondary binding site exists in this area (58), a phosphatidylcholine-binding site postulated to be formed by a conformational change upon membrane adsorption by the tetrad core (25, 136, 614). Though a tryptophan residue is located in the N-terminus, it is not believed to mediate the secondary membrane binding (58, 361), since such binding occurs in a hydrophobic, calcium-independent manner (58, 473). Unlike AnxA2, in which two p36, or one tetramer, links two membranes (338), it is thought that AnxA1 laterally self-associates on a membrane surface (58), and that one layer of AnxA1 monomer links two apposing membranes (58, 136, 365). The presence of the secondary binding site is therefore probably essential to the ability of AnxA1 to mediate aggregation.

Further evidence of a role for AnxA1 in cell trafficking is provided by reports of intracellular colocalization or direct interaction with several cytoskeletal proteins, including F-actin, profilin, cytokeratins 8 and 18, tubulin, dynein, actin, tubulin, kinesin, vinculin and spectrin (18, 127, 191, 314, 436, 558). In particular, AnxA1 was shown to induce bundling of F-actin (191) and to abrogate the inhibitory effect of profilin on actin polymerization (19). These observations suggest that AnxA1 may regulate membrane-cytoskeleton reorganization during trafficking events.

1.3.5.2.2. Anti-inflammation. CMV infection results in pro-inflammatory effects in the host cell, which may be exploited by the virus to enhance infection (3, 397, 502). The anti-inflammatory properties AnxA1 could play an important role in inhibition of CMV. AnxA1 has been implicated as a mediator of glucocorticoid inhibition of inflammation. Exposure of cells to glucocorticoid treatment has been reported to induce AnxA1 expression (17, 125, 186, 360, 378, 434, 527, 545, 579, 601, 613), translocation to the cell surface, (122, 360, 514, 541, 542, 545), and secretion (4, 85, 90, 91, 111, 211, 353, 358, 377, 378, 514, 579). Pro-inflammatory stimuli also alter AnxA1 expression, including induction upon tissue injury (111, 316, 577) and inhibition of expression by IL-1β (123).

Induction and translocation of AnxA1 has been proposed as a mechanism by which glucocorticoids mediate their anti-inflammatory effect. Though several AnxA1-independent anti-inflammatory actions have been observed for glucocorticoids (68, 88, 354, 434, 545, 578), in cells, AnxA1 has been observed to reproduce the inhibitory effect of glucocorticoids in response to various pro-inflammatory stimuli, including the attenuation of induction and activation of PLA2 (123, 579), inducible nitric oxide synthase (iNOS) (376, 604, 613) and cyclooxygenase-2 (COX-2) (376). AnxA1 has also been shown to mimic the inhibitory effect of glucocorticoids on the secretion of stress-response hormones (540, 541, 543), arachidonic acid and its derivatives (17, 124, 376, 529), and hormones from the anterior pituitary such as prolactin (542, 545) and thyroid-stimulating hormone (543). AnxA1 has also been shown to be inhibitory to *in vitro* models of inflammation, including cell recruitment and attachment to endothelium (424, 515).

In addition to cells, AnxA1 has also been shown to mimic the inhibitory effect of glucocorticoids on pro-inflammatory events in animal models of inflammation. Administration of AnxA1 was observed to attenuate generation of reactive oxygen species (611) and arachidonic acid (81, 612), as well as induction of iNOS (72). Indicating a physiological role in inflammation in animal models, AnxA1 was shown to inhibit the inhibition of recruitment of neutrophils and eosinophils to sites of inflammation (14, 107, 128, 183, 202, 217, 330, 353, 402, 416, 417, 420, 421, 424, 547, 611), as well as swelling of skin and joints (5, 150, 202, 241, 339, 611) and ischemia/reperfusion injury (128, 315). Mouse AnxA1 knockouts were recently generated, which provided more insight into the possible role of AnxA1 in inflammation. The knockout mice had altered expression of other annexins, COX-2, and PLA2, exaggerated inflammatory responses including increased cell migration and IL-1β generation, resistance to the anti-inflammatory effect of glucocorticoids, and anomalies in phagocytosis (213).

The exact mechanism of AnxA1 as an anti-inflammatory agent is under debate, and it is possible that AnxA1 may exert more than one activity since different domains of the protein have been shown to mediate anti-inflammatory effects. AnxA1 has been observed to inhibit PLA2 activity *in vitro* by direct interaction using amino acids 275-346 (287, 288, 290). There is some evidence that domain IV of the tetrad core may be important for PLA2 (290, 374), though domain I has also been implicated (400). The discrepancy may be due to the fact that PLA2 inhibition has been suggested as a more general characteristic of annexins (159), consisting of substrate depletion, whereby the phospholipid is bound by the annexin and protected from lysis, rather than by direct

inhibition of the enzyme (42, 74, 205). AnxA1 may inhibit PLA2 by more than one mechanism, including substrate depletion.

The N-terminus of AnxA1 has also been implicated in anti-inflammatory activity, since peptides corresponding to amino acids 1-188 inhibited hormone release (117, 541, 542, 543) and iNOS induction (604). A peptide corresponding to amino acids 2-26 inhibited release of hormones (246), arachidonic acid and its derivatives (161, 376), iNOS induction and nitric oxide release (264, 376), as well as PLA2 activity (481). A peptide corresponding to amino acids 13-25 inhibited arachidonic acid release (124) and cPLA2 activation (123), by blocking Grb2, p21, and Raf recruitment to EGFR (122). A core peptide corresponding to amino acids 18-25, including Tyr-21, was shown to be indispensable, though insufficient, to inhibit arachidonic release (121). Together, these observations suggest that AnxA1 may contain many anti-inflammatory domains, including in the N-terminus and tetrad core.

In addition to acting intracellularly, AnxA1 is believed to mediate its anti-inflammatory effects by a paracrine or juxtacrine mechanism, whereby AnxA1 is released from one cell type to act on neighbouring cells. In support of this theory, several cell types have been identified that possess the ability to bind AnxA1, both constitutive (103, 155, 200, 410, 557), and inducible, following pro-inflammatory stimulation (155, 482). There is evidence that the AnxA1 cellular receptors for anti-inflammatory activity consist of the formylated peptide receptor (315, 423, 587) and its analogue, the lipoxin A_4 receptor (174), both of which are involved in transmission of inflammatory signals. Collectively, these data provide several models by which AnxA1 mediates its many anti-inflammatory activities.

1.3.5.2.3. Signalling. AnxA1 has been implicated in regulation of intracellular signalling, a property that could affect CMV infection events. AnxA1 has been shown to inhibit cellular proliferation (263, 289) and specifically to attenuate the pro-mitotic ERK signalling cascade (15). Interestingly, there is evidence that AnxA1 may also augment proliferation by stimulating insulin secretion in pancreatic cells (231, 600). Together with its ability to inhibit cell growth, AnxA1 has also been shown to bind RNA and DNA (224) and to possess DNA-unwinding and re-annealing activity (225), with the possible implication that AnxA1 may regulate DNA-related signalling events.

AnxA1 has also been reported to mediate pro-apoptotic events. AnxA1 has been observed to delay necrosis and promote apoptosis (478). AnxA1-induced apoptosis has been observed to be associated with elevation of intracellular calcium, shedding of L-selectin, and activation of caspase-3 (511, 512) and, in an apparent contradiction to its known properties, increased PLA2 activation (80, 511), with a peptide corresponding to amino acids 1-144 mimicking the effect of full-length AnxA1 (511). Interestingly, there is also a report of AnxA1 as an inhibitor of apoptosis; purified AnxA1 was shown to inhibit TNFα-induced apoptosis of leukemic cells by inhibiting PLA2 activation (606). To explain the apparent paradox in AnxA1 activity, it has been reported that Tyr-21 phosphorylation of AnxA1 leads to its activation of PLA2, while the unphosphorylated form is inhibitory (134). This report is in agreement with the localization of the anti-proliferative activity in the N-terminus, specifically on the Tyr-21 residue, implicating phosphorylation in the regulation of the effect of AnxA1 on proliferation (126, 263, 370).

In contrast to reports implicating the N-terminus in regulation of signalling, proliferation was reported to be inhibited by peptide 27-346, but not 1-26 (194). ATP, which is required for DNA unwinding (225), has been shown to bind to AnxA1 in domain I of the core (212). It is possible that, as in inflammation, different domains of AnxA1 may mediate distinct anti-proliferative and pro-apoptotic effects, and the effects of AnxA1 may vary according to stimulus or cell type.

1.3.5.3. Regulation.

1.3.5.3.1. Calcium. Like all annexins, some AnxA1 functions are regulated by calcium, allowing AnxA1 to respond to intracellular calcium concentration fluctuations. In addition to membrane binding, purified AnxA1 has been shown to undergo a calcium-dependent conformational change in the presence of calcium (470, 471, 490), unmasking new sites for binding of calcium (472).

There is evidence that separate calcium-binding sites in the tetrad core control binding and aggregation, as well as phospholipid specificity. The type II site in domain II is essential for liposome binding but not directly involved in aggregation, while loss of the type II binding sites in domains III and IV resulted in loss of liposome aggregation (56). Interestingly, when all type II sites were mutated, the intact type III sites provided residual binding and aggregation, but mutation of all type III sites had little effect on these parameters (56), further contradicting the dogma regarding type III sites as the phospholipid-binding sites. Calcium-binding sites may also be responsible for

phospholipid and membrane specificity. A site in domain I has been shown to be responsible for phosphatidylserine preference (556), while a site in domain II was important for endosomal localization (461). In addition to the core-domain calcium binding, the N-terminus calcium-binding sites have also been shown to influence endosomal localization (461, 495).

Calcium also regulates the association between AnxA1 and S100C. Unlike the p36-p11 interaction, the AnxA1-S100C complex formation is dependent on the presence of calcium (389). AnxA1 contains a calcium-dependent conformational switch, resulting in a conformational change (470, 471, 490) and unmasking of the binding site for S100C (471), located in a hydrophobic cluster in amino acids 1-12 (350, 462, 494). Reciprocally, S100C also undergoes a conformational change upon calcium binding, resulting in the unmasking of the hydrophobic area that constitutes the AnxA1 binding site, amino acids 91-94 (16, 143, 494).

In addition to association with membranes and S100A11, calcium also regulates other AnxA1 activities, including association with F-actin (490), transglutaminase-mediated crosslinking of AnxA1 (22), DNA unwinding and annealing (225), and proteolytic cleavage (85).

1.3.5.3.2. S100C. Like p11, S100C is a member of the S100 family of EF-hand calcium-binding proteins. Though crystal structures have intimated that a tetrameric AnxA1 (AnxA1t) complex may form, similar in structure to the AnxA2t (325), the functional characteristics of AnxA1t are still mostly unknown. AnxA1 and S100C have colocalized in cells at early endosomes and on the cell surface, though they were both detected

separately as well (53, 495), suggesting that formation of the AnxA1t complex may regulate cellular localization. In addition, only the tetrameric form of AnxA1 exhibited DNA binding, unwinding and reannealing, as well as ATP-hydrolyzing activity (224, 225). While the possible roles of AnxA1t have not yet been fully explored, the data currently available suggest that S100C binding may functionally regulate AnxA1 properties.

1.3.5.3.3. Post-translational modifications. Like AnxA2, AnxA1 activity is regulated by phosphorylation of both tyrosine and serine residues. AnxA1 is a tyrosine phosphorylation substrate of EGFR (232, 490) and hepatocyte growth factor receptor kinase (507), and has been reported to be tyrosine phosphorylated upon cellular stimulation with growth hormone, angiotensin II, arginine-vasopressin, and endothelin I (479, 480). The phosphorylation site for EGFR exists at Tyr-21 in the N-terminus (193, 574). Tyrosine phosphorylation has been reported to modulate several properties of AnxA1, including conversion of calcium-dependent to calcium-independent binding to intracellular membranes (172), sensitivity to proteolytic cleavage (21, 22, 205) and inhibition of arachidonic acid release (124). These observations suggest tyrosine phosphorylation may regulate the activity of AnxA1 in cell trafficking and inflammation.

Like AnxA2, AnxA1 is also a substrate for PKC serine phosphorylation, at Ser-27 in the N-terminus (574, 597). Serine-phosphorylated AnxA1 exhibits normal membrane binding (251), but is less able to aggregate liposomes and requires more calcium to do so, due to a conformational change (251, 438, 590, 591). While serine phosphorylation does not affect self-association (438), phosphorylated AnxA1 interacts with the non-

phosphorylated form and interferes with its ability to aggregate liposomes (590). Serine phosphorylation has also been shown to alter intracellular membrane targeting, including cell-surface translocation (53, 246, 313, 513), perhaps by disrupting the AnxA1-S100C complex formation, since S100C protects AnxA1 from PKC phosphorylation (389). Since binding, aggregation, and intracellular localization are important factors in the role of AnxA1, the collected evidence therefore suggests that serine phosphorylation regulates the trafficking and anti-inflammatory properties of AnxA1.

There are reports in the literature that AnxA1 may be phosphorylated at other sites, including Thr-24 and Ser-28 by PKC (286, 591), Thr-216 by cAMP-dependent kinase (574), and an unidentified histidine residue in the tetrad core (387). While the functional consequences of these phosphorylations are unknown, it is interesting to note that some these phosphorylations occur in the tetrad core of the molecule, suggesting possible involvement in calcium or phospholipid binding.

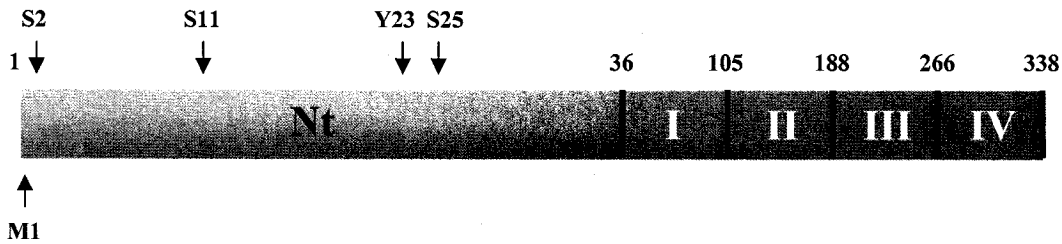
In addition to phosphorylation, AnxA1 also undergoes proteolytic cleavage at Leu-8, Lys-9, Trp-12, and Lys-26 (21, 104, 205, 335, 384, 385, 591). Proteolytic cleavage of AnxA1 has been reported to be calcium- and phospholipid-dependent, with the phosphorylated form preferentially cleaved over the non-phosphorylated form (104, 490). Proteolytic cleavages of AnxA1 have been reported to affect calcium binding, with increased sensitivity conferred following cleavage at Leu-8 and Lys-26 (384, 591), and decreased sensitivity conferred following cleavage at Trp-12 (591). Truncation has been reported to affect intracellular localization (85, 186, 385), and may promote stability of the protein (205). Interestingly, peptides corresponding to the N-terminus have exhibited

anti-inflammatory activity, suggesting that proteolytic cleavage may also regulate that activity of AnxA1.

1.4. OBJECTIVES.

To clarify the apparent discrepancy between the biochemical and the cellular literature, the first objective of this study was to systemically investigate the role of AnxA2 at various steps within the CMV cell infection cycle. AnxA5 has been shown to inhibit AnxA2-mediated fusion of CMV with liposomes (458), and both AnxA5 and AnxA1 bind directly to AnxA2 (69, 323). The second objective of this study was therefore to determine the effect of AnxA5 and AnxA1 on CMV infection, and whether any effect was related to their interaction with AnxA2.

A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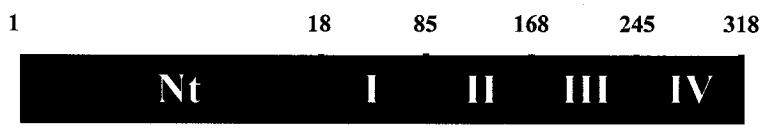
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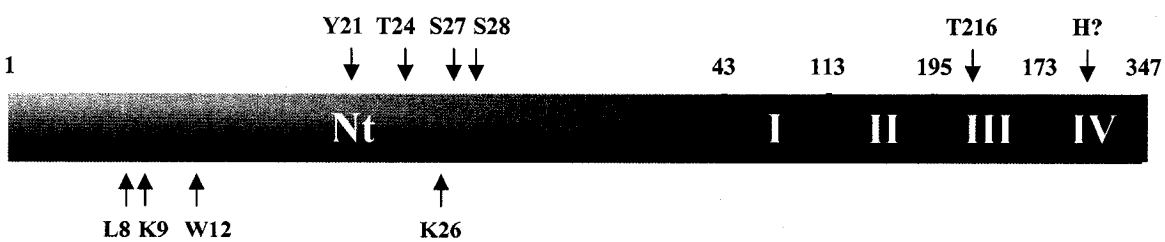
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T216
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43
113
195
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347
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L8 K9 W12
K26

Figure 4. Post-translational modifications of annexins. Some annexins contain sites for post-translational modifications in their unique N-termini or their C-terminal tetrad cores, such as phosphorylation (black), acetylation (red), or proteolytic cleavage (blue). A. AnxA2. B. AnxA5. C. AnxA1.

2. MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Cells. All cells were grown in incubators at 37°C, 5% CO_2, 95% humidity. Cells were passaged at confluence by first rinsing in warmed phosphate-buffered saline (PBS, 10.14 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 150 mM NaCl, 2.68 mM KCl, pH 7.2). Monolayers were incubated with warmed 0.025% trypsin containing 0.03% EDTA, and flasks were rocked until cells detached. Cells were diluted in culture medium, pipetted to break up cell clumps, and seeded into new flasks or plates.

2.1.1.1. Human foreskin fibroblasts (HFF). HFF, the well-characterized cell model commonly used for studying CMV biochemistry and infection, were obtained commercially from the American Tissue Culture Collection (ATCC) and were used between passages 5 and 12 only. HFF were grown and maintained in Basal Medium Eagle (BME) supplemented with 5% bovine calf serum, 14 μM L-glutamine, and 1 U/ml gentamycin. Upon passaging, cells were split in a 1 to 5 ratio. For cells seeded into 24- or 48-well plates, 10% of the contents of one T-175 flask were diluted to 25 ml BME and seeded into the culture plates

2.1.1.2. HepG2 cells. Native HepG2 cells, a human epitheloid hepatocellular carcinoma cell line, were obtained from ATCC (HB-8065). HepG2 transfected with p36 were a kind

gift from Dr. Alain Puisieux (Centre Anti cancer Léon-Berard, Lyon, France). All HepG2 lines were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 14 μM L-glutamine, and 1 U/ml gentamycin. Transfected cell lines were maintained in MEM containing 0.4 mg/ml geneticin to keep a selection pressure on the transfected cells. Upon passaging, cells were split in a ratio of 1 to 10. For cells seeded in 48-well plates, 50% of the contents of one T-75 flask was diluted to 25 ml and seeded into the culture plates.

2.1.1.3. Mycoplasma testing. To confirm that cells were free of mycoplasma contamination, cells were subjected to nuclear DNA stain and immunofluorescence microscopy every 2 weeks. Cells were seeded onto coverslips in 6-well plates. At a minimum of 3 days post-seeding, cells were washed once in PBS, fixed in 4% (w/v) paraformaldehyde in PBS for 10 minutes, washed 3 times in PBS, stained with 4',6-diamidino-2-phenylindole (0.0125 μg/ml in PBS) for 5 minutes, and washed once in PBS. Coverslips were placed cell side down on a 10 μl droplet of glycerol, and examined by immunofluorescence microscopy. Contamination was identified by a speckled staining across the cells, while non-contaminated cells stained only in the nucleus. Contaminated cells were either discarded, or maintained in the normal medium supplemented with 25 μg/ml plasmocin (Cedarlane) for 2 weeks to eliminate the contamination.

2.1.2. CMV. CMV (AD-169 strain) was propagated in HFF, purified by tartrate glycerol centrifugation, and quantified by electron microscopy and plaque assay, as previously described (442). Briefly, to prepare stock inoculum for propagation, wells in a 24-well

plate of HFF were inoculated with 1 infectious particle per well, at an approximate multiplicity of infection (MOI, infectious particles per cell) of 0.0004, in order to obtain genetically identical progeny. On day 7 post-infection, the supernatant was collected and used to inoculate a T-75 flask of HFF at an MOI of 0.001. Supernatant was collected on day 14 post-infection, centrifuged for clarification, and stored at -80°C. To prepare purified CMV, HFF were inoculated at an MOI of 0.0001. On day 17 post-infection, following visual inspection for cytopathic effect, cells were scraped from the flasks. Cells and supernatant were clarified by centrifugation, and supernatants were collected for further purification. Pellets were lysed by three freeze-thaw cycles and reclarified by centrifugation. Combined supernatants were centrifuged at 27,000 *g*, and the resulting pellets were collected and stored at -80°C until purification. Pellets were thawed, resuspended, overlaid on a tartrate-glycerol gradient, and centrifuged at 70,000 *g*. The middle band, consisting of virus particles, was collected, washed in ice-cold PBS, and stored at -80°C. CMV concentration (virus particles/ml) was assessed by electron microscopy (EM). Typical virus particle concentration was approximately 1-5 x 10^{11} vp/ml. The virus preparations normally contained less than 10% dense bodies and cell debris. To determine infectivity (plaque-forming units/ml), cytopathic plaque assays were conducted. The ratio of virus particles to plaque forming units was typically 10,000:1.

2.1.3. Annexins. Human recombinant AnxA5 was obtained commercially from Pharmingen. Purified bovine AnxA2t and p11, derived in large quantity from readily available lung tissue, and human recombinant human p36 were prepared and characterized as previously described (267, 285) and were a kind gift from Dr. David

Waisman (U. Calgary, Alberta, Canada). Bovine AnxA2t and human recombinant p36 were used in all purified AnxA2 experiments, except where specified, where human placental AnxA2 was used. Human AnxA2 and AnxA1 were purified from human placenta as previously described (232, 458). Briefly, human placentas were homogenized and calcium-dependent proteins were eluted using EDTA. Eluates were separated by a DEAE-Cellulose column and the entire wash-through was collected until just before the flow began to turn pink. The sample was concentrated, dialyzed into 2-(N-Morpholino)ethanesulfonic buffer, and separated by an S-Sepharose cation exchange column using a sodium chloride gradient for elution. Fractions were tested for PLA2 inhibition, since the ability of annexins to inhibit PLA2 is well-documented, and the presence of annexins was confirmed by Western blot analysis. The fractions from the second inhibition peak were collected, concentrated, and separated by a Mono-S column using a sodium chloride gradient. At approximately 23% sodium chloride, when a protein peak corresponding to AnxA2 began to elute, the gradient was held to that level until the peak was completely eluted. The protein peak was pooled, concentrated, and separated on an AnxA1 immuno-affinity column. The flow-through consisting of AnxA2 was collected and stored at -80°C. Bound AnxA1 was eluted using 3 M potassium isothiocyanate, and was changed into HEPES-buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.2) using Centricon-10 filters. AnxA1 was stored at -80°C. Protein concentration was determined by spectrophotometry. The absorbance of protein samples at 280 nm was determined, and the protein concentration was calculated using an extinction coefficient of 0.68 $mg/ml^{-1}cm^{-1}$ for AnxA2 and 0.55 $mg/ml^{-1}cm^{-1}$ for AnxA1 (C. Restall, personal communication).

2.1.3.1. Phospholipase A2 inhibition assay. To detect the presence of annexins, PLA2 inhibition assays were performed to exploit the well-known ability of annexins to inhibit PLA2 activity, as previously described (232, 458). Briefly, assays were performed in a buffer containing 100 mM Tris, 10 mM $CaCl_2$, pH 8. Purified PLA2 (25 ng) was added to 50 µl of each fraction to be tested. The assay was begun with the addition of 50 µl ^{3}H-labelled *E. coli*, the lipid substrate. After 5 minutes, the assay was stopped with the addition of 100 µl hydrochloric acid 2 N. 100 µl bovine serum albumin (BSA, 100 mg/ml) was added to each tube, and all tubes were centrifuged at 13,000 *g* for 5 minutes. Supernatant was removed and subjected to scintillation counting.

2.1.3.2. *E. coli* labelling. To prepare the ^{3}H-labelled lipid substrate, LB broth (50 ml) was combined with 20 µl *E. coli* culture and incubated at 37°C with shaking for 2 hours. Following the incubation, 100 µCi of ^{3}H-oleic acid were added; the mixture was incubated at 37°C for 4 hours, then autoclaved. The mixture was spun for 20 minutes at 1,500 *g*. A 50 µl aliquot of the supernatant was subject to scintillation counting, and the pellet was resuspended in Tris/BSA buffer (200 mM Tris, 10 mg/ml BSA, pH 8). The pellet was washed until the supernatant no longer contained detectable radioactivity. The *E. coli* substrate was stored at 4°C in Tris/BSA buffer containing 0.1% (w/v) sodium azide.

2.1.4. Phospholipid vesicles. Small unilamellar phospholipid vesicles, containing a 75:25 mixture of phosphatidylcholine and phosphatidylserine were prepared and quantified as

previously described (306). Briefly, purified phosphatidylcholine and phosphatidylserine, in methanol, were dried under nitrogen flow, and were resuspended in HBS. The solution was sonicated and centrifuged at 115,000 *g* for 30 minutes, then at 150,000 *g* for 3 hours. The top 6-7 ml of the phospholipid sample, at the top of the tube, was collected and stored at 4°C. To determine phosphate concentration, the vesicle preparation was combined with a solution containing perchloric acid and sulfuric acid and heated. To each tube was added molybdic acid and elon for development of colour. Absorbance of the samples was read at 700 nm, and the phosphate concentration was determined using potassium phosphate as a standard.

2.1.5. Other materials. Rabbit polyclonal anti-p11 antibody was generated and purified as previously described (274, 426) and was a kind gift from Dr. David Waisman (U. Calgary, Alberta). Briefly, the rabbit host was inoculated with a synthetic peptide corresponding to residues 21-38 of p11 linked to keyhole limpet hemocyanin. The anti-p11 antibody was subsequently affinity-purified using the 21-38 peptide. Rabbit polyclonal anti-p36 antibody, specific to a peptide corresponding to residues 9-30, was obtained from BioDesign. Non-immune rabbit IgG was obtained from Sigma-Aldrich. Mouse monoclonal antibodies (mAbs) directed against the following antigens were obtained commercially: AnxA1 (Translab), p36 (Oncogene, Translab, or Zymed as indicated), p11 (Translab) AnxA5 (Alexis), NF-κB p65 subunit (Translab), CMV IE72 gene product (Accurate Scientific), and β-actin (Sigma-Aldrich). Non-immune mouse immunoglobulins G1 (IgG1) and G2a (IgG2a) were obtained from Sigma-Aldrich. ^{35}S-methionine and Na^{125}I were obtained from Amersham-Pharmacia. Low-molecular weight

heparin (average molecular weight = 3 kDa) was obtained from Sigma-Aldrich.

2.2. METHODS.

2.2.1. Electrophoretic analysis.

2.2.1.1. SDS-PAGE. Protein samples were diluted in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.5 M Tris, 2% sodium dodecylsulfate, 10% glycerol, 0.01% bromophenol blue, 12.5 mg/ml dithiothreitol) and boiled for 2 minutes (protein samples) or 10 minutes (cell or virus lysates). Samples were resolved on polyacrylamide gels (12% acrylamide, 0.375 M Tris, 0.1% sodium dodecylsulfate, 0.07% N,N,N',N'-tetramethylethylenediamine, 0.07% ammonium persulfate).

2.2.1.2. Western blots. Samples were transferred to polyvinylidine difluoride (PVDF) membranes in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11) for 60 minutes at 400 mA at 4°C. Detection of bands by Western blot was performed according to the enhanced chemiluminescence method (ECL, Amersham-Pharmacia, all antibodies except anti-AnxA5 and -NF-κB p65 subunit antibodies) or the SuperSignal West Femto method (Pierce, anti-AnxA5 and -NF-κB p65 subunit antibodies). Briefly, PVDF membranes were blocked in 5% skim milk powder (ECL) or 1% BSA (SuperSignal) in Tris-buffered saline (0.05 M Tris, 150 mM NaCl, pH 8) containing 0.05% (w/v) Tween-20 (TBST) for 60 minutes at room temperature.

Membranes were incubated with primary antibody in blocking buffer for 60 minutes at room temperature, washed in TBST, incubated with secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson) in blocking buffer for 30 minutes at room temperature, and washed again in TBST. Western blots were developed on X-OMAT autoradiography film as per the manufacturer's instructions. Following development, bound proteins on the membrane were visualized using Coomassie Blue membrane staining solution (0.01% Coomassie Blue R-250, 8% acetic acid, 40% methanol) for 5 minutes at room temperature, followed by membrane destaining solution (8% acetic acid, 50% methanol) for 5 minutes at room temperature.

2.2.1.3. Autoradiography. Gels were dried under vacuum between gel-bond (Pierce) and filter paper, or were transferred to PVDF membrane, as indicated. Gels or membranes were exposed to an X-ray film in a cassette at -80°C with an intensifying screen (BioRad). The cassette and its contents were warmed to room temperature before developing.

2.2.2. Cell surface expression of annexins. HFF or HepG2 were grown to confluence in T-25 culture flasks (approximately 4 x 10^5 cells per flask). Cells were washed three times in serum-free BME and incubated in serum-free BME in the presence or absence of 20 mM EGTA for 30 minutes at 37°C. Supernatants were clarified in an Allegra-6 centrifuge at 500 *g* for 5 minutes and diluted in SDS-PAGE sample buffer. Cell lysates were obtained by washing confluent cells in serum-free BME, then dissolving in SDS-PAGE sample buffer. All samples were subsequently resolved by SDS-PAGE, and subjected to

Western blot analysis using mAbs directed against AnxA1, AnxA5 or p36, as well as NF-κB p65 subunit as an intracellular control. To compare expression levels, all lanes were corrected for protein content by quantification of Coomassie blue staining intensity of the entire lane, using Northern Eclipse imaging software (Empix).

2.2.3. Radiolabelling.

2.2.3.1. ^{35}S-labelling of CMV. CMV was labelled with ^{35}S-methionine on day 7 post-infection with 1.25 mCi per T-175 flask (8 flasks total), and purified as previously described (442). Following gradient centrifugation, because the virus band was not visible due to the small amount being purified, the gradient volume was collected in 1-ml fractions, and small aliquots were subjected to scintillation counting. The middle peak of activity was pooled and stored at -80°C.

2.2.3.2. ^{125}I-labelling of AnxA2. AnxA2 was labelled with ^{125}I using the Iodogen method (Pierce). To prepare iodination tubes, Iodogen reagent was dissolved in chloroform (1 mg/ml) and was distributed into glass test tubes (100 µg/tube). Chloroform was dried under nitrogen flow, and tubes were stored at 4°C under desiccation until use. For iodination, approximately 50 µg AnxA2 and approximately 2.4 x 10^7 cpm Na^{125}I were combined in an iodination tube in a total volume of 100 µl, and incubated on ice for 15 minutes. The mixture was removed from the tube and centrifuged at 13,000 *g* for 5 minutes to clarify. To determine specific activity (cpm/µg), approximately 1 µg protein was combined in a total volume of 100 µl in HBS containing 10% tri-chloroacetic acid

and 0.5 mg/ml BSA, and incubated on ice for 15 minutes. The mixture was centrifuged at 13,000 *g* for 5 minutes. Both the pellet and the supernatant were subjected to gamma-counting, and specific activity and percentage of free ^{125}I were determined. Specific activity was generally 100,000-300,000 cpm/μg, and free ^{125}I was typically less than 10%.

2.2.4. Infection assays.

2.2.4.1. Inoculations (antibodies). All incubations were performed in serum-free BME containing 1 mg/ml BSA (BME/BSA). Where indicated, antibodies and purified proteins were pre-incubated at room temperature for 30 minutes prior to incubating with cells and virus. Cells were pre-washed twice in BME/BSA containing 20 mM EGTA (206) and washed twice in BME/BSA alone. Both cells and virus were separately pre-incubated, at 37°C and on ice respectively, with the indicated concentrations of antibody for 60 minutes. Solutions were removed from the cells, replaced with the virus inoculum (final volume of 200 μl) at the indicated MOI, and incubated for 90 minutes at 37°C. Cells were then washed three times in BME and cultured in BME to allow infection to develop.

2.2.4.2. Inoculations (heparin, heparinase, EGTA). In all infection assays, HFF were grown to approximately 80% confluence. All incubations were performed in BME/BSA. Where indicated, cells were pre-treated with a cocktail containing 10 U/ml heparinase I and 1 U/ml heparinase III in BME/BSA, or mock-treated with BME/BSA alone, for 2 hours at 37°C. Cells were pre-washed twice in BME/BSA containing 10 mM EGTA

(206), then twice in BME/BSA alone. When the cells were inoculated in the presence of EGTA, the final wash contained 10 mM EGTA. Where indicated, both the cells and virus were pre-incubated, at 37°C and on ice respectively, with low-molecular weight heparin. Solutions were removed from the cells, replaced with the virus inoculum (final volume of 200 µl) at the indicated MOI, and incubated for 90 minutes at 37°C. Cells were then washed three times in BME and cultured in BME to allow infection to develop.

2.2.4.3. Inoculations (purified proteins). All incubations were performed in BME/BSA containing 2 mM $CaCl_2$ (BME/BSA/Ca). Where indicated, purified proteins were pre-incubated at room temperature for 30 minutes prior to incubating with cells and virus. Cells were pre-washed twice in BME/BSA/Ca. Both cells and virus were separately pre-incubated, at 37°C and on ice respectively, with the indicated concentrations of protein for 60 minutes. Solutions were removed from the cells, replaced with the virus inoculum (final volume of 200 µl) at the indicated MOI, and incubated for 90 minutes at 37°C. Cells were then washed three times in BME and cultured in BME to allow infection to develop. In HepG2 experiments, inoculations were performed in MEM containing 1 mg/ml BSA and 2 mM $CaCl_2$ (MEM/BSA/Ca), with CMV-infected HFF supernatant (442) at the indicated MOI.

2.2.4.4. 2-step IE72 expression. Infectious progeny release from host cells was measured by assessing infected cell supernatants for the ability to generate the IE72 gene product. Cells were inoculated and infection was allowed to develop for 7 days, with medium replacement on days 3 and 5 post-infection. On day 7 post-infection, supernatants from

infected cells were collected and used to inoculate fresh HFF monolayers for 90 minutes at 37°C. At 20 hours post-infection, the cells were lysed in sample buffer, resolved by SDS-PAGE, and subject to anti-IE72 Western blot analysis. Blots were subsequently reprobed for β-actin. IE72 band intensity, relativized to actin band intensity, was quantified using Northern Eclipse imaging software (Empix). To compare between experimental conditions, lysates were run together on the same gel and quantified together. Where lysates were run on separate gels, a standard IE72 positive control was used to normalize band intensity from gel to gel.

2.2.4.5. 2-step plaque formation. Infectious progeny release was measured by plaque assay of infected cell supernatants, adapted from a previously described protocol (280). Cells were inoculated and fed in BME, and infection was allowed to develop for 7 days, with medium replacement on days 3 and 5 post-infection to remove virions released into the extracellular space. On day 7 post-infection, supernatants from infected cells were collected and used to inoculate fresh HFF monolayers for 90 minutes at 37°C, and infection was allowed to develop with medium replacement on days 3 and 5 post-infection. Plaques were counted on day 6 post-infection by visual microscopic examination.

2.2.4.6. 1-step IE72 expression. Early post-entry infection events were evaluated by IE72 expression after primary inoculation. A dose-response curve was performed prior to beginning the study, and a virus concentration was chosen to fall within the linear range for IE72 band intensity. HFF cells were inoculated and infection was allowed to develop

for 20 hours. IE72 expression was quantified as described for the 2-step IE72 expression assays.

2.2.4.7. 1-step plaque formation. Early infection events leading up to and including CMV cell entry were measured by changes in plaque number (203). Cells were inoculated as described for the 2-step plaque assays, and infection was allowed to develop, with medium replacement on days 3 and 5 post-infection. Plaques were counted on day 6 post-infection by visual microscopic examination.

2.2.4.8. ^{35}S-CMV-cell binding. HFF or HepG2 were grown to approximately 80% confluence. Cells were chilled on ice for 20 minutes, pre-washed twice in ice-cold BME/BSA/Ca containing 20 mM EGTA, and twice in ice-cold BME/BSA/Ca. Cells and ^{35}S-CMV were pre-incubated on ice with the indicated concentrations of reagent for 90 minutes. Monolayers were washed three times in ice-cold BME/BSA/Ca to remove unbound virus, and dissolved in solubilization buffer at 60°C for 60 minutes. The amount of cell-bound ^{35}S-CMV was quantified. To determine the number of cells per well, a control well was incubated with 0.025% trypsin at 37°C for 3 minutes, and the cells were quantified and evaluated for viability by Trypan Blue exclusion assay.

2.2.5. Binding of ^{125}I-AnxA2 to HFF. HFF, at a confluence of approximately 80% in 24-well plates, were chilled on ice for 5 minutes, then washed twice in ice-cold PBS-S (PBS containing 1 mM $MgCl_2 \cdot 6H2O$, 1 mg/ml glucose, 5 mg/ml BSA, 2 mM $CaCl_2$, and 10 mM KI), and once in ice-cold PBS-S containing 10 mM EGTA. Cells were then

incubated in PBS-S containing 10 mM EGTA on ice for 30 minutes to allow calcium-dependently bound AnxA2 to elute from the cells surface (206), then washed in ice-cold PBS-S. Where indicated, when the cells were incubated with ^{125}I-AnxA2 in the presence of EGTA, the final wash contained 10 mM EGTA. The indicated concentration of ^{125}I-p36 or ^{125}I-AnxA2t was incubated with the cells for 90 minutes at 4°C. Cells were then washed three times in ice-cold PBS-S, then taken up in solubilization buffer at 60°C for 1 hour. Solubilized monolayers were subjected to gamma-counting.

2.2.6. Co-immunoprecipitation. ^{35}S-CMV particles (10^8 virus particles/ml) were lysed in immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Nonidet P-40, pH 7.5) for 30 minutes on ice. Lysates were incubated with 100 nM AnxA2t in immunoprecipitation buffer containing 0.2 mg/ml ovalbumin for 60 minutes at RT. To these mixtures, polyclonal anti-p11 antibody (D. Waisman) or non-immune rabbit IgG were added to a final concentration of 0.1 mg/ml and incubated overnight at 4°C. To each tube, 10 µl of a 50% suspension of Sepharose beads covalently linked to protein A and protein G, to bind antibody, was added for an incubation of 60 minutes at room temperature. Beads were washed three times, using the following protocol: a centrifugation at 13,000 *g* for 5 minutes, the removal of the supernatant, and the addition of fresh immunoprecipitation buffer. Following a fourth centrifugation and removal of supernatant, proteins bound to the beads were eluted using reducing SDS-PAGE sample buffer for 15 minutes at 100°C, resolved by SDS-PAGE, and transferred to PVDF. The membrane was subjected to anti-p36 immunoblot analysis and autoradiography.

2.2.7. Solution-phase crosslinking. CMV particles (5 x 10^7 virus particles/ml) were incubated with ^{125}I-labelled purified placental AnxA2 (100 nM) in the presence of phospholipid vesicles (2 μM phospholipid) at 37°C for 60 minutes. Equilibrium interactions were trapped using the chemical crosslinker bis[2-(Sulfosuccinimidooxycarbonyloxy) ethyl]sulfone (sulfo-BSOCOES) at a final concentration of 200 μM, and the resulting AnxA2-CMV protein complexes were lysed in reducing sample buffer and separated by 4-15% SDS-PAGE. Gels were dried and subjected to autoradiography.

2.2.8. Ligand blot. Purified CMV (10^9 virus particles/lane) was resolved by SDS-PAGE, transferred to PVDF membrane, and blocked in 5% skim milk powder in HBS. Membranes were subsequently incubated with ^{125}I-labelled purified human AnxA2 (100 nM) in HBS containing 0.05% Tween-20 and 1 mM $CaCl_2$ (HBST) at room temperature for 30 minutes. The chemical crosslinker dithiobis(succinimidylpropionate) (DSP) was added to a final concentration of 6 mM to the binding buffer and incubated at room temperature for 30 minutes. Membranes were then rinsed twice in HBST and were washed in HBST 6 times for 5 minutes. Membranes were dried and subjected to autoradiography.

2.2.9. Surface plasmon resonance. A Biacore 1000 (Biacore) was used to study protein-protein interactions by surface plasmon resonance (SPR). A flow cell of research-grade CM5 sensor chip (Biacore), consisting of carboxymethylated dextran, was activated with

40µl activation buffer (1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide: N-hydroxy-succinimide, 1:1), coated with approximately 1000 response units (RU, 1pg protein/mm^2) of AnxA5 (0.5 mg/ml in 10 mM sodium acetate buffer) by covalent amine coupling, and blocked with 30µl ethanolamine (1 M, pH 8.5). All binding injections were performed in SPR running buffer (HBS containing 2 mM $CaCl_2$ and 0.005% P-20). 30 µl p36 or AnxA2t were injected at the indicated concentrations, and the RU change at the end of the injection period was noted. Between injections, the chip was regenerated with an injection of 1 M sodium chloride to remove bound AnxA2 through high ionic force.

2.3. DATA ANALYSIS.

Percentage of inhibition of infection was determined using the following formula: % inhibition = 100 – (% expression). To determine maximum inhibition (I_{max}) and concentrations at half-maximal inhibition (IC50), all titration data were fit by non-linear least squares to a simple mathematical model that assumed a single class of binding (i.e. a rectangular hyperbola), using SigmaPlot graphing software (Jandel Scientific). Statistical significance was further analyzed using a two-tailed T-test (>95% confidence), by comparing individual points to the control.

The AnxA2 homology model was obtained with Swiss-Model software (204, 414, 493), using Swiss-Prot protein accession number P07355, and allowing automatic template matching. The AnxA2 homology model and the structures for AnxA1 (protein database ID 1AIN) and AnxA5 (protein database ID 1ANX) were visualized using Protein Explorer software (Eric Martz, www.proteinexplorer.org).

3. RESULTS.

3.1. EXPRESSION OF ANNEXINS IN CELLS AND VIRUS.

For AnxA2, AnxA5 or AnxA1 to affect CMV infection, they must be available on the host cell surface to interact with other cellular and/or viral species during infection. AnxA2 (39, 70, 86, 105, 106, 157, 207, 307, 342, 344, 426), AnxA5 (305, 413, 451), and AnxA1 (85, 91, 117, 513) have been reported on the surface of various cell types. Additionally, CMV propagated in HFF has been previously reported to have AnxA2 in its envelope (430, 458, 602); both monomeric AnxA2 (p36) and p11 were detected, implying the presence of tetrameric AnxA2 (AnxA2t, $p36_2p11_2$) (429, 458). However, it is unknown whether AnxA2, AnxA5 or AnxA1 are available on the surface of cells to be used in the current study, HFF and HepG2 cells. Since annexins interact with cell membranes in a calcium-dependent manner, we sought to determine if they could be detected in the extracellular medium following EGTA elution from the cell surface (207, 426), using Western blot analysis.

For HFF, the well-characterized cell model commonly used for studying CMV biochemistry and infection, AnxA5, AnxA1 and p36 were all detected in total cell lysates (figure 5). All three annexins were also present in the EGTA eluates of cells, but not in the control calcium eluate, indicating they interacted with the cell surface and required calcium to do so. Surface annexin expression constituted approximately 1-2% of total cellular annexin expression (table 1). NF-κB p65 subunit, an intracellular protein serving as a negative control for surface expression, was detected in neither calcium nor EGTA eluates (figure 5), even after long film exposures, indicating that EGTA elution of

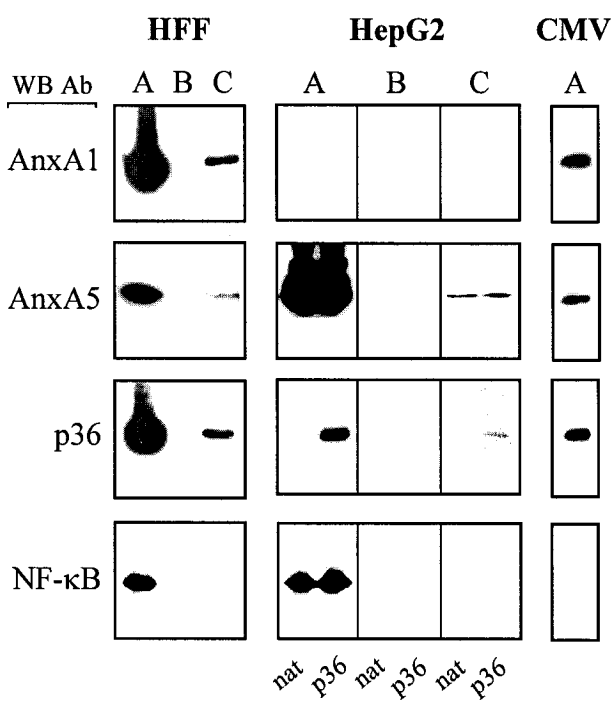

HFF
HepG2
CMV
WB Ab
A B C
A B C
A
AnxA1
AnxA5
p36
NF-κB
nat
p36
nat
p36
nat
p36

Figure 5. Cell surface expression of AnxA5 and AnxA1. SDS-PAGE and immunoblot analysis was conducted on total protein extracts (A) from HFF, native HepG2 (nat), p36-transfected HepG2 (p36) or purified CMV, or cell supernatants following 30 minutes at 37°C in serum-free culture medium containing 1.8 mM calcium (B) or supplemented with 20 mM EGTA (C), using antibodies to AnxA5, AnxA1, p36, or NF-κB p65 subunit.

	HFF			Nat HepG2			p36 HepG2		
Species	lysate	Ca^{2+}	EGTA	lysate	Ca^{2+}	EGTA	lysate	Ca^{2+}	EGTA
AnxA1	100.0[a]	-[b]	1.0	-	-	-	-	-	-
AnxA2	100.0	-	0.9	-	-	-	100.0	-	0.9
AnxA5	100.0	-	2.3	100.0	-	1.0	100.0	-	1.4

Table 1. Surface expression of annexins in HFF, HepG2, and CMV.

[a]Percent total cellular expression

[b]No detectable expression

of annexins was not due to unintentional cell lysis and detection of intracellular proteins. These results demonstrate that AnxA5 and AnxA1 are present on the surface of HFF and are therefore available during CMV infection. In addition, the presence of AnxA2 on the HFF surface was confirmed as a positive control (426).

A second cell model used in the current study is HepG2 cells, a cell line that does not express p36. In addition to confirming that HepG2 cells are p36-deficient (443), Western blot analysis showed that AnxA1 was not detected in either cell line. AnxA5 was expressed in both cell lines and was also detected in the EGTA eluates, but not the calcium eluates (figure 5). Surface AnxA5 constituted approximately 1% of total cellular AnxA5 expression (table 1), and HepG2 AnxA5 expression was approximately 60% that of HFF (table 2). However, as expected, p36 was detected in p36-transfected cells only, and was detected in the EGTA eluates, but not the calcium eluates (figure 5). Surface p36 constituted approximately 1% of total cellular p36 expression (table 1), and HepG2 p36 expression was approximately 8% of that of HFF (table 2). The lack of detectable NF-κB p65 subunit in either calcium or EGTA eluates confirmed that intracellular proteins were not being detected.

In addition to host cells, AnxA1, p36, and AnxA5 were all detected in the lysate of purified virus preparations (figure 5). As expected, NF-κB was not detected in CMV. The presence of annexins in the envelope reflects the ability of CMV to retain them in its envelope following egress from the host cell. Upon normalization to total protein content, CMV was observed to contain 19% AnxA1, 72% p36, and 37% AnxA5 of the levels of annexins observed in HFF (table 2). These data show that AnxA5 and AnxA1 are present in the CMV preparation and confirm the presence of AnxA2 (430, 458, 602). In addition,

Species	HFF	nat HepG2	p36 HepG2	CMV
AnxA1	100[a]	-[b]	-	19
AnxA2	100	-	8	72
AnxA5	100	59	56	37

Table 2. Relative expression of annexins in HFF, HepG2, and CMV.

[a]Percent expression level relative to HFF, corrected for total protein content.

[b]No detectable expression

these results indicate that the levels of the annexins in the CMV envelope are not directly proportional to host cell expression. Collectively, these results indicate that all three annexins are available for interactions with other species on the cell and/or virus.

3.2. ANXA2 IN CMV INFECTION.

3.2.1. HFF. HFF are the standard cell model used in investigations of CMV biochemistry and infectivity. To account for the possibility that AnxA2 may act at multiple stages of the CMV life cycle, and to avoid inadvertently excluding any step that may be affected by AnxA2, we evaluated the effect of AnxA2 at various stages in the infection cycle. Both endogenous and exogenous AnxA2 were evaluated for their ability to affect CMV infection of HFF.

3.2.1.1. Endogenous AnxA2. In order to evaluate the effect of endogenously expressed AnxA2 in the cell, we evaluated the ability of antibodies directed against both p11 and p36 to modulate CMV infection.

3.2.1.1.1. Anti-AnxA2 polyclonal antibodies. Two polyclonal antibodies were evaluated for their effect on CMV infection. The polyclonal anti-p11 antibody was raised to a peptide corresponding to amino acids 21-38, while the polyclonal anti-p36 antibody was raised to a peptide corresponding to amino acids 9-30, in the N-terminus. Supernatants from HFF infected in the presence of either anti-p11 or anti-p36 antibody were used to inoculate fresh HFF in a second round of infection (i.e. 2-step infection), and immediate-

early 72 (IE72) antigen expression was quantified by immunoblot analysis. IE72 is the first CMV gene expressed upon infection, and is a convenient, quantitative marker for infection (112). These experiments demonstrated that both anti-p11 and anti-p36 antibodies inhibited 2-step IE72 expression by approximately 95% (figure 6A). Non-immune rabbit IgG, as well as anti-p11 and anti-p36 that had been pre-adsorbed with AnxA2t, had no significant effect on IE72 expression (figure 6A inset), indicating that the inhibition by anti-p11 and anti-p36 antibodies was antigenically specific and reversible.

To confirm the 2-step IE72 expression assays, 2-step plaque formation assays were conducted to determine the effect of AnxA2 on the complete infection cycle. When supernatants from cells inoculated in the presence of either anti-p11 or anti-p36 were used to inoculate fresh HFF, second-generation CMV plaque formation was inhibited by approximately 70% (figure 6B), corroborating the 2-step IE72 experiments. Neither non-immune rabbit IgG nor AnxA2t-adsorbed anti-p11 or anti-p36 had a significant effect on plaque generation (figure 6B inset), demonstrating the antigenic specificity and reversibility of the inhibition. Thus endogenous p36 and p11 are implicated in the overall mechanism leading to infectious CMV progeny production. Though it cannot be concluded which form of AnxA2 is involved, the participation of p11 strongly supports a role for AnxA2t.

To determine the stage of infection influence by anti-p11 or anti-p36 antibody, experiments were conducted to assess their effect on early CMV infection events, i.e. 1-step infection events. The involvement of endogenous AnxA2 on primary IE72 expression was evaluated using anti-p11 or anti-p36 antibody, which inhibited

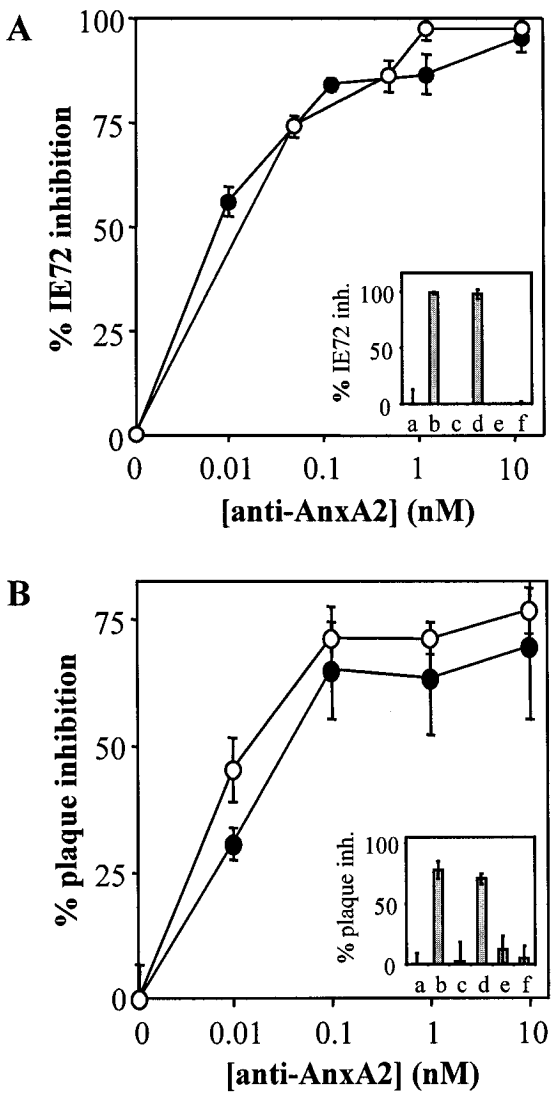

A
100
75
50
25
0
% IE72 inhibition
0
0.01
0.1
1
10
[anti-AnxA2] (nM)
% IE72 inh.
100
50
0
a b c d e f
B
75
50
25
0
% plaque inhibition
0
0.01
0.1
1
10
[anti-AnxA2] (nM)
% plaque inh.
100
50
0
a b c d e f

Figure 6. Effect of anti-AnxA2 antibodies (polyclonal) in productive infection in HFF. HFF were inoculated with CMV in the presence of the indicated concentrations of anti-p11 (•) or anti-p36 (○), at an MOI of 0.0003. On day 7 post-infection, supernatants were collected and used to inoculate fresh HFF. A. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point represents the average change in IE72 expression in lysates of HFF from two separate wells. Error bars represent standard error; $p<0.05$. *Inset,* no antibody (a), 100 nM anti-p11 (b), 100 nM anti-p11 + 100 nM AnxA2t (c), 100 nM anti-p36 (d), 100 nM anti-p36 + 100 nM AnxA2t (e), 100 nM non-immune rabbit IgG (f). B. Plaques were quantified on day 6 post-infection. *Inset,* no antibody (a), 100 nM anti-p11 (b), 100 nM anti-p11 + 100 nM AnxA2t (c), 100 nM anti-p36 (d), 100 nM anti-p36 + 100 nM AnxA2t (e), 100 nM non-immune rabbit IgG (f). Each point represents the average change in plaque number from two separate wells. Error bars represent standard error; $p<0.05$.

approximately 90% of IE72 expression following primary inoculation (figure 7A). Demonstrating specificity and reversibility, neither non-immune rabbit IgG nor anti-p11 or anti-p36 antibody that had been pre-incubated with AnxA2t had a significant effect on IE72 expression (figure 7A inset). These observations suggest that endogenous p36 or p11 enhance CMV infection at or prior to IE72 expression.

To further locate the stage of infection enhanced by AnxA2, primary plaque formation was followed. This assay is typically used as a measurement of virus-cell entry but includes subsequent steps obligate for virus production causing cytopathic effects (203). A specific role for endogenous AnxA2 was demonstrated by observing a 50% reduction in 1-step plaque number by anti-p11 or anti-p36 antibody (figure 7B). Non-immune rabbit IgG had no effect on plaque number, nor did anti-p11 or anti-p36 antibody that had been pre-incubated with AnxA2t (figure 7B inset), indicating that the inhibition by anti-p11 and anti-p36 antibodies was antigenically specific and reversible. These data show that endogenous p11 and p36 play a specific role to enhance CMV infection prior to detection of cytopathicity.

Purified AnxA2t and p36 have each been shown to enhance binding of CMV to model phospholipid membranes (458). To investigate the implied role for cellular AnxA2, CMV-cell attachment was assessed by following ^{35}S-CMV binding to HFF at 4°C, in the presence or absence of anti-p11 or anti-p36 antibody. The finding that ^{35}S-CMV binding was not significantly affected by either anti-p11 or anti-p36 antibody (figure 7C), at the same concentration observed to inhibit later stages of infection, suggests that endogenous AnxA2 does not participate in CMV-cell attachment. However, a notable difference compared to the previous experiments is that the current binding

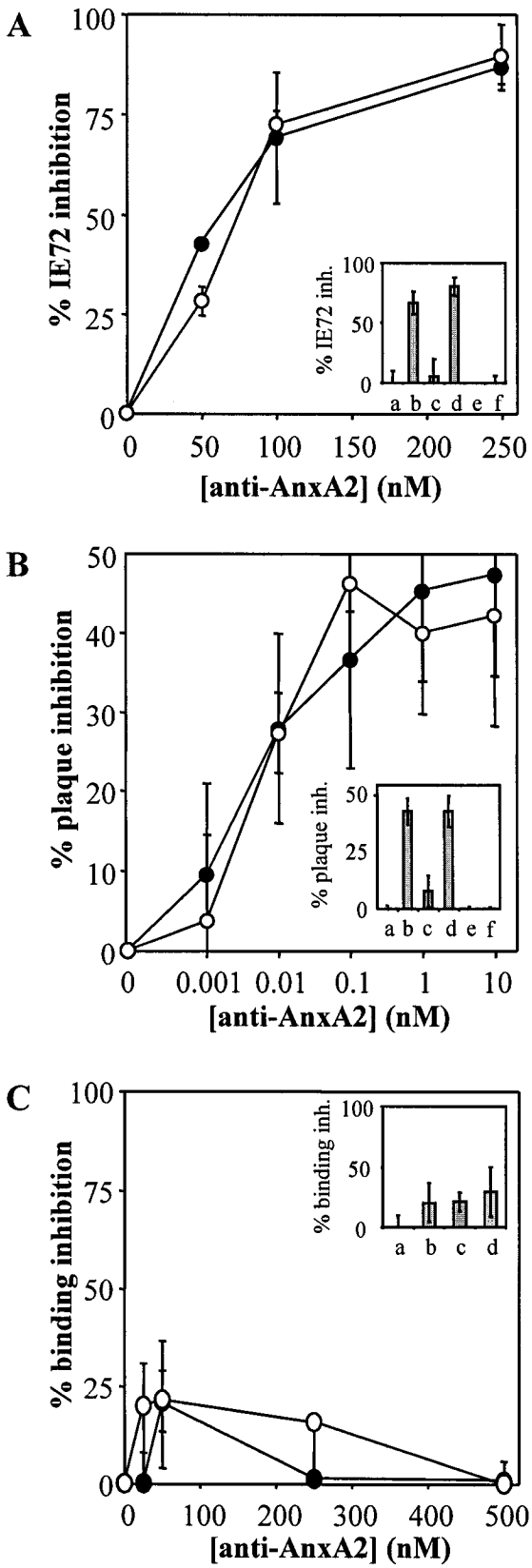

A
100
75
50
25
0
% IE72 inhibition
0
50
100
150
200
250
[anti-AnxA2] (nM)
% IE72 inh.
100
50
0
a b c d e f
B
50
40
30
20
10
0
% plaque inhibition
0
0.001
0.01
0.1
1
10
[anti-AnxA2] (nM)
% plaque inh.
50
25
0
a b c d e f
C
100
75
50
25
0
% binding inhibition
0
100
200
300
400
500
[anti-AnxA2] (nM)
% binding inh.
100
50
0
a b c d

Figure 7. Anti-AnxA2 antibodies (polyclonal) in early infection events in HFF. A. HFF were inoculated with CMV in the presence of the indicated concentrations of anti-p11 (•) or anti-p36 (○), at an MOI of 0.003. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point corresponds to the average change in IE72 expression from two separate experiments. Error bars represent standard error; $p<0.05$. *Inset,* no antibody (a), 100 nM anti-p11 (b), 100 nM anti-p11 + 100 nM AnxA2t (c), 100 nM anti-p36 (d), 100 nM anti-p36 + 100 nM AnxA2t (e), 100 nM non-immune rabbit IgG (f). B. HFF were inoculated with CMV in the presence of the indicated concentrations of anti-p11 (•) or anti-p36 (○), at an MOI of 0.0003. Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from four separate experiments comprising the average of two wells each. Error bars represent standard deviation; $p<0.05$. *Inset,* no antibody (a), 100 nM anti-p11 (b), 100 nM anti-p11 + 100 nM AnxA2t (c), 100 nM anti-p36 (d), 100 nM anti-p36 + 100 nM AnxA2t (e), 100 nM non-immune rabbit IgG (f). C. HFF and ^{35}S-CMV ($5x10^8$ virus/ml) were incubated at 4°C in the presence of the indicated concentrations of anti-p11 (•) or anti-p36 (○). HFF were solubilized and bound ^{35}S-CMV was quantified. Each point represents the average change in binding from three separate wells. Error bars represent standard deviation; $p<0.05$. *Inset,* no antibody (a), 100 nM anti-p11 (b), 100 nM anti-p36 (c), 100 nM non-immune rabbit IgG (d).

studies were conducted at 4°C to prevent virus-cell fusion, but under this condition membrane trafficking events are inhibited, such as the previously observed translocation of AnxA2 (426).

3.2.1.1.2. Anti-AnxA2 monoclonal antibodies. To test whether specific epitopes on AnxA2 were responsible for enhancing CMV infection, a panel of mouse monoclonal antibodies (mAbs) was evaluated, consisting of an anti-p11 antibody raised against the full-length protein (Translab) and three anti-p36 antibodies raised against either the full-length protein (Oncogene, Zymed) or a peptide corresponding to amino acids 123-328, spanning domains II-IV (Translab). In contrast to the polyclonal antibodies, none of the monoclonal antibodies had any significant effect on either primary IE72 expression (figure 8A) or primary plaque formation (figure 8B).

Taken together, these immunoinhibition data demonstrate that endogenous p11 and p36 enhance CMV infection of host HFF, presumably complexed as AnxA2t. While polyclonal anti-p11 and anti-p36 antibodies were inhibitory, various mAbs to p11 and p36 had no effect on either IE72 expression or plaque formation, which may suggest that that specific epitopes on p36 and p11 may be involved (table 3). While we cannot exclude the possibility that additional downstream sites are affected by AnxA2, the earliest stage precedes IE72 expression and the detection of cytopathicity, but occurs after initial host cell attachment.

3.2.1.1.4. Relationship between IE72 expression and MOI. IE72 has been previously used to assess penetration of CMV into the host cell (112). To determine whether IE72

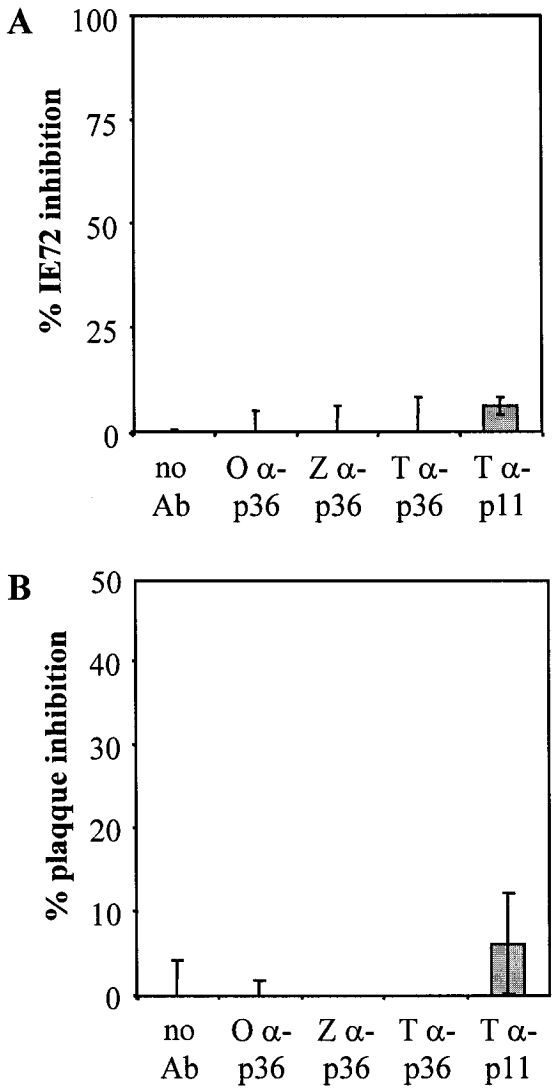

A
100
75
50
25
0
% IE72 inhibition
no Ab
O α-p36
Z α-p36
T α-p36
T α-p11
B
50
40
30
20
10
0
% plaque inhibition
no Ab
O α-p36
Z α-p36
T α-p36
T α-p11

Figure 8. Anti-AnxA2 antibodies (monoclonal) in early infection stages in HFF. A. HFF were inoculated with CMV in the presence of 100 nM anti-AnxA2 mAbs from Oncogene (O), Zymed (Z), or Translab (T), at an MOI of 0.003. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point represents the average change in IE72 expression from two separate experiments comprising the average of two wells each. Error bars represent standard error; $p<0.05$. B. HFF were inoculated with CMV in the presence of 10 nM anti-AnxA2 mAbs from Oncogene (O), Zymed (Z), or Translab (T), at an MOI of 0.0003. Plaques were quantified on day 6 post-infection. Each point represents the average change in plaque number from two separate wells. Error bars represent standard error; $p<0.05$.

Protein	Source	Type	Isotype	Antigen	Inhibition
p11	D. Waisman	polyclonal	N.A.	21-38[a]	Y
p36	BioDesign	polyclonal	N.A.	9-30	Y
p11	Translab	monoclonal	IgG1	F.L.[b]	N
p36	Translab	monoclonal	IgG1	123-328	N
p36	Oncogene	monoclonal	IgG2a	F.L.	N
p36	Zymed	monoclonal	IgG1	F.L.	N

Table 3. Comparison of anti-AnxA2 antibodies.

[a]Amino acid range of the peptide.

[b]Full-length protein.

expression was quantitatively proportional to virus infection, HFF were infected with CMV at different concentrations of virus. IE72 expression increased proportionally with multiplicity of infection (MOI), reaching a plateau starting at an MOI of approximately 0.01 (figure 9A). These results indicate that Western blot analysis of IE72 expression serves as a convenient, quantitative way of assessing virus penetration into the host cell. Since all virus concentrations in the current study fell within the linear range of IE72 expression, these data confirm that observed changes IE72 in expression reflect changes in virus infection levels.

It has been previously reported that, like CMV-cell attachment, IE72 expression in host cells is dependent on the availability of HSP on the cell surface (112). To confirm the validity of the IE72 expression assay, and determine whether IE72 expression is indeed dependent on HSP, HFF were inoculated with CMV either in the presence of 0.01 mM heparin, or following treatment of the cells with a heparinase cocktail to remove cell-surface HSP. The addition of soluble heparin resulted in 99% abrogation of IE72 expression, while the pre-treatment of cells with the heparinase cocktail led to a 97% decrease in IE72 expression (figure 9B). These data confirm that early infection events, as assessed by IE72 expression, are dependent on the presence and availability of HSP, and confirm the validity of the IE72 expression assay.

AnxA2 is bound to the cell surface in part by a calcium-dependent interaction. In order to determine whether IE72 expression in host cells is calcium-dependent, HFF were inoculated in the presence of EGTA, which resulted in a 75% inhibition of IE72 expression (figure 9B). These data indicate that early CMV entry events up to and including gene expression are partly calcium-dependent. In HFF that had been pre-treated

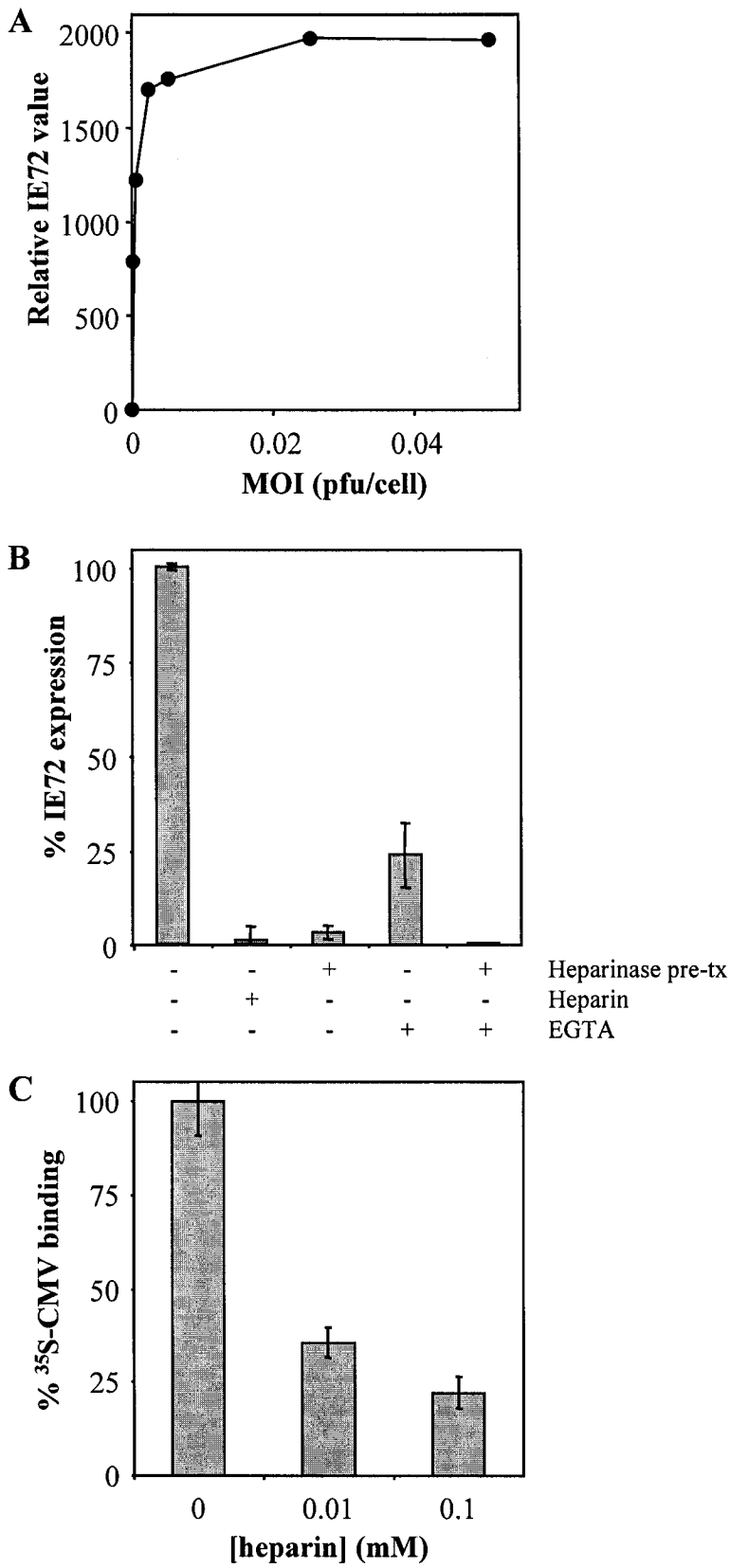

A
Relative IE72 value
2000
1500
1000
500
0
0
0.02
0.04
MOI (pfu/cell)
B
% IE72 expression
100
75
50
25
0
- - + - +
- + - - -
- - - + +
Heparinase pre-tx
Heparin
EGTA
C
% 35S-CMV binding
100
75
50
25
0
0
0.01
0.1
[heparin] (mM)

Figure 9. Validation of the IE72 expression and ^{35}S-CMV binding assays in HFF. A. HFF were inoculated with CMV at the indicated MOI. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. B. HFF were pre-treated with a cocktail containing 10 U/ml heparinase I and 1 U/ml heparinase III (heparinase pre-tx), or were mock-treated. HFF were inoculated with CMV in the presence or absence of 0.1 mM heparin or 20 mM EGTA, as indicated, at an MOI of 0.003. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point represents the average change in IE72 expression from two separate experiments. Error bars correspond to standard error; $p<0.05$. C. HFF were incubated with ^{35}S-CMV (5×10^8 virus particles/ml) in the presence of the indicated concentration of heparin. HFF were washed and solubilized, and bound ^{35}S-CMV was quantified. Each point represents the average change in binding from three separate wells. Error bars correspond to standard deviation; $n=3$; $p<0.05$.

with heparinase, EGTA lowered IE72 expression below the limit of detection, indicating that the infection pathways mediated by HSP and by calcium are separate. This latter notion is consistent with the hypothesis that CMV employs a calcium-dependent pathway, i.e. consistent with AnxA2, separate from the HSP-mediated pathway.

3.2.1.1.5. Validation of the ^{35}S-CMV-HFF binding assay. It has been previously reported that CMV binds to host cells using cell-surface HSP (112). To confirm the validity of the binding assay, ^{35}S-CMV was incubated with host HFF at 4°C in the presence of heparin. Binding of ^{35}S-CMV to HFF was inhibited by approximately 80% at 0.1 mM heparin (figure 9C), a value consistent with that previously reported (112), confirming the validity of the ^{35}S-CMV binding assay. Consistent with previous reports (112, 270), even very high concentrations of heparin could not completely inhibit binding, indicating the presence of a heparin-insensitive cell-surface CMV receptor.

3.2.1.2. Purified AnxA2. To confirm the effect of endogenously expressed AnxA2 in CMV infection, and to determine whether the monomeric or tetrameric forms of AnxA2 are involved, the effect of purified AnxA2t, p11 or p36 was explored. These experiments assumed that the amount of endogenous AnxA2 was insufficient to maximize the infection of CMV. To evaluate the effect of exogenous AnxA2 on the entire CMV infection cycle, we assessed the effect of purified AnxA2t, p36, and p11 on 2-step CMV infection, that is, the ability of supernatants from CMV-infected HFF to generate either IE72 expression or plaque formation in fresh HFF. The presence of purified AnxA2t or p11 during the initial inoculation enhanced 2-step IE72 generation in a dose-dependent,

saturable manner, to a maximum of approximately 5-fold (figure 10A). Confirming the 2-step IE72 expression assays, purified AnxA2t and p11 each enhanced 2-step plaque formation in a dose-dependent, saturable manner, to a maximum of approximately 7-fold (figure 10B). No significant effect was observed in the presence of p36, indicating that the enhancement was antigenically specific. Since p11 is not known to interact with membranes separately from the AnxA2t complex (621), these data suggest that AnxA2t increases productive CMV infection.

To identify the earliest stages of infection at which purified AnxA2 acts, experiments were conducted to assess the effect of purified AnxA2t, p11 or p36 on primary CMV infection. HFF were inoculated in the presence of purified AnxA2t, p36, or p11. The lysates were collected shortly after inoculation and subjected to anti-IE72 Western blot, in order to analyze the effect of purified AnxA2 on infection steps up to and including gene expression. Exogenous AnxA2t and p11 each enhanced IE72 expression by approximately 3-fold (figure 11A). However, exogenous p36 had no significant effect. These results indicated that exogenous AnxA2t specifically enhances CMV infection at or before immediate-early gene expression.

To further locate the stage of infection that is affected by exogenous AnxA2t, primary cytopathic plaque assays were conducted. Unlike endogenous AnxA2, which was observed to participate in CMV plaque formation in 1-step experiments, no significant effect on plaque number was observed in the presence of purified AnxA2t, p11, or p36 (figure 11B). These data highlight a difference between endogenous and purified AnxA2. Since plaque number is typically used as a measure of cell entry (203),

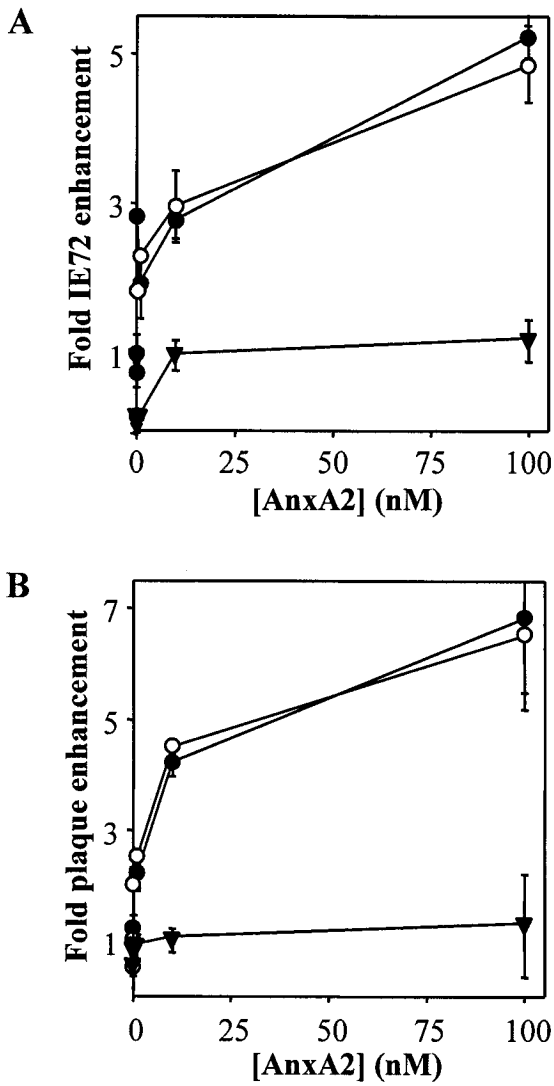

A
Fold IE72 enhancement
5
3
1
0
25
50
75
100
[AnxA2] (nM)
B
Fold plaque enhancement
7
5
3
1
0
25
50
75
100
[AnxA2] (nM)

Figure 10. Purified AnxA2 in productive infection in HFF. HFF were inoculated with CMV in the presence of the indicated concentrations of AnxA2t (●), p11 (○), or p36 (▼), at an MOI of 0.0003. On day 7 post-infection, supernatants were collected and used to inoculate fresh HFF. A. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point represents the average change in IE72 expression from two separate wells. Error bars represent standard error; $p<0.05$. B. On day 7 post-infection, supernatants were collected and used to inoculate fresh HFF. Plaques were quantified on day 6 post-infection. Each point represents the average change in plaque number from two separate wells. Error bars represent standard error; $p<0.05$.

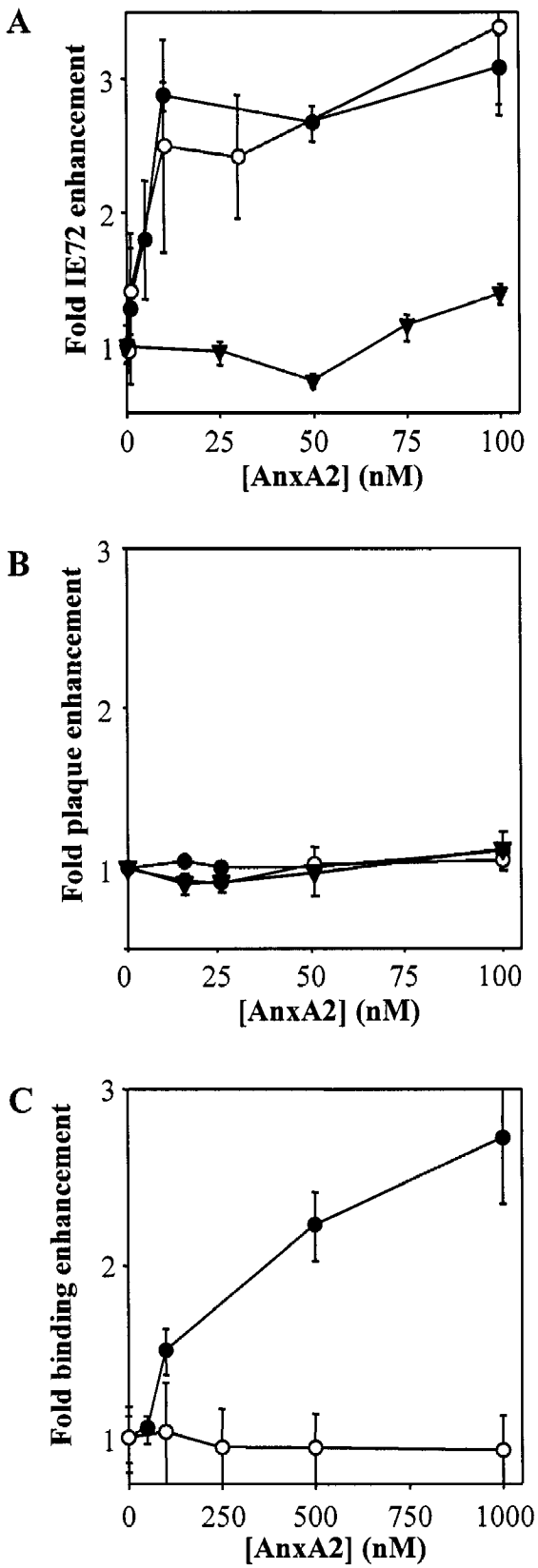

A
Fold IE72 enhancement
3
2
1
0
25
50
75
100
[AnxA2] (nM)
B
Fold plaque enhancement
3
2
1
0
25
50
75
100
[AnxA2] (nM)
C
Fold binding enhancement
3
2
1
0
250
500
750
1000
[AnxA2] (nM)

Figure 11. Purified AnxA2 in early infection stages in HFF. A. HFF were inoculated with CMV in the presence of the indicated concentrations of AnxA2t (●), p11 (○), or p36 (▼), at an MOI of 0.003. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point represents the average change in IE72 expression from two separate experiments. Error bars represent standard error; $p<0.05$. B. HFF were inoculated with CMV in the presence of the indicated concentrations of AnxA2t (●), p11 (○), or p36 (▼), at an MOI of 0.0003. Plaques were quantified on day 7 post-infection. Each point represents the average change in plaque number from two separate experiments comprising the average of two wells each. Error bars represent standard error; $p<0.05$. C. HFF and ^{35}S-CMV ($5x10^8$ virus/ml) were incubated at 4°C in the presence of the indicated concentrations of AnxA2t (●) or p36 (○). HFF were solubilized and bound ^{35}S-CMV was quantified. Each point represents the average change in binding from three separate wells. Error bars represent standard deviation; $p<0.05$.

these results suggest that the earliest enhancing effect of exogenous AnxA2t and p11 on CMV infection occurs between virus-cell entry and IE72 production.

Our laboratory has shown that both AnxA2t and p36 can increase binding of CMV to anionic phospholipid-containing vesicles. In light of this observation, and to complete the comparison of exogenous to endogenous AnxA2 in CMV infection, virus-cell binding was followed in the presence or absence of purified AnxA2t or p36. Since there was no distinguishable effect on plaque number, a similar observation was anticipated for binding. Surprisingly, the addition of purified AnxA2t specifically enhanced ^{35}S-CMV binding to host HFF, while p36 had an insignificant effect on binding (figure 11C). Taken together, these data delineated a gap in the presumed linear model of CMV infection, which appears to exclude effects of purified AnxA2t on primary plaque formation, but not the steps measured before or after. These results therefore suggest the existence of a pathway separate from entry which is enhanced by purified AnxA2t. Purified p36 had no significant effect at any stage of infection, confirming the specificity of exogenous AnxA2t.

3.2.1.4.1. ^{125}I-AnxA2-HFF binding. Although p36 had no discernable effect on any stage of CMV infection, p36 has been previously reported to bind to the surface of endothelial cells (206). To confirm that the lack of effect of p36 was not due to a failure to bind HFF, binding assays were conducted using ^{125}I-p36 or ^{125}I-AnxA2t with HFF at 4°C. Both ^{125}I-p36 and ^{125}I-AnxA2t bound equivalently to HFF in a dose-dependent manner (figure 12A). As reported for endothelial cells (206), binding of both p36 and AnxA2t to HFF was sensitive to EGTA chelation, with binding inhibited by approximately 40%

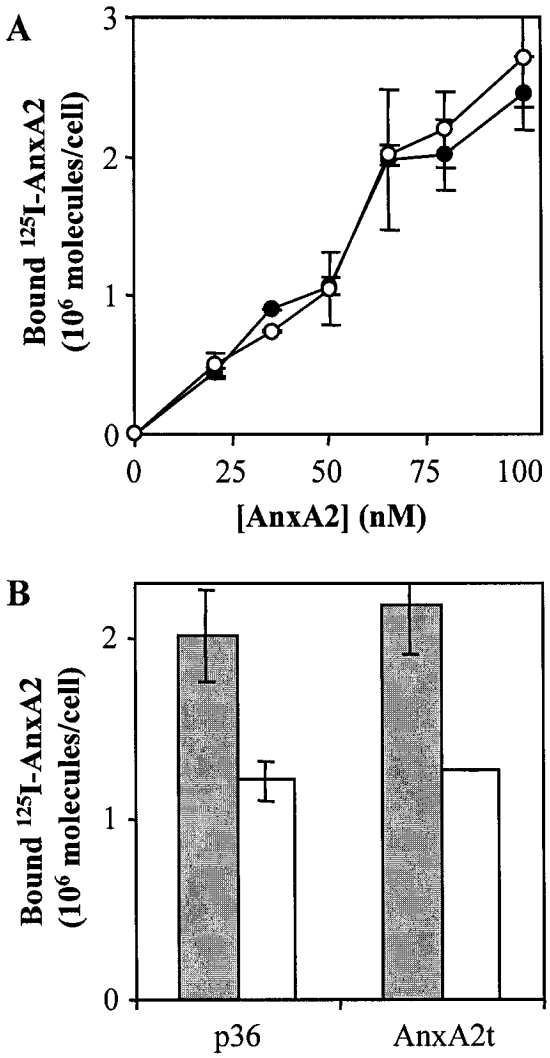
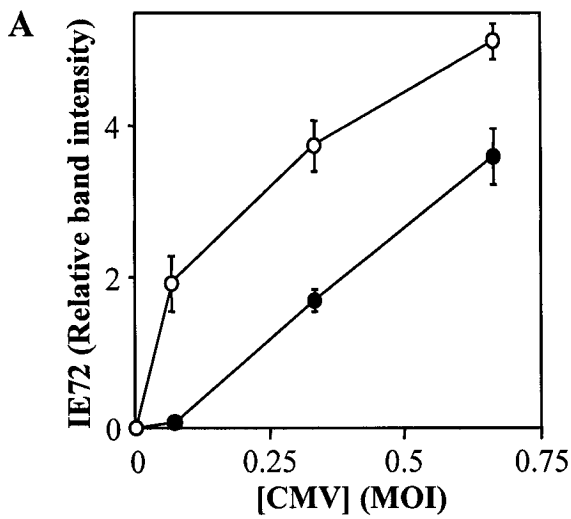

A
Bound ^{125}I-AnxA2 (10^6 molecules/cell)
3
2
1
0
0
25
50
75
100
[AnxA2] (nM)
B
Bound ^{125}I-AnxA2 (10^6 molecules/cell)
2
1
0
p36
AnxA2t

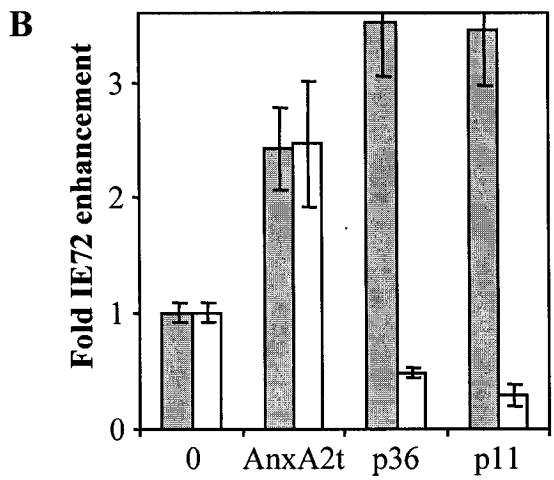
Figure 12. Binding of ^{125}I-AnxA2 binding to HFF. A. HFF were incubated at 4°C with the indicated concentrations of ^{125}I-p36 (●) or ^{125}I-AnxA2t (○). HFF were washed and solubilized, and bound ^{125}I-AnxA2 was quantified. Each point represents the average change in binding from two separate wells. Error bars correspond to standard error; $p<0.05$. B. HFF were incubated at 4°C with 80 nM ^{125}I-p36 or ^{125}I-AnxA2t in the presence of either 2 mM Ca^{2+} (gray bars) or 10 mM EGTA (white bars). HFF were washed and solubilized, and bound ^{125}I-AnxA2 was quantified. Each point represents the average change in binding from two separate wells. Error bars correspond to standard error; $p<0.05$.

(figure 12B), indicating that AnxA2 binding to HFF is partly calcium-dependent. Since a significant proportion of AnxA2 binding was not inhibited by EGTA, these data may indicate that binding of AnxA2 to HFF has a calcium-independent component. These data confirm that added purified p36 could bind HFF and was available to CMV during infection, demonstrating that the enhancing effect of AnxA2t was antigenically specific.

*3.2.2. **HepG2.*** To substantiate the role of AnxA2t in CMV infection, an additional cell model was employed. HepG2 cells were chosen because they are the only documented cell line that does not express either p36 mRNA or p36 antigen. While low levels of p11 mRNA and protein are detectable, transfection of HepG2 with the p36 gene (443) causes upregulation of p11 protein, resulting in probable assembly of AnxA2t. Although neither native nor p36-transfected HepG2 cells was permissive for productive CMV infection, both were permissive for CMV infection steps at least up to IE72 expression. The p36-transfected HepG2 cell line is therefore a useful tool for assessing the role of AnxA2 at early steps in CMV infection.

To determine whether p36 expression enhances early events in CMV infection, 1-step IE72 expression assays were conducted. At low CMV concentrations, detection of IE72 antigen was increased by an average of 3-fold in p36-transfected HepG2 cells, compared to native HepG2 (figure 13A), confirming observations obtained with HFF. These data also suggest that the cellular pool of AnxA2, and not just that endogenous to the virus surface, enhances CMV infection processes. The finding that native HepG2, having no p36 or AnxA2t, were permissive for IE72 expression, indicates that endogenous cellular AnxA2 enhances, but is not obligate for CMV infection under these


A
IE72 (Relative band intensity)
0
2
4
0
0.25
0.5
0.75
[CMV] (MOI)


B
Fold IE72 enhancement
0
1
2
3
0
AnxA2t
p36
p11

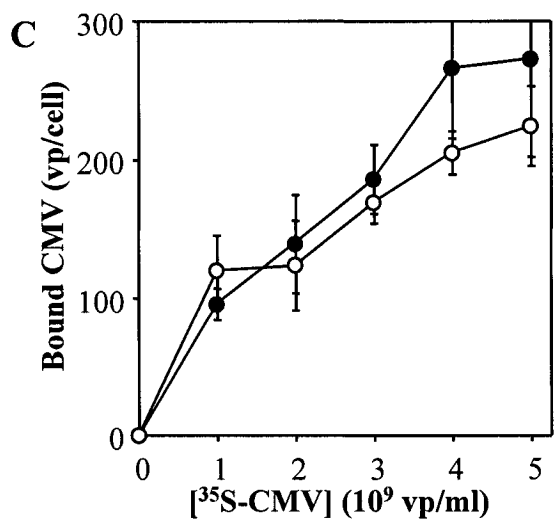

C
Bound CMV (vp/cell)
0
100
200
300
0
1
2
3
4
5
[35S-CMV] (10^9 vp/ml)

Figure 13. AnxA2 in early infection stages in HepG2. A. Native (●) or p36-transfected (○) HepG2 cells were inoculated with CMV at the indicated MOI. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point represents the average IE72 expression from two separate wells. Error bars represent standard error; $p<0.05$. B. Native (filled bars) or p36-transfected (open bars) HepG2 cells were inoculated with CMV in the presence of 100 nM exogenous purified AnxA2t, p11, or p36, at an MOI of 0.5. At 20 hours post-infection, lysates were collected and subject to anti-IE72 Western blot analysis. Each point represents the average change in IE72 expression from three separate experiments comprising the average of two wells each. Error bars represent standard error; $p<0.05$. C. Native (●) or p36-transfected (○) HepG2 cells were incubated at 4°C with ^{35}S-CMV at the indicated concentration. HepG2 were solubilized and bound ^{35}S-CMV was quantified. Each point represents the average change in binding from three separate wells. Error bars represent standard deviation; $p<0.05$.

experimental conditions.

Investigations into the effect of purified AnxA2 in early stages of CMV infection showed that purified AnxA2t caused a 2-fold increase in IE72 expression in both native and p36-transfected HepG2 (figure 13B), suggesting that the AnxA2t produced endogenously by the transfected cells was insufficient to saturate the effect. The addition of purified p11 or p36 increased IE72 expression in native HepG2 by 3-fold, possibly by forming AnxA2t with endogenously expressed components on the cell or virus. For as yet unknown reasons, IE72 expression by p36-transfected HepG2 was inhibited by the addition of either purified p11 or p36 (figure 13B). However, this observation is consistent with an inhibitory effect we observed on HFF, at very low (<0.1 nM) concentrations of purified p11 or p36 (figure 10A, 9B). These data suggest that under these conditions, equilibria with other cellular or viral constituents may alter the overall enhancing effects of p11 or p36. It is possible that purified p11 or p36, while unable to enhance events leading to IE72 expression, competed with endogenous AnxA2t for binding to CMV.

To assess whether AnxA2 enhances CMV-cell attachment in HepG2, ^{35}S-CMV binding was evaluated. These experiments showed that ^{35}S-CMV binding was equivalent to native and p36-transfected HepG2 (figure 13C). Confirming the observations made in HFF, these data indicate that endogenously expressed AnxA2 had no effect on CMV binding to HepG2 at 4°C.

Collectively, these data indicate that AnxA2t, but not p36, enhances CMV infection (table 4).

Treatment	Infection stage				
	IE72 (2-step)[b]	Plaques (2-step)	IE72 (1-step)[c]	Plaques (1-step)	Binding
Anti-p11	5 ± 3	30 ± 14	14 ± 4	47 ± 14	NE[d]
Anti-p36	3 ± 1	32 ± 3	11 ± 8	53 ± 16	NE
AnxA2t	520 ± 30	680 ± 20	310 ± 30	NE	270 ± 40
p11	480 ± 50	650 ± 10	340 ± 60	NE	-[e]
p36	NE	NE	NE	NE	NE

Table 4. Summary of the effect of AnxA2 on CMV infection[a].

[a]Percent of untreated.

[b]In 2-step experiments, HFF were inoculated in the presence of the indicated treatment. Supernatants of infected cells were subsequently evaluated for presence of infectious progeny.

[c]In 1-step experiments, HFF were inoculated in the presence of the indicated treatment, and the effect on primary infection events was evaluated.

[d]No effect.

[e]Not determined.

3.3. ANXA2-CMV INTERACTION.

It has been previously reported that AnxA2 is found both on the cell surface (39, 70, 86, 105, 106, 157, 207, 307, 342, 344, 426) and associated with the CMV envelope (430, 458, 602). While AnxA2 may bind to the anionic phospholipids present in the virus envelope (602), it has also been shown that the interaction between AnxA2 and CMV is only partially calcium-dependent (430), suggesting that a subpopulation of AnxA2 might target a non-phospholipid component. Glycoprotein B (gB), the major protein species in the virus envelope, has been suggested as a ligand for AnxA2 (61, 430). Several approaches were undertaken to identify viral ligands for AnxA2.

3.3.1. CMV-AnxA2 co-immunoprecipitation. The first approach taken to identify CMV ligands for AnxA2 consisted of the incubation of purified AnxA2t with lysates of ^{35}S-CMV particles, followed by immunoprecipitation using a polyclonal anti-p11 antibody. ^{35}S-CMV proteins that bound AnxA2 were then visualized by SDS-PAGE and autoradiography. The data show a major band at 55 kDa, with minor bands at 70 kDa and 150 kDa, in virus that had been incubated with AnxA2t, as well as in virus alone (figure 14A). Non-immune rabbit IgG did not immunoprecipitate these bands, indicating that their detection was due specifically to interactions with a p11-containing species, i.e. AnxA2t. Anti-p36 Western blot confirmed the immunoprecipitation of p36 by anti-p11 antibody, but not by rabbit IgG. These data reveal potential CMV ligands may form a protein complex with AnxA2t. These results also show that AnxA2t endogenous to the virus, as well as purified AnxA2t, can interact with CMV species.

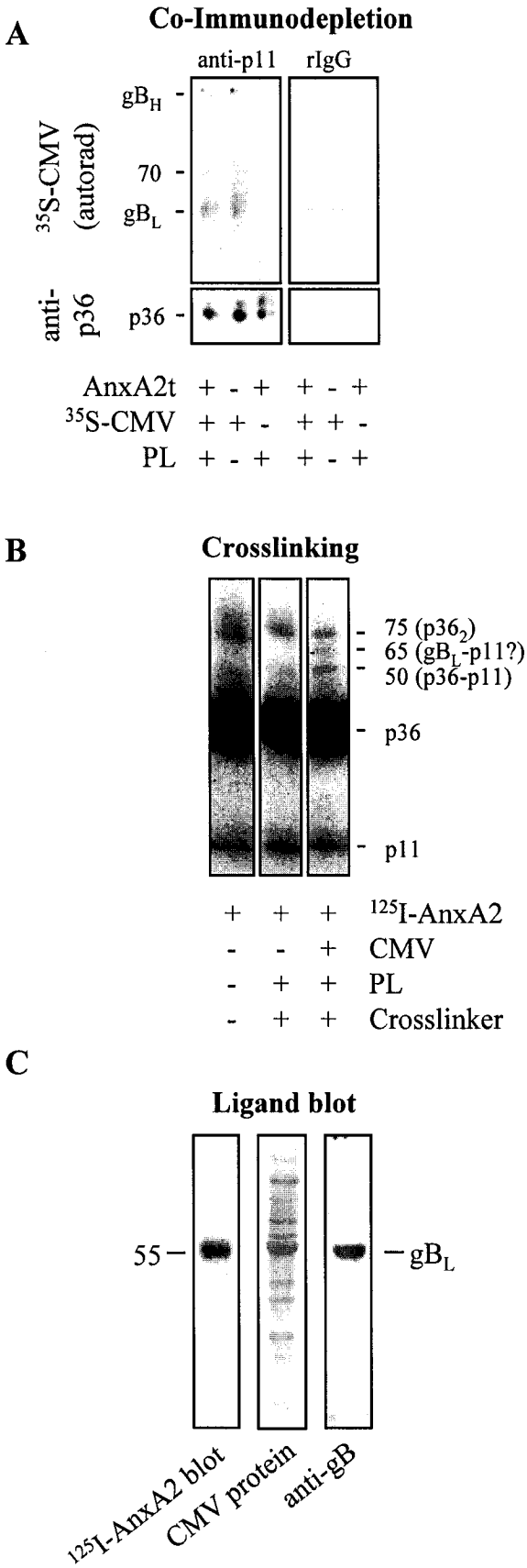

A
Co-Immunodepletion
anti-p11
rIgG
gB_H
70
gB_L
35S-CMV (autorad)
anti-p36
p36
AnxA2t + - + + - +
35S-CMV + + - + + -
PL + - + + - +
B
Crosslinking
75 (p36_2)
65 (gB_L-p11?)
50 (p36-p11)
p36
p11
+ + + 125I-AnxA2
- - + CMV
- + + PL
- + + Crosslinker
C
Ligand blot
55
gB_L
125I-AnxA2 blot
CMV protein
anti-gB

Figure 14. CMV ligands for AnxA2. A. Lysed ^{35}S-CMV particles (10^8 virus/ml) were incubated with 100 nM AnxA2t and phospholipid vesicles (PL, 2 μM phospholipid). The mixtures were subject to immunoprecipitation using anti-p11 antibody or non-immune rabbit IgG (rIgG). Protein complexes were lysed in SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred to PVDF, and subject to autoradiography and anti-p36 Western blot analysis. B. CMV particles ($5x10^7$ virus/ml) were incubated with 100 nM ^{125}I-labelled purified placental AnxA2 pre-bound to PL vesicles (2 μM phospholipid). Equilibrium interactions were trapped using 200 μM of the chemical crosslinker sulfo-BSOCOES. The mixtures were lysed in SDS-PAGE sample buffer, resolved by SDS-PAGE, and subject to autoradiography. C. CMV (10^9 virus particles/lane) was resolved by SDS-PAGE, transferred to PVDF, and either incubated with 100 nM ^{125}I-labelled purified placental AnxA2 and subject to autoradiography, stained with Coomassie Blue protein stain, or subject to anti-gB light chain Western blot analysis, as indicated.

3.3.2. ^{125}I-AnxA2-CMV solution-phase crosslinking. A second approach to identify AnxA2 ligands on CMV consisted of the chemical crosslinking of intact CMV virions to phospholipid-bound ^{125}I-labelled purified placental A2. The resulting ^{125}I-AnxA2-CMV complexes were lysed, resolved by SDS-PAGE and visualized by autoradiography. A unique complex at 65 kDa was observed only in the presence of both phospholipid-bound ^{125}I-AnxA2 and CMV (figure 14B). The specificity of this 65-kDa complex was demonstrated by its absence when either CMV or crosslinker was not present. Since any radiolabelled complex must necessarily comprise ^{125}I-AnxA2, the 65-kDa complex is consistent in size with a complex between p11 and a 55-kDa species, confirming the results from the immunodepletion experiment.

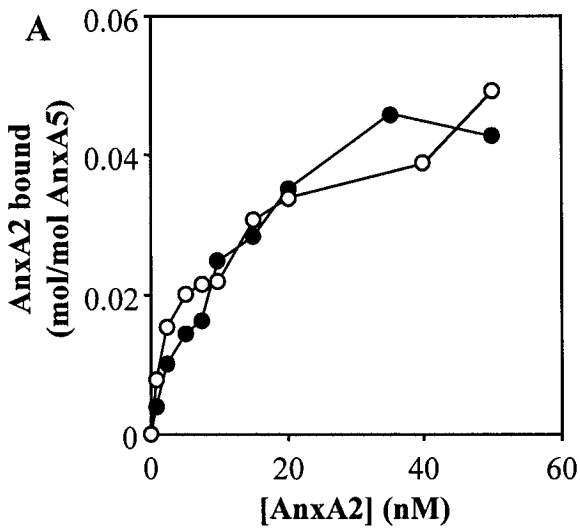
3.3.3. ^{125}I-AnxA2 ligand blot. A final approach to identify AnxA2 ligands on CMV consisted of the electrophoretic separation and transfer to a PVDF membrane of purified CMV. The CMV proteins were then incubated with phospholipid-bound ^{125}I-labelled purified placental AnxA2, and subject to autoradiography to identify AnxA2-interacting species. ^{125}I-AnxA2 interacted with a 55-kDa protein associated with the purified CMV preparation (figure 14C), confirming the results from the immunodepletion and solution-phase crosslinking experiments. Furthermore, the 55-kDa AnxA2 ligand co-migrated with the major band in CMV, the light chain of glycoprotein B (gB_L), as demonstrated by both Coomassie blue protein stain and anti-gB Western blot, suggesting an interaction between gB_L and membrane-bound AnxA2. Collectively, these data suggest an interaction between glycoprotein B and AnxA2, consistent with previous reports (61, 430). In addition, it has been shown for the first time that the gB-AnxA2 interaction

likely occurs between gB_L and p11, and that membrane-bound AnxA2 may participate in these interactions. These data are consistent with our observations that cellular AnxA2t, but not p36, can facilitate CMV infection.

3.4 ANXA2-ANXA5 INTERACTION.

It has been previously shown in our laboratory that AnxA5, a protein homologous to AnxA2, has no significant effect on CMV-phospholipid binding and fusion, but inhibits AnxA2-mediated CMV-phospholipid fusion (458). These results suggest an interaction between AnxA2 and AnxA5. An investigation in our laboratory using fluorescence quenching demonstrated an interaction between FITC-labelled AnxA5 and purified AnxA2 (69). To confirm and further characterize the AnxA2-AnxA5 interaction, surface plasmon resonance (SPR) was undertaken.

AnxA5 was covalently linked to an SPR dextran chip. Soluble p36 or AnxA2t was flowed over the chip at different concentrations, and total binding was noted at the end of the injection. Both AnxA2t and p36 bound in equivalent molar amounts (figure 15A), with dissociation constants of approximately 5-10 nM, consistent with those observed in the fluorescence experiments (69). Neither soluble AnxA5 nor ovalbumin bound in detectable amounts to linked AnxA5, demonstrating the specificity of the AnxA2-AnxA5 interaction (figure 15B). These data confirm the high-affinity AnxA2-AnxA5 interaction, and further show that both AnxA2t and p36 may interact with AnxA5.


A
0.06
0.04
0.02
0
AnxA2 bound
(mol/mol AnxA5)
0
20
40
60
[AnxA2] (nM)

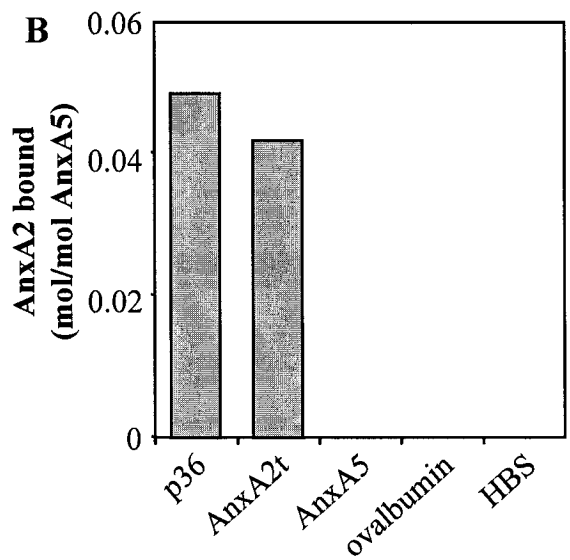

B
0.06
0.04
0.02
0
AnxA2 bound
(mol/mol AnxA5)
p36
AnxA2t
AnxA5
ovalbumin
HBS

Figure 15. Interaction between AnxA2 and AnxA5. Surface plasmon resonance was employed to study the interaction between AnxA2 and AnxA5. A. Purified AnxA2t (●) or p36 (○) were injected at the indicated concentration over a sensor chip covalently linked to AnxA5. Each point represents the change in response units (RU) at the end of the injection period. B. 50 nM purified p36, AnxA2t, AnxA5, ovalbumin, or HBS alone was injected over a sensor chip covalently linked to AnxA5. Each point represents the change in RU at the end of the injection period.

3.5. ANXA5 AND ANXA1 IN CMV INFECTION.

Our laboratory has shown that AnxA5 inhibits AnxA2-mediated fusion between the CMV envelope and anionic phospholipid-containing membranes (458). We hypothesized that the inhibition of fusion was due to the direct interaction between AnxA2 and AnxA5 (69), and that AnxA5 may comprise a natural line of defense by the host cell against CMV invasion. AnxA1 has been also reported to bind AnxA2 in a calcium-dependent manner (323), though no link has been reported between this interaction and CMV infection. We therefore hypothesized that AnxA5 or AnxA1 could inhibit CMV by binding AnxA2 and interfering with its enhancement of infection.

3.5.1. Inhibition of CMV infection. To determine whether AnxA5 and AnxA1 decrease CMV infection, we assessed the effect of both exogenously added purified proteins and endogenously expressed proteins, using antibodies.

3.5.1.1. Purified AnxA5 and AnxA1. To evaluate the effect of AnxA5 or AnxA1 on the entire CMV infection cycle, we assessed the effect of purified AnxA5 and AnxA1 on 2-step CMV infection, i.e. the ability of supernatants from CMV-infected HFF to generate either IE72 expression or plaque formation in fresh HFF. When either AnxA5 or AnxA1 was present during the initial inoculation, IE72 expression in fresh HFF was inhibited in a dose-dependent manner to a maximum of approximately 80% (figure 16A). Confirming the 2-step IE72 expression assays, the presence of AnxA5 or AnxA1 during the initial inoculation significantly decreased 2-step plaque formation in a dose-dependent manner to a maximum of approximately 45% (figure 16B). Demonstrating the antigenic

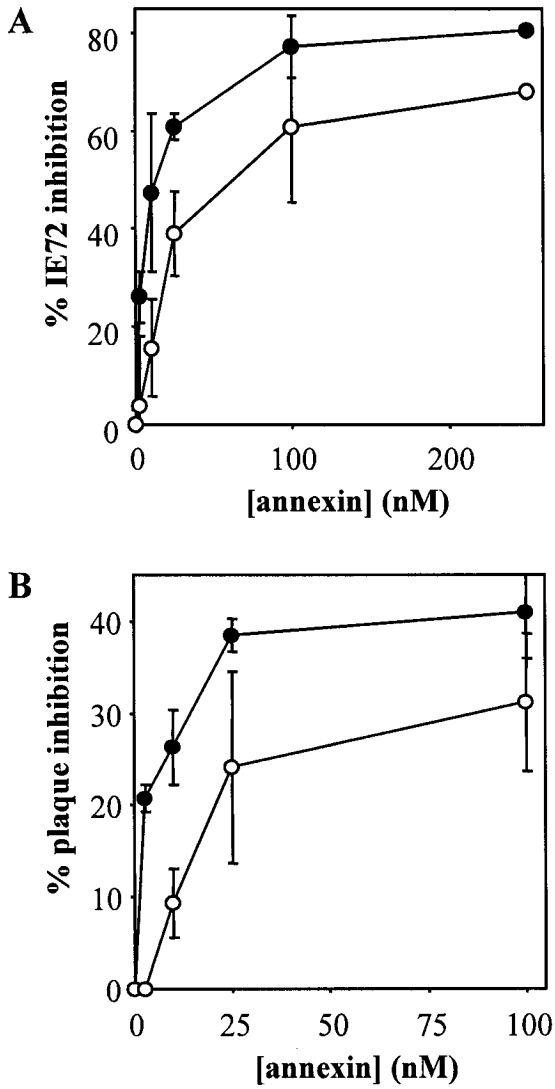

A
% IE72 inhibition
0
20
40
60
80
0
100
200
[annexin] (nM)
B
% plaque inhibition
0
10
20
30
40
0
25
50
75
100
[annexin] (nM)

Figure 16. AnxA5 and AnxA1 in productive infection in HFF. HFF were inoculated with CMV in the presence of the indicated concentrations of AnxA5 (○) or AnxA1 (●). On day 7 post-infection, supernatants were collected and used to inoculate fresh HFF. A. At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point corresponds to the average change in plaque number from two separate experiments comprising the average of two wells each. Error bars represent standard error; $p<0.05$. B. Plaques were quantified 6 days after the second inoculation. Each point corresponds to the average change in plaque number from three separate experiments comprising the average of two wells each. Error bars represent standard deviation; $p<0.05$.

specificity of the inhibition, no significant effect was observed in the presence of p36 (figure 10A, 9B). These data demonstrate that both AnxA5 and AnxA1 can inhibit production of infectious CMV progeny.

To pinpoint at what stage of the CMV life cycle AnxA5 and AnxA1 inhibit CMV infection, the effect of purified AnxA5 and AnxA1 on various steps of the infection cycle was assessed. AnxA5 and AnxA1 each inhibited 1-step IE72 expression by approximately 60% (figure 17A). However, exogenous p36 had no significant effect (figure 11A). These results indicate that AnxA5 and AnxA1 specifically decrease CMV infection at or before immediate-early gene expression.

To further locate the stage of infection that is affected by AnxA5 and AnxA1, primary cytopathic plaque assays were conducted. Both AnxA5 and AnxA1 significantly inhibited primary plaque number by approximately 45% (figure 17B). No significant effect was observed in the presence of p36 (figure 11B). Since primary plaque formation assays are used to measure pre-entry events in infection (203), these data indicate that AnxA5 and AnxA1 both specifically inhibit CMV infection at a stage prior to CMV-cell entry.

To evaluate the effect of AnxA5 and AnxA1 on CMV-cell attachment, their effect on ^{35}S-CMV-cell binding was investigated. The addition of either AnxA5 or AnxA1 had no significant effect on ^{35}S-CMV binding to host HFF (figure 17C). These data indicate that the inhibition occurs at a step occurring after CMV-cell attachment.

3.5.1.2. Anti-AnxA5 and -AnxA1 monoclonal antibodies. To determine whether the inhibition AnxA5 and AnxA1 could be reversed, and to test the involvement of

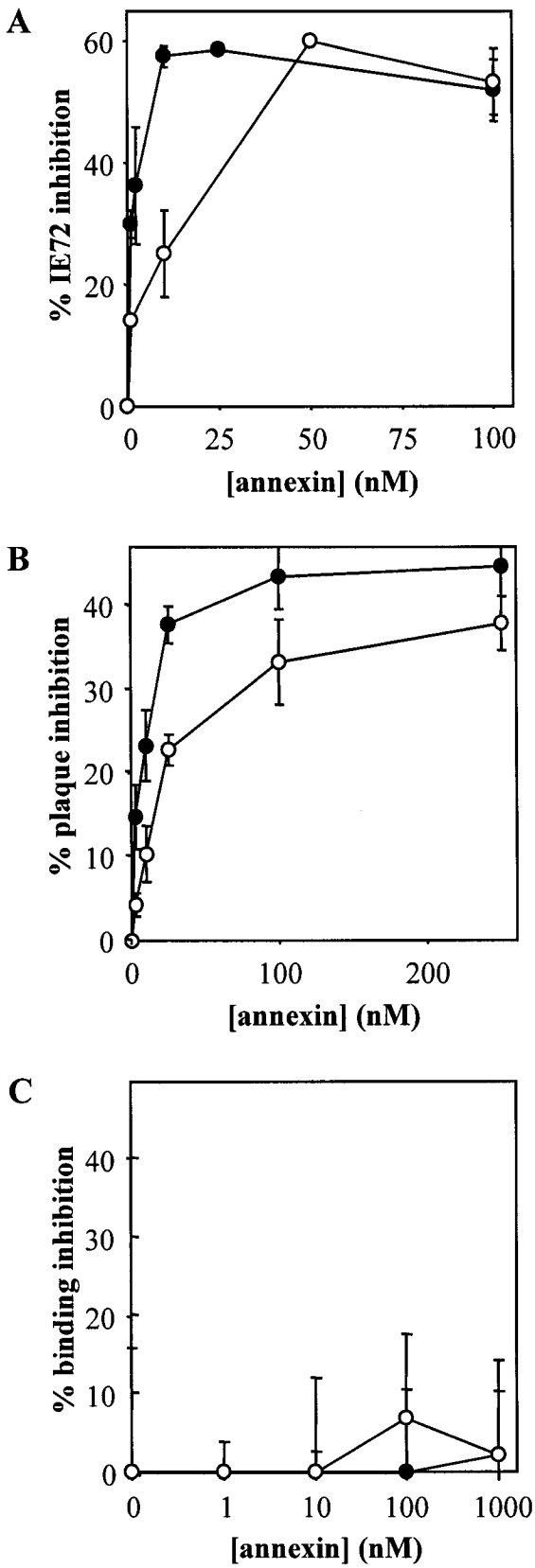

A
% IE72 inhibition
0
20
40
60
0
25
50
75
100
[annexin] (nM)
B
% plaque inhibition
0
10
20
30
40
0
100
200
[annexin] (nM)
C
% binding inhibition
0
10
20
30
40
0
1
10
100
1000
[annexin] (nM)

Figure 17. AnxA5 and AnxA1 in early infection stages in HFF. A. HFF were inoculated with CMV at an MOI of 0.003 in the presence of the indicated concentrations of AnxA5 (○) or AnxA1 (●).At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point corresponds to the average change in plaque number from two separate experiments comprising the average of two wells each. Error bars represent standard error; $p<0.05$. B. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentrations of AnxA5 (○) or AnxA1 (●). Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from four separate experiments comprising the average of two wells each. Error bars represent standard deviation; $n=4$; $p<0.05$. C. HFF and ^{35}S-CMV were incubated at 4°C in the presence of the indicated concentrations of AnxA5 (○) or AnxA1 (●). After washing, HFF were solubilized and subject to scintillation counting. Each point corresponds to the average change in CMV binding from three wells. Error bars represent standard deviation; $p<0.05$.

endogenous AnxA5 and AnxA1, HFF were inoculated in the presence of mAbs directed against either AnxA5 or AnxA1. Neither mAb had any effect on primary plaque formation when added alone (figure 18A, 18B). However, anti-AnxA5 and -AnxA1 mAbs each abrogated the effect of purified AnxA5 and AnxA1, respectively (figure 18A, 18B). Neither non-immune mouse IgG2a nor non-immune mouse IgG1, the respective isotype controls, had any effect on primary plaque formation alone, nor did they significantly affect inhibition by AnxA5 or AnxA1, respectively (figure 18A, 18B), demonstrating that the effect of the antibodies on reversal of inhibition was specific. These data indicate first that inhibition by purified AnxA5 and AnxA1 is antigenically reversible, and therefore specific. However, the lack of effect by the mAbs alone suggests that endogenous AnxA5 and AnxA1 may not behave in the same manner as exogenously added AnxA5 and AnxA1.

Taken together, these data indicate that purified AnxA5 and AnxA1 specifically inhibit a stage of infection that occurs prior to the detection of cytopathicity, but after attachment (table 5).

3.5.2. Mechanism of CMV inhibition by AnxA5 and AnxA1. Having determined that both AnxA5 and AnxA1 inhibit CMV infection, the mechanism of inhibition was investigated. Interference with AnxA2 activity was postulated, since 1) AnxA5 and AnxA1 are documented to interact with AnxA2, and 2) AnxA5 and AnxA1 inhibit the same stages of CMV infection as are enhanced by endogenous AnxA2t, that is, a post-attachment, pre-entry step of infection.

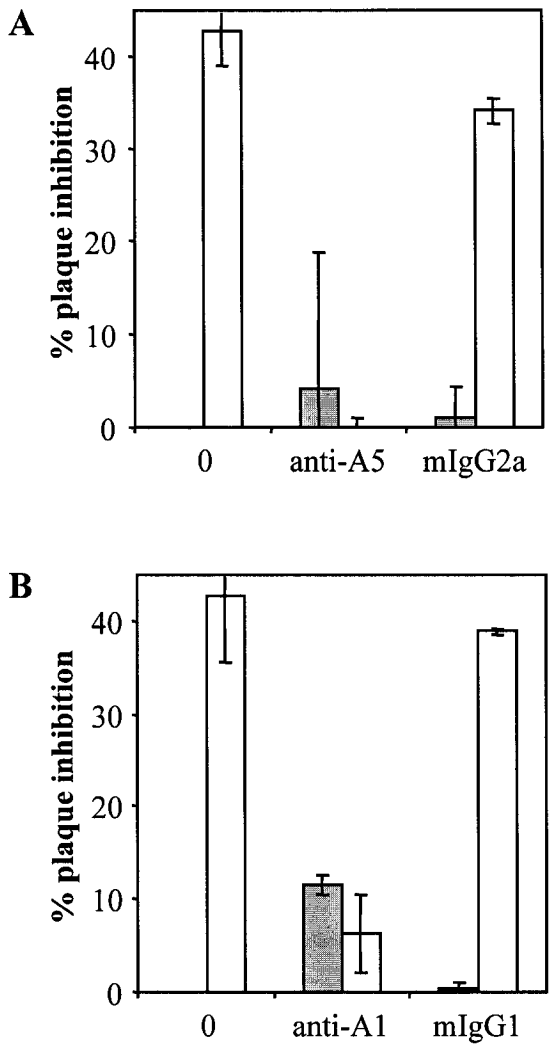

A
% plaque inhibition
40
30
20
10
0
0
anti-A5
mIgG2a
B
% plaque inhibition
40
30
20
10
0
0
anti-A1
mIgG1

Figure 18. Anti-AnxA5 and -AnxA1 antibodies in plaque formation in HFF. A. HFF were inoculated with CMV in the presence of 25 nM anti-Anx5 antibody or non-immune mouse IgG2a alone (gray bars) or in the presence of 50 nM AnxA5 (white bars), at an MOI of 0.0003. Plaques were quantified on day 6 post-infection. Each point represents the average change in plaque number from two separate wells. Error bars represent standard error; $p<0.05$. B. HFF were inoculated with CMV in the presence of 25 nM anti-AnxA1 antibody or non-immune mouse IgG1 alone (gray bars) or in the presence of 50 nM AnxA1 (white bars), at an MOI of 0.0003. Plaques were quantified on day 6 post-infection. Each point represents the average change in plaque number from two separate wells. Error bars represent standard error; $p<0.05$.

	1-step plaques[a]		1-step IE72		2-step plaques[b]		2-step IE72	
	I_{max}[c]	IC50[d]	I_{max}	IC50	I_{max}	IC50	I_{max}	IC50
AnxA1	43	5.0	58	1.1	42	3.4	81	6.0
AnxA5	45	24	64	11	44	39	77	29

Table 5. Summary of the effects of annexins 1 and 5 on CMV infection

[a]In 1-step experiments, HFF were inoculated in the presence of the indicated treatment, and the effect on primary infection events was evaluated.

[b]In 2-step experiments, HFF were inoculated in the presence of the indicated treatment. Supernatants of infected cells were subsequently evaluated for presence of infectious progeny.

[c]Inhibition = 100 - percent of untreated control.

[d]Concentration at half-maximal inhibition (nM).

3.5.2.1. Combined AnxA5 and AnxA1. Comparison of the inhibition by AnxA5 and AnxA1 showed that the IC50 of AnxA1 is approximately 5- to 10-fold lower than that of AnxA5 (table 5), suggesting that the mechanism of inhibition may not be identical. Therefore, to determine whether AnxA5 and AnxA1 act on the same pathway of infection, HFF were inoculated in the presence of either AnxA5 or AnxA1, or both, and primary plaque formation was quantified. In the presence of 50 nM AnxA5 (i.e. 50% of maximal inhibition alone), the apparent IC50 of AnxA1 was decreased, due to an additive effect with AnxA5 (figure 19A). Similarly, in the presence of 5 nM (i.e. 50% of maximal inhibition alone) AnxA1, the apparent IC50 of AnxA5 was decreased due to an additive effect with AnxA1 (figure 19B). However, the maximum inhibition observed (I_{max}) for AnxA5 and AnxA1 together was the same as for either reagent alone. These data show AnxA5- and AnxA1-dependent inhibition of CMV production was additive at sub-maximal concentrations, but that at saturating concentrations of either AnxA5 or AnxA1, no significant additional inhibition was observed. Therefore, AnxA5 and AnxA1 appear to function independently, but affect the same saturable stage of the CMV infection pathway.

In light of our finding that AnxA5 and AnxA1 use a similar mechanism to inhibit CMV infection, and that the step(s) affected is the same as that enhanced by endogenous AnxA2t, we therefore investigated whether AnxA1- and AnxA5-mediated inhibition of CMV infection was dependent on AnxA2.

3.5.2.2. AnxA5 and AnxA1 with purified AnxA2. The first approach taken consisted of the pre-incubation of AnxA5 or AnxA1 with purified AnxA2 prior to inoculation. Since

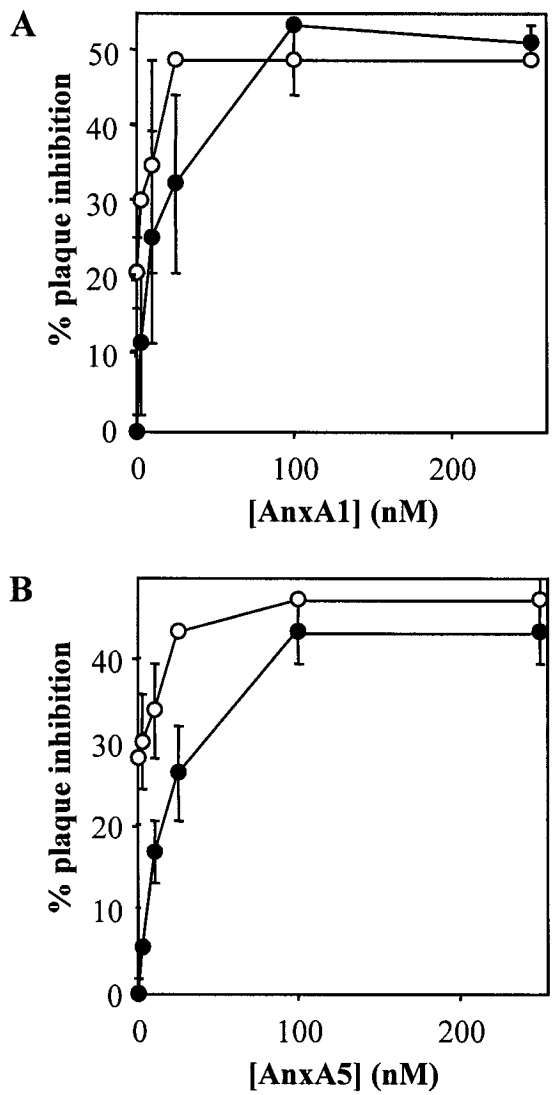

A
% plaque inhibition
0
10
20
30
40
50
0
100
200
[AnxA1] (nM)
B
% plaque inhibition
0
10
20
30
40
0
100
200
[AnxA5] (nM)

Figure 19. Combined AnxA5 and AnxA1 in plaque formation in HFF. A. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentration of AnxA1 alone (•) or with 50 nM AnxA5 (○). Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$. B. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentration of AnxA5 alone (•) or with 5 nM AnxA1 (○). Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$.

both AnxA5 and AnxA1 have been reported to interact with AnxA2 in purified systems (69, 323), we hypothesized that the addition of AnxA2 to AnxA5 or AnxA1 would prevent their binding to endogenously expressed AnxA2 on the host cell surface, thus attenuating inhibition. Purified AnxA2t, p11, or p36 alone had no observable effect on primary plaque formation (figure 11B), thereby simplifying the interpretation of effects that the purified forms of AnxA2 may have on AnxA5 or AnxA1. These experiments showed that AnxA5-mediated inhibition of CMV infection could be completely reversed by pre-incubation with purified AnxA2t, but was not significantly affected by the addition of purified p11 or p36 (figure 20A), demonstrating the specificity of the effect of AnxA2t on AnxA5. Restoration of expected inhibition was achieved in the presence of a 10-fold molar excess of AnxA5 (250 nM) over AnxA2t (25 nM), but not a 4-fold molar excess (100 nM). Similar to AnxA5, AnxA1-mediated inhibition could be completely reversed by pre-incubation with purified AnxA2t. However, unlike AnxA5, both p11 and p36 also reversed the inhibitory effect of AnxA1 (figure 20B). Also unlike AnxA5, a 4-fold molar excess of AnxA1 (100 nM) was sufficient to fully restore the expected extent of inhibition. These reversal data show that the addition of purified AnxA2t to either AnxA5 or AnxA1 abrogated their ability to decrease infection, suggesting that inhibition occurred via interactions with endogenously expressed AnxA2 on the host cell surface. That purified p36 and p11 abrogated inhibition by AnxA1, but not AnxA5, implies that the binding site on AnxA2 for AnxA1 may differ from that of AnxA5.

3.5.2.3. AnxA5 and AnxA1 with inhibitory anti-AnxA2 antibodies. A second approach taken to determine whether AnxA1- and AnxA5-mediated inhibition occurred

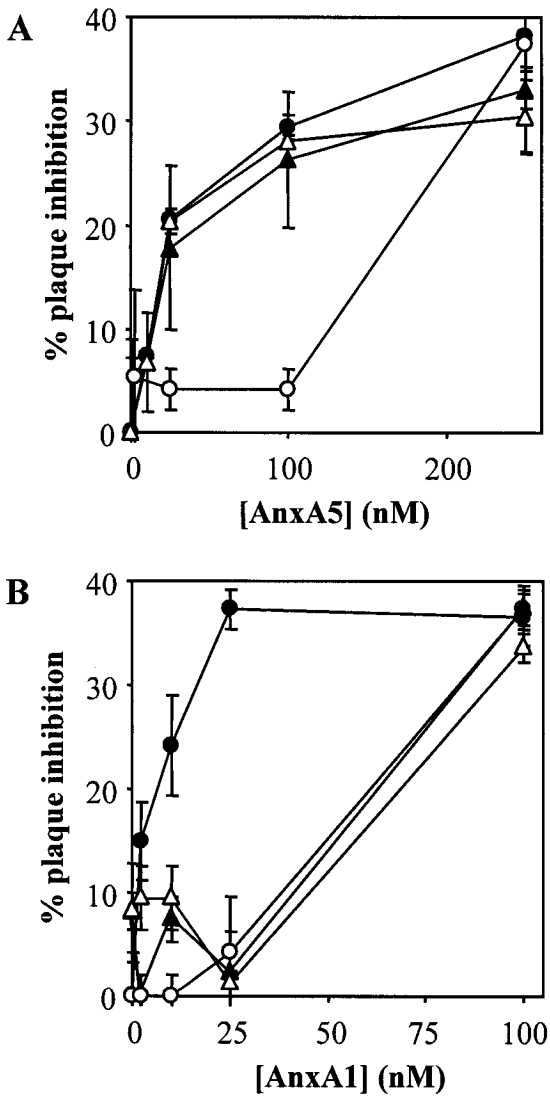

A
40
30
20
10
0
% plaque inhibition
0
100
200
[AnxA5] (nM)
B
40
30
20
10
0
% plaque inhibition
0
25
50
75
100
[AnxA1] (nM)

Figure 20. AnxA5 and AnxA1 with purified AnxA2 in plaque formation in HFF. A. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentration of AnxA5 alone (●) or pre-mixed with 25 nM AnxA2t (○), p11 (▲), or p36 (Δ). Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$. B. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentration of AnxA1 alone (●) or pre-mixed with 25 nM AnxA2t (○), p11 (▲), or p36 (Δ). Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$.

through interference with cellular AnxA2 consisted of the use of anti-p11 and anti-p36 antibodies, which alone are inhibitory to CMV plaque formation (figure 7B). We hypothesized that the addition of AnxA5 or AnxA1 to these antibodies would not result in additional inhibition, since all are postulated to inhibit AnxA2-dependent CMV infection events. HFF were inoculated with CMV in the presence of anti-AnxA2 antibodies with either AnxA5 or AnxA1, and primary plaque formation was quantified. In the presence of 0.01 nM (i.e. 50% of maximal inhibition alone) anti-p11 or anti-p36, the apparent IC50 for AnxA5 was observed at a lower concentration, due to an additive effect with each of the antibodies (figure 21A). Similarly, the apparent IC50 for AnxA1 was also observed at a lower concentration in the presence of anti-p11 or anti-p36, due to an additive effect (figure 21B). However, the addition of antibodies did not significantly alter the I_{max} for either AnxA5 or AnxA1. As expected, non-immune rabbit IgG alone had no effect on infection, nor did it alter AnxA5 or AnxA1 activity (figure 21A inset, 21B inset). These observations suggest AnxA5, AnxA1, and anti-AnxA2 antibodies each target a similar CMV infection pathway for inhibition, further supporting the possibility that AnxA5 and AnxA1 inhibit infection by interfering with endogenous AnxA2-dependent events.

3.5.2.4. AnxA5 and AnxA1 with non-inhibitory anti-AnxA2 antibodies. An additional approach taken to determine whether AnxA1- and AnxA5-mediated inhibition occurred through interference with AnxA2 activity consisted of the use of non-inhibitory anti-p11 and anti-p36 mAbs, which alone had no significant effect on primary plaque formation (figure 8B). We hypothesized that the addition of these antibodies might protect AnxA2

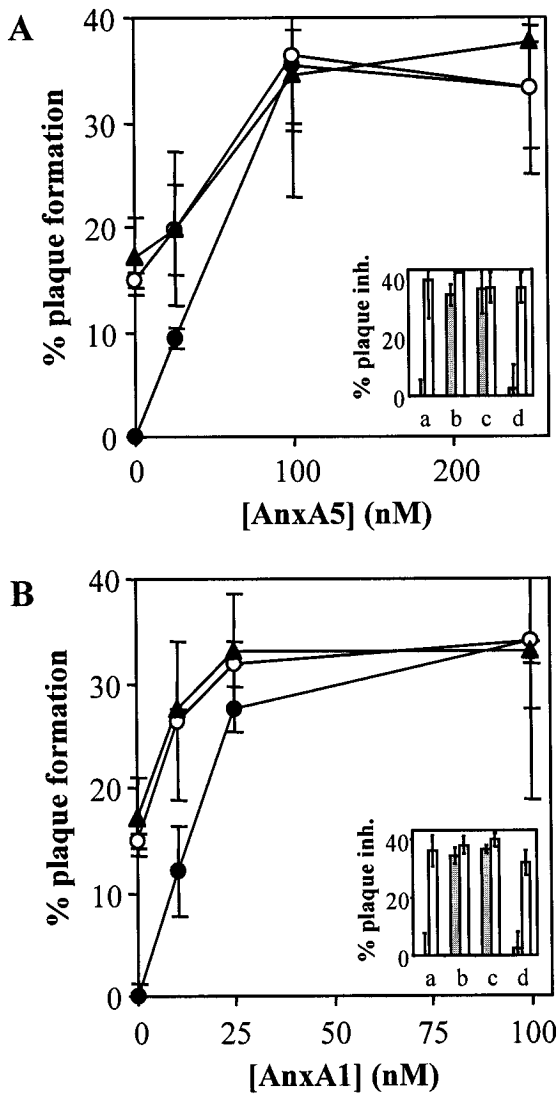

A
40
30
20
10
0
% plaque formation
0
100
200
[AnxA5] (nM)
% plaque inh.
40
20
0
a b c d
B
40
30
20
10
0
% plaque formation
0
25
50
75
100
[AnxA1] (nM)
% plaque inh.
40
20
0
a b c d

Figure 21. AnxA5 and AnxA1 with inhibitory anti-AnxA2 in plaque formation in HFF. A. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentration of AnxA5 alone (●) or with 0.01 nM anti-p11 (○) or anti-p36 (▲) antibody. Plaques were quantified on day 6 post-infection. Error bars represent standard error; n=2; $p<0.05$. *Inset*, HFF were inoculated with CMV at an MOI of 0.0003 in the absence (gray bars) or presence (white bars) of AnxA5 (100 nM) with no antibody (a), 10 nM anti-p11 (b), 10 nM anti-p36 (c), or 10 nM non-immune rabbit IgG (d). Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$. B. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentration of AnxA1 alone (●) or with 0.01 nM anti-p11 (○) or anti-p36 (▲) antibody. Plaques were quantified on day 6 post-infection. Error bars represent standard error; n=2; $p<0.05$. *Inset*, HFF were inoculated with CMV at an MOI of 0.0003 in the absence (gray bars) or presence (white bars) of AnxA1 (25 nM) with no antibody (a), 10 nM anti-p11 (b), 10 nM anti-p36 (c), or 10 nM non-immune rabbit IgG (d). Plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$.

from the inhibitory effects of AnxA5 and AnxA1 by interfering with binding. HFF were inoculated with CMV in the presence of these "non-inhibitory" anti-AnxA2 mAbs with either AnxA5 or AnxA1, and primary plaque formation was quantified. As expected, none of the antibodies alone had any significant effect on primary plaque formation (figure 22A-D). However, though AnxA5-mediated inhibition was unaffected by Translab anti-p11 (figure 22A), inhibition was attenuated in a dose-dependent manner by Oncogene anti-p36 (figure 22B), Translab anti-p36 (figure 22C) and Zymed anti-p36 (figure 22D). Interestingly, AnxA1-mediated inhibition was attenuated by a somewhat different set of anti-AnxA2 antibodies: both Translab anti-p11 (figure 22A) and Oncogene anti-p36 (figure 22B) reversed AnxA1-mediated inhibition, but Translab anti-p36 (figure 22C) and Zymed anti-p36 (figure 22D) had no significant effect. Neither mouse IgG1 (figure 22E), the isotype control for the Translab and Zymed mAbs, nor mouse IgG2a (figure 22F), the isotype control for the Oncogene mAb, had any effect on AnxA5 or AnxA1, indicating that the effects of the anti-AnxA2 mAbs were specific. That the AnxA5- and AnxA1-mediated inhibition could be abrogated by the addition of anti-AnxA2 antibodies, which alone had no effect on CMV plaque formation, suggests that AnxA5 and AnxA1 inhibition is dependent upon an interaction with AnxA2. Since the various AnxA2-specific antibodies differentially affected CMV infection in the presence of AnxA5 or AnxA1, distinct epitopes on AnxA2 may be involved in CMV infection and in interactions with AnxA1 and AnxA5.

3.5.2.5. AnxA5 and AnxA5 in CMV infection of HepG2. To corroborate our HFF studies showing that AnxA5 or AnxA1 inhibit CMV infection, and to further investigate

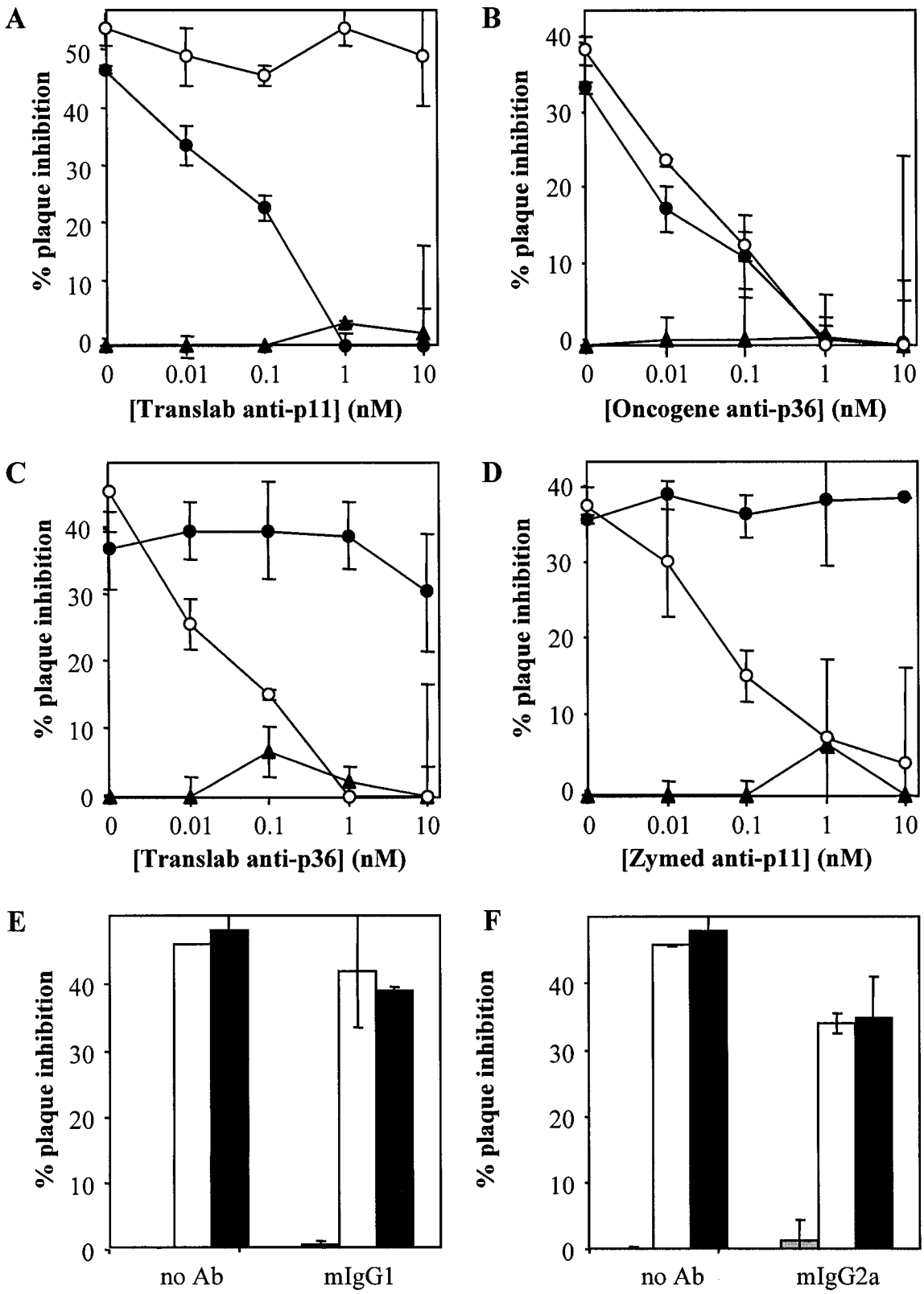

A
% plaque inhibition
50
40
30
20
10
0
0
0.01
0.1
1
10
[Translab anti-p11] (nM)
B
% plaque inhibition
40
30
20
10
0
0
0.01
0.1
1
10
[Oncogene anti-p36] (nM)
C
% plaque inhibition
40
30
20
10
0
0
0.01
0.1
1
10
[Translab anti-p36] (nM)
D
% plaque inhibition
40
30
20
10
0
0
0.01
0.1
1
10
[Zymed anti-p11] (nM)
E
% plaque inhibition
40
30
20
10
0
no Ab
mIgG1
F
% plaque inhibition
40
30
20
10
0
no Ab
mIgG2a

Figure 22. AnxA5 and AnxA1 with non-inhibitory anti-AnxA2 in plaque formation in HFF. A-D. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of the indicated concentration of anti-AnxA2 antibody alone (▲) or with 250 nM AnxA5 (○) or 100 nM AnxA1 (●). Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$. E, F. HFF were inoculated with CMV at an MOI of 0.0003 in the presence of 10 nM non-immune mouse IgG1 (E) or IgG2a (F) either alone (gray bars), with 250 nM AnxA5 (white bars) or with 100 nM AnxA1 (black bars). All plaques were quantified on day 6 post-infection. Each point corresponds to the average change in plaque number from two wells. Error bars represent standard error; $p<0.05$.

the possible AnxA2 dependence of this inhibition, a second cell model was used. Here the effect of AnxA5 and AnxA1 in the p36-null cell line, HepG2, was followed. Transfection of p36 into HepG2 cells increased primary IE72 expression by approximately 3-fold (figure 13A), indicating that there is an AnxA2-dependent component to CMV-cell interplay.

To determine the influence of p36 expression on AnxA5- and AnxA1-mediated inhibition of CMV infection, HepG2 cells were inoculated in the presence of AnxA5 or AnxA1, and primary IE72 expression was quantified. In native HepG2 cells, neither AnxA5 nor AnxA1 had any significant effect on IE72 expression (figure 23A). However, in HepG2 that had been transfected with p36, both AnxA5 and AnxA1 significantly inhibited IE72 expression by approximately 80% (figure 23B). These results show that in HepG2, expression of p36 is required for AnxA5 and AnxA1 to mediate inhibition, indicating that that AnxA1- and AnxA5-mediated inhibition of CMV is dependent on the presence of cellular AnxA2, and providing further evidence that CMV inhibition is AnxA2-dependent.

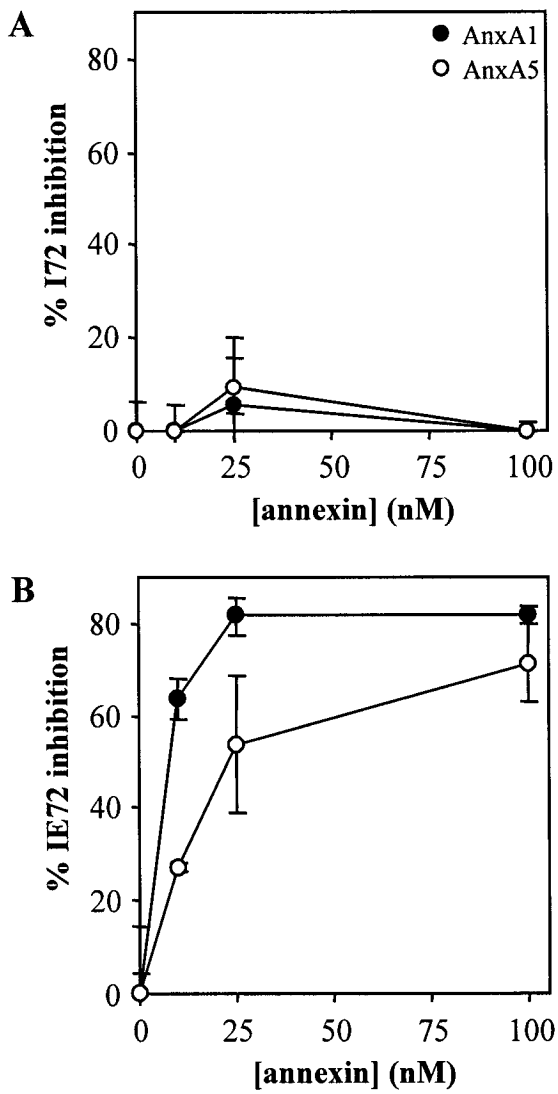

A
AnxA1
AnxA5
% I72 inhibition
80
60
40
20
0
0
25
50
75
100
[annexin] (nM)
B
% IE72 inhibition
80
60
40
20
0
0
25
50
75
100
[annexin] (nM)

Figure 23. AnxA5 and AnxA1 in primary IE72 expression in HepG2. Native (A) or p36-transfected (B) HepG2 were inoculated in the presence of the indicated concentrations of either AnxA5 (○) or AnxA1 (●).At 20 hours post-infection, lysates were collected and subjected to Western blot analysis for IE72 expression. Band intensities were quantified and normalized to β-actin levels. Each point corresponds to the average change in plaque number from two separate experiments comprising the average of two wells each. Error bars represent standard error; $p<0.05$.

4. DISCUSSION

4.1. EXPRESSION OF ANNEXINS IN CELLS.

The goal of this study was to investigate the roles of different annexins, specifically AnxA2, AnxA5 and AnxA1, in CMV infection. For any species to affect CMV pre-entry infection events, it must be available on the cell surface or on the virus envelope to interact with other cellular and/or viral species during infection. Though AnxA2 (39, 70, 86, 105, 106, 157, 207, 307, 342, 344, 426), AnxA5 (305, 413, 451), and AnxA1 (85, 91, 117, 513) have been detected on the surface of various cell types, whether these species are present on the surface of the cell types used in the current study, HFF and HepG2, or on the CMV envelope is unknown. Here we report that in HFF cells, the standard cell model used in investigations of CMV biochemistry and infectivity, AnxA5 and AnxA1 were specifically detected on the cell surface following EGTA elution. p36 was also detected on the surface of HFF, confirming an earlier report (426). In HepG2 cells, AnxA5 was detected on the surface of both native and p36-transfected cells, AnxA1 was not detected in lysates of either cell line, and p36 was detected on the surface of AnxA2 transfectants only, as expected (443). Surface annexin expression comprised approximately 1-2% of total cellular expression (summarized in table 1), similar to a previous report (206). Since EGTA elution removes only calcium-dependently bound proteins from the surface, a calcium-independent pool of AnxA1, AnxA2 and AnxA5 may also exist on the cell surface, since all have been reported to interact with phospholipid and cell membranes in a calcium-independent manner (215, 296, 430, 473).

AnxA1, AnxA5, and AnxA2, but not NF-κB p65, were all detected in the CMV lysate. However, the respective amounts detected did not correspond to their relative levels in HFF (summarized table 2), the cells in which CMV was propagated. This may suggest selectivity in inclusion of cellular proteins in the CMV envelope, leading to speculation that CMV may derive an advantage in infection by altering the relative levels of annexins in its envelope.

4.2. ANXA2 IN CMV INFECTION.

Investigating the effect of AnxA2 on CMV infection is complicated by: 1) the ligand p11, which enables the concurrent availability of functionally different monomeric (p36) and tetrameric (A2t; $p36_2p11_2$) configurations; 2) sources endogenous to both the host cell (39, 70, 86, 105, 106, 157, 207, 307, 342, 344, 426) and purified virus (430, 458, 602); 3) known functional changes caused by regulated phosphorylation of AnxA2 (250, 254, 460); and 4) signal-induced redistribution of the intracellular AnxA2, some of which is translocated to the cell surface (158, 426, 476). The latter involves membrane trafficking, and requires physiological temperature to ensure membrane fluidity. Combined with the knowledge that CMV can facilitate cell signalling (64, 505, 616, 617), AnxA2 may thus participate at numerous points along the infection pathway, inside and outside of cells. In the current work, immunoinhibition, purified proteins, and cell transfection studies were employed to systematically evaluate the effect of both endogenous and exogenous AnxA2 (summarized in table 4). Our findings that, 1) certain antibodies to p36 or p11 inhibit all steps of CMV infection we examined subsequent to virus-cell attachment, and 2) purified AnxA2t or p11, but not p36, enhance infection at

all but one of the steps, strongly support a role for AnxA2t. These observations confirm the previous evidence implicating AnxA2 in the CMV infection mechanism (430, 458, 602, 603).

4.2.1. Endogenous AnxA2. When HFF, the standard cell model for studying CMV infection, were inoculated in the presence of antibodies directed against p11 or p36, productive CMV infection was inhibited at a stage occurring prior to expression of the first viral gene product, IE72. To confirm the involvement of endogenous AnxA2 at an early stage of infection using a different host cell model, we employed HepG2 cells, the only cell line known not to express detectable p36 mRNA or antigen. HepG2 cells constitutively express p11, which has been shown to be upregulated by p36 transfection, implying AnxA2t formation (443). In HepG2, p36 expression also increased early CMV infection events prior to or at IE72 expression, confirming the role for endogenous AnxA2 in CMV infection we identified using inhibitory antibodies. These transfection experiments also support a role for cellular AnxA2, although the source known to be associated with the virus (430, 458, 602) may also be involved. Our observation that binding to neither HFF nor HepG2 was affected by endogenous AnxA2, suggests that the earliest effect of AnxA2 in this complex mechanism is downstream of virus-cell attachment but earlier than IE72 expression. Nevertheless, it must be emphasized that to inhibit virus-cell membrane fusion, binding studies were conducted at 4°C. This will preclude signal-mediated modification or relocalization of endogenous AnxA2, which may be required for an experimental effect. Therefore a function for AnxA2 in binding cannot be unambiguously excluded by these experiments.

In contrast to the inhibitory effect of the polyclonal antibodies, several mAbs tested had no effect on either primary plaque formation or primary IE72 expression. While the epitopes targeted by these mAbs have not been identified (table 3), their failure to inhibit infection, combined with the inhibition by the polyclonal antibodies, indicates that specific domains in AnxA2 may be responsible for enhancing CMV infection, particularly the amino acids 21-38 in p11, and amino acids 9-30 in p36 in the unique N-terminal domain. Since the sites responsible for AnxA2t complex formation reside in amino acids 85-86 of p11 and 1-9 of p36, the inhibitory antibodies probably did not prevent the p11-p36 association, but rather interfered with some other activity of AnxA2t.

4.2.2. Purified AnxA2. In support of a role for endogenous AnxA2 in CMV infection, the addition of exogenous AnxA2t or p11 was observed to enhance production of progeny virus and IE72 expression after primary inoculation. However, purified p36 had no effect on any aspect of CMV infection tested, making p36 a useful negative control for the specific effects we observed for both AnxA2t and p11. These observations suggest that endogenous AnxA2t, rather than p36, was inhibited in our anti-p36 antibody experiments. Similar to HFF, purified AnxA2t also increased IE72 expression in both native and p36-transfected HepG2. Combined, these data demonstrate that AnxA2 assists in the production of CMV and that the p11 constituent of the tetrameric form is necessary. Unlike HFF, purified p11 and p36 both enhanced IE72 expression in native HepG2, but were inhibitory in p36 transfectants. While a definitive explanation cannot be provided at this time, we speculate that in native HepG2, the exogenous p11 and p36 might each combine with endogenous elements to form AnxA2t, thereby enhancing IE72 expression.

In p36 HepG2, however, it is possible that purified p11 or p36, while unable to enhance events leading to IE72 expression, competed with endogenous AnxA2t for binding to CMV. This latter observation is consistent with an inhibitory effect we observed on HFF, at very low (<0.1 nM) concentrations of purified p11 or p36 (figure 10A, 10B). These data suggest that under these conditions, equilibria with other cellular or viral constituents may alter the overall enhancing effects of p11 or p36.

Both p36 and AnxA2t bound equivalently to HFF cells, confirming that purified p36 was available on cells during infection. The failure of p36 to increase CMV infection therefore reflects the specificity for the observed enhancement by AnxA2t and p11, rather than an inability to bind host cells. Confirming a previous report (206), approximately 40% of binding could be abrogated in the presence of EGTA, indicating that the AnxA2-HFF interaction is partly calcium-dependent. These data suggest that AnxA2 may interact with more than one species on the HFF surface. Though AnxA2 is a documented phospholipid-binding protein, it has also been reported to interact with cell membranes in a calcium-independent manner (256, 337, 559, 564). Potential cell-surface ligands for AnxA2 include tenascin-C (105, 106), pro-cathepsin B (348, 349), HSPs (167, 276), β2-microglobulin I (345) and cholesterol (116). AnxA2 has also been detected in clathrin-coated vesicles (564), caveolae (569), and lipid microdomains (31, 216). Any of these interactions could potentially contribute to the calcium-independent binding of AnxA2 to the cell surface.

4.2.3. Differences between endogenous and purified AnxA2. An interesting discrepancy was noted in the current study between the effects of endogenous and purified AnxA2 on

CMV infection. Although not yet investigated, this may be accounted for by different AnxA2 functional states known to be caused by cell-induced modification, such as phosphorylation (250, 254, 460). The discrepancy concerns primary plaque formation, which was inhibited by antibodies specific for p11 or p36, but not affected by purified AnxA2t, p11 or p36. Since all steps we measured preceding and following primary plaque formation were augmented by purified AnxA2t and p11, a "gap" in the exogenous AnxA2t-dependent mechanism was revealed. This gap suggests that exogenous AnxA2t facilitates downstream effects separate from entry to increase infectious viral yield. In agreement with this hypothesis, CMV has been shown to rapidly initiate several second-messenger pathways (10, 33, 247), some of which may be mediated through virus surface gB (64, 505, 616, 617), a documented AnxA2 ligand (61, 430). Thus, purified AnxA2t appears to function at discrete steps in the infection mechanism, but may only indirectly participate in later processes through intracellular signalling. Previous evidence has suggested that signalling is important for replication of homologous Herpesviruses, including herpes simplex virus (HSV) type 1 (477, 586) and varicella-zoster virus (219), and also for unrelated virus families (32, 203).

4.2.4. Reconciling controversial results. The observation that AnxA2 plays a role in CMV infection is controversial. Despite considerable biochemical evidence implicating AnxA2 in CMV infection in the past, a more recent study concluded that AnxA2 had no direct effect on either entry or spread of CMV in host cells (429). However, the seemingly contradictory results reported in the previous study may be explained by differing methodology. 1) The previous study focused mainly on p36. In our hands,

purified p36 had no significant effect on any phase of infection tested, even at high concentrations (100 nM). 2) Antibody inhibition assays conducted previously used either a commercially available mAb to p11, or an in-house polyclonal antibody to p36. Our data obtained with the p11 mAb were identical to those previously reported and showed no effect on CMV plaque formation or IE72 expression. The polyclonal anti-p36 antibody was not evaluated during this study. While evaluation of as many antibodies as possible can be useful, four anti-p36 antibodies were already tested, of which only one was inhibitory. Several explanations exist for why the anti-p36 antiserum used in the previous study may not have had an effect. First, it was raised against a His-tagged recombinant protein that had been denatured and refolded. Second, whether this antibody recognizes native cellular AnxA2 was not evaluated. Third, the inhibitory antibody used in our study was raised against a synthetic peptide corresponding to amino acids 9-30 of the N-terminus of p36, and may therefore recognize a vital epitope with higher efficacy. The most important difference in methodology, and the most likely basis for the seemingly contradictory results, is that in the earlier study, cells and virus were pre-equilibrated at 4°C to subsequently facilitate synchronous entry at 37°C. In agreement with the previous study, we also found that under these temperature conditions anti-AnxA2 antibodies had no effect (data not shown). While speculative, an emerging model we are investigating predicts that CMV-dependent cell priming (112) may be affected by the 4°C pre-treatment. The potential priming event of interest is signal-mediated translocation of AnxA2 and p11 to the HFF cell surface, which has been observed at 37°C (158, 426, 476), but not at 4°C (data not shown). Together with reports suggesting that gB is a ligand for AnxA2 (61, 430) and that CMV induces a cell-surface receptor,

potentially for gB (63, 112, 270), the consequences of signaling may explain why pre-treatment at 4°C, but not at 37°C, precludes detectable effects of AnxA2.

IE72 expression has been used in the past as a quantitative indicator of CMV-cell entry (112). We now show that IE72 expression may not necessarily reflect of numbers of infectious virus entering cells. First, purified AnxA2t and p11 enhanced primary IE72 expression and productive virus infection, but had no effect on primary plaque number, typically used as a quantitative indicator of entry (203). This failure of AnxA2t and p11 to affect plaque number suggests that extracellular factors, which do not affect entry, may modulate intracellular events in CMV infection, leading to altered IE72 expression. A second indication that IE72 did not necessarily reflect infectious virus entry was that IE72 expression was proportional to multiplicity of infection (MOI) only up to a plateau at an MOI of approximately 0.01, i.e., one infectious virus per 100 cells. Since all HFF in a monolayer are infectible, saturation would be expected at an MOI of 1 or more. A possible explanation may lie in the fact that the ratio of virus particles to plaque-forming particles is typically 10,000:1 in our preparations, meaning that an MOI of 0.01 is equivalent to 100 virus particles per cell. Moreover, some CMV particles may enter the cell and induce and saturate IE72 expression, but be unable to replicate. Examples of cells that express immediate-early products upon infection, but do not support productive infection, have been reported in the current study in HepG2 cells, and also in the literature (317, 321, 585). In addition, several inhibitors of CMV have been shown to prevent DNA replication while not affecting immediate-early gene expression (372, 441, 508). The notion of non-productive but immediate-early-inducing infection of cells by CMV is therefore consistent with our observation that an MOI far below 1 saturates IE72

expression in host cells. Collectively, these data show that IE72 expression may be affected separately from either CMV-cell entry or from production of infectious progeny, and must therefore be considered a distinct stage in infection.

4.2.5. Molecular mechanism of enhancement. Our data indicate that p11 is the important moiety in AnxA2t that functions in CMV infection. Indeed, the observation that p36 had no effect on any stage of CMV infection shows that the enhancement of CMV infection by AnxA2t is specific, and not due solely to phospholipid-binding properties typical of annexins. Because p36 is endogenous to the cell surface, it is unknown whether the effect of exogenous p11 is independent of p36. p11 function apart from the AnxA2t complex has not been reported. Furthermore, there is evidence that p11 can bind to phospholipid membranes only as an AnxA2t subunit (621). For these reasons, it is probable that in our experiments, exogenous p11 exerts an enhancing effect on CMV infection by forming AnxA2t with endogenous p36 that may be present on the cell or virus surface (406).

The observation that purified AnxA2t, but not p36, specifically enhanced CMV-cell binding is in contrast to a previous report from our laboratory, showing that both AnxA2t and p36 could enhance CMV binding to synthetic anionic phospholipid membranes with a reported apparent Kd of 40 nM (458). p36 has also been shown to bind to fibroblasts in the current study, and previously to endothelial cells (206), with similar affinity. Given that AnxA2t and p11 increase infection with half-maximal enhancement at 5-10 nM, this suggests that in our cellular models, the AnxA2t interaction with the cell, and the subsequent enhancement of CMV infection, is not mediated solely by the

AnxA2t-phospholipid interaction. In light of the known property of p36 to aggregate phospholipid membranes and of findings from our laboratory showing that p36 can enhance CMV binding to phospholipid membranes (458), the inability of p36 to enhance CMV-cell binding further argues against mechanisms involving solely phospholipid, but rather suggests that AnxA2t may interact with other cellular or viral constituents.

The inferences that p11 is the important moiety in AnxA2t for enhancing infection, and that species other than phospholipid interact with AnxA2 during infection, are compatible with our observation of an interaction between AnxA2t and a viral species, which we have tentatively identified as the light chain of gB (figure 24). The immunodepletion experiment indicates that several species consistent in size with both the light chain (gB_L) and the heavy chain (gB_H) of gB were complexed to AnxA2t, both endogenous to the virus preparation and liposome-bound purified AnxA2t. While an additional minor band was detected at 70 kDa, the absence of this species in the other experiments suggests it may have interacted with the gB-AnxA2t complex, and not specifically with AnxA2t itself. The solution-phase crosslinking experiment showed that a complex, consistent in size with a p11-gB_L adduct, contained elements from the liposome-bound ^{125}I-AnxA2, rather than the viral source of AnxA2, since the latter was unlabelled. This experiment suggests that CMV may be able to bind cell-bound AnxA2t. Finally, the ligand blot showed that the AnxA2-binding species co-migrated with gB_L, identified by Coomassie blue staining and immunoblot analysis. Our tentative supposition of a p11-gB_L interaction is supported by previous reports of an AnxA2-gB complex (61, 430), and further localizes the interaction to specific chains within each complex. Since gB is a known fusogen for CMV (391, 561, 562) and mediates

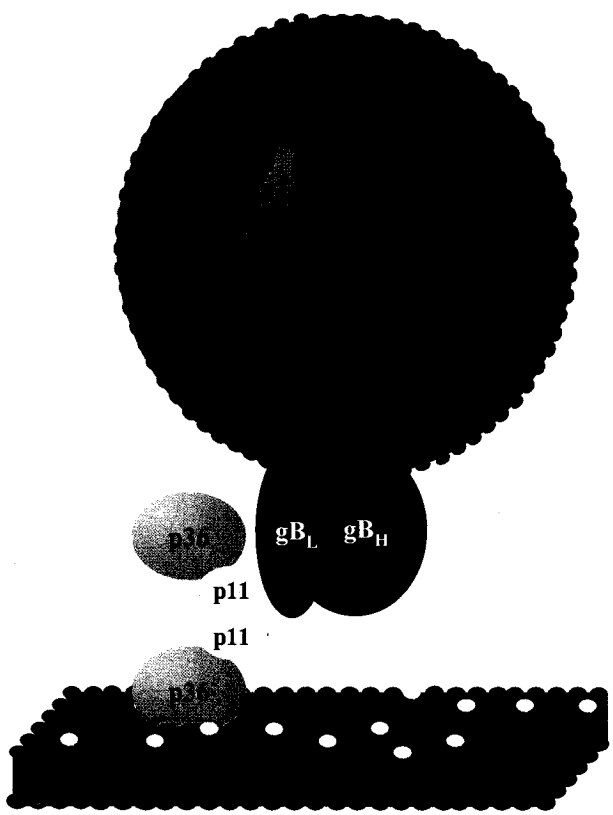
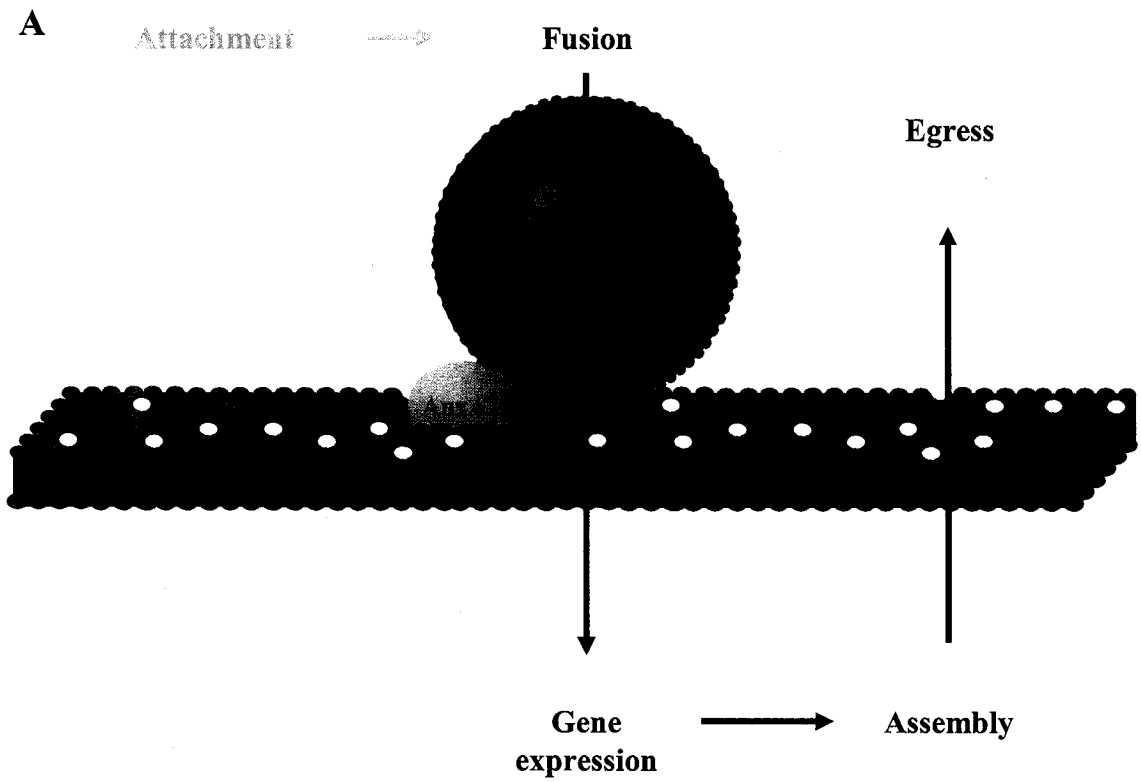

p36
gB$_L$
gB$_H$
p11
p11
p36

Figure 24. Interaction between AnxA2t and glycoprotein B. The AnxA2t binds to CMV using an interaction between p11 and the light chain of glycoprotein B (gB_L).

intracellular signalling even in the absence of entry (64), an AnxA2-gB interaction is also consistent with the putative dual role of AnxA2 at a post-attachment, pre-entry step, as well as in a parallel but separate role in cell stimulation.

The mechanism by which AnxA2t enhances CMV infection of cells is not yet determined. A role of AnxA2t in virus-cell fusion (figure 25A) is suggested by previous data from our laboratory showing that AnxA2t, but not p36, promotes fusion of the CMV envelope with synthetic phospholipid membranes (458), and by our confirmation of independent reports of an interaction between AnxA2 and gB (61, 430). Our data also suggest a second, intracellular pathway by which AnxA2t augments infection (figure 25B). Because AnxA2t is endogenous to both the cell and the purified virus, it is unclear whether AnxA2t is obligate for CMV infection. Several reports in the literature have argued against the necessity for AnxA2 in CMV infection in cultured cells (154, 429). In the current study, the existence of an AnxA2-independent infection pathway is implied by the observations that inhibition by antibodies was incomplete, and that p36-null HepG2 cells were still permissive for IE72 expression. While it is possible that separate AnxA2-dependent and -independent mechanisms exist, given its known interactions with HSP (167, 276) and gB (61, 430), it is more likely that AnxA2t participates in the modulation of the classical infection pathways. It is tempting to speculate that a gB-AnxA2-HSP ternary complex may be formed, with HSP acting as the scaffold on which the complex is built (figure 26A). Both gB and the gH component of gCIII stimulate cellular transcription events in host cells (64, 505, 616, 617), leading to the possibility that the viral fusogens may act together to mediate CMV-cell infection, and that AnxA2t may participate in this process (figure 26B). Thus AnxA2t is likely an auxiliary, rather


A
Attachment
Fusion
Egress
Gene expression
Assembly

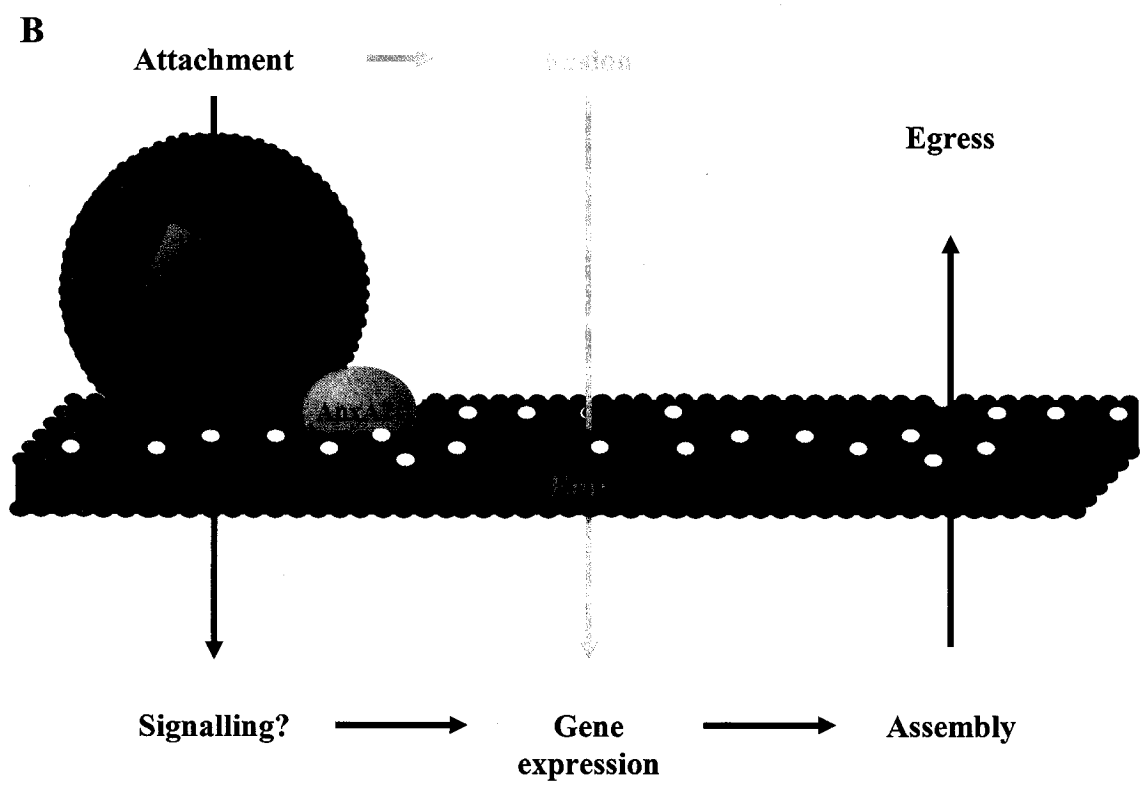
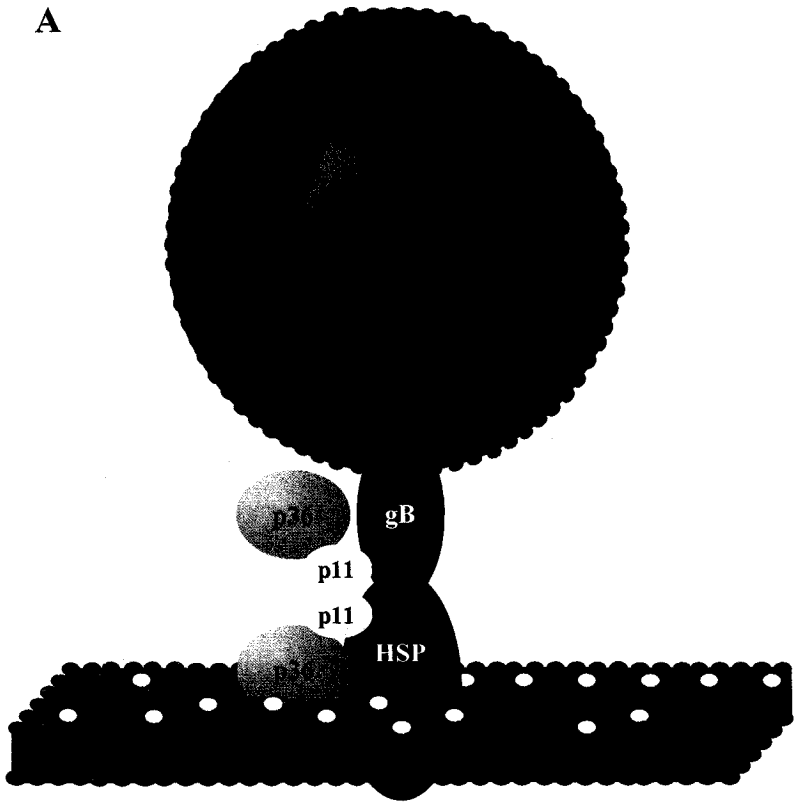

B
Attachment
Egress
Signalling?
Gene expression
Assembly

Figure 25. Proposed infection stages enhanced by AnxA2t. AnxA2t augments CMV infection by at least two pathways. A. AnxA2t enhances an entry step occurring after CMV-cell attachment but preceding gene expression, possibly fusion. B. AnxA2t increases infection by a pathway separate from entry, potentially by acting intracellularly.

A



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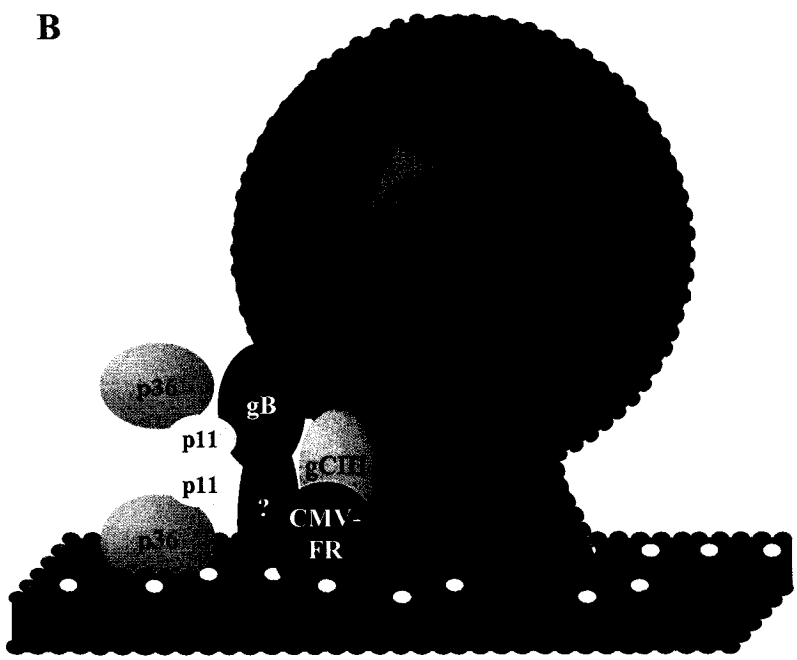


Figure 26. Potential molecular mechanisms of AnxA2t enhancement of infection. AnxA2t is likely an auxiliary, rather than a critical, component of the infection machinery. A. AnxA2t may bind gB and HSP simultaneously, aiding in HSP-mediated intracellular signalling or other "priming". B. Through its interaction with gB, AnxA2t may also participate in CMV-cell fusion, mediated by the known fusogens gB, gCIII, and CMVFR.

than a critical, component of the infection machinery of cultured cells, which may confer an essential kinetic advantage to the virus in the context of the host immune system *in vivo*.

4.3. ANXA2-ANXA5 INTERACTION.

Our laboratory has previously observed that AnxA5 inhibited AnxA2-dependent fusion of the CMV envelope with model phospholipid membranes, but had no effect on CMV-phospholipid binding (458). We therefore hypothesized an interaction between AnxA5 and AnxA2. Previous work in our laboratory has described a calcium-dependent, phospholipid-independent AnxA2-AnxA5 interaction (69). Forming the basis of the surface plasmon resonance (SPR) studies presented in the paper, in the current study dextran-linked AnxA5 binding to both p36 and AnxA2t was shown to have similar affinity and maximum binding. This suggested that AnxA5 interacts with the p36 moiety. Our laboratory has demonstrated that heparin inhibited the AnxA2-AnxA5 interaction (69). Both AnxA2 and AnxA5 have been previously shown to interact with heparin and HSP (82, 83, 167, 244, 276). CMV employs HSP on the cell surface for host entry (112), suggesting that the AnxA5-AnxA2 interaction may interfere with HSP-mediated infection events. Apart from CMV infection, an AnxA2-AnxA5 interaction may also have consequences on the properties of AnxA2 during infection or during the disposal of normal physiological functions, and may constitute a mode of regulation.

4.4. ANXA5 AND ANXA1 IN CMV INFECTION.

Annexin-annexin interactions have been widely reported in the literature, often with functional consequences (119, 333, 367, 369, 458, 528, 618, 619). In particular, AnxA5 has been shown to inhibit AnxA2-mediated fusion between the CMV envelope and anionic phospholipid-containing membranes (458). The inhibition of fusion was proposed to be due to the direct interaction between AnxA2 and AnxA5 (69), and we hypothesized that AnxA5 may comprise a natural line of defense by the host cell against CMV invasion. AnxA1 has been also reported to bind AnxA2 in a calcium-dependent manner (323), though no link has been reported between this interaction and CMV infection. We therefore hypothesized that AnxA5 or AnxA1 might affect CMV infection of host cells by binding AnxA2 and interfering with its activity. Using purified proteins, antibodies, and transfection studies, we now report for the first time that AnxA5 and AnxA1 each inhibit CMV infection of host cells by a similar mechanism, likely by interfering with AnxA2-dependent infection events.

4.4.1. AnxA5 and AnxA1 inhibition of infection. When HFF were inoculated in the presence of either AnxA5 or AnxA1, CMV infection was significantly inhibited in a dose-dependent, saturable manner. Production of infectious CMV progeny, primary IE72 expression and primary plaque formation were inhibited, but CMV-cell attachment was not affected. The specificity of the inhibition was demonstrated by the failure of p36 to affect any stage of infection tested, in agreement with a previous report (429), and by the ability of antibodies directed against AnxA5 and AnxA1 to prevent inhibition. The stage

of infection inhibited occurs prior to gene expression, but after CMV-cell attachment, the same enhanced by endogenous AnxA2t.

The IC50 values for AnxA1 were consistently 5-10-fold lower than those of AnxA5 (summarized in table 5), suggesting that the inhibition mechanism is not identical. However, the effects of AnxA5 and AnxA1 were additive at sub-maximal concentrations, but did not induce additional inhibition at saturation. This observation, combined with the finding that maximal inhibition was identical for AnxA5 and AnxA1 for a given stage of infection (table 5), is consistent with each annexin functioning independently, but inhibiting the same part of the mechanism.

That the anti-AnxA1 and -AnxA5 mAbs alone had no effect on CMV infection was surprising, since they were able to reverse the inhibition of purified AnxA5 and AnxA1, respectively. Endogenous AnxA5 and AnxA1 are present both on the HFF host cell surface and in the CMV envelope, implying that they are available for antibody binding. The discrepancy is therefore likely due to differing interactions with various cellular components. Thus purified AnxA5 and AnxA1 may present an available epitope, which may be masked in their cell-bound counterparts. Alternatively, the lack of effect of the mAbs may indicate that purified AnxA5 and AnxA1 may behave differently than the endogenous proteins during CMV infection, a phenomenon also observed for AnxA2t. Epitopes involved in one inhibition pathway may not necessarily overlap with those involved in other mechanisms, in which case the antibodies may not be able to exert any detectable effect on endogenous AnxA1 or A5.

4.4.2. AnxA2 dependence of inhibition. The stage inhibited by AnxA5 and AnxA1, occurring post-attachment but preceding IE72 gene expression, is consistent with inhibition of endogenous AnxA2, since anti-AnxA2 antibodies inhibited infection at this same step. Several lines of evidence presented here indicate that AnxA5 and AnxA1 inhibit CMV by interfering with AnxA2-dependent events: 1) the effects of AnxA5 and AnxA1 could each be reversed by the addition of purified AnxA2t, the form of AnxA2 observed to specifically enhance CMV infection. This observation suggests purified AnxA2t may saturate AnxA5 or AnxA1 binding sites for endogenous AnxA2t. 2) Several anti-AnxA2 mAbs, which alone had no effect, variously abrogated the inhibition by either AnxA5 or AnxA1, implying that the mAbs impede access to AnxA2. 3) In the presence of polyclonal anti-AnxA2 antibodies, which alone were inhibitory, no additional inhibition was observed, implying that anti-AnxA2 antibodies targeted the same pathway as AnxA5 and AnxA1, i.e. endogenous AnxA2. 4) AnxA5 and AnxA1 could only inhibit the CMV infection process in HepG2 transfected with AnxA2, but not in AnxA2-null HepG2, indicating that the expression of cellular AnxA2 is required for the inhibitory effect. Together, these observations indicate that AnxA5 and AnxA1 inhibit infection by interfering with cellular AnxA2t activity. The exact stage of infection inhibited is not yet known. AnxA5 has been previously reported to inhibit AnxA2t-mediated fusion of CMV with model phospholipid membranes in a cell-free system, without affecting either p36- or AnxA2t-dependent CMV-phospholipid binding (458). Combined with our observation in the current study that neither AnxA1 nor AnxA5 affect CMV-cell attachment at 4°C, it is likely that AnxA5 and AnxA1 interfere with AnxA2t-dependent CMV infection by inhibiting a stage downstream of attachment, such as fusion or intracellular signalling.

4.4.3. Interactions with AnxA2. Combined with reports that AnxA5 and AnxA1 each directly interact with AnxA2 (69, 323), evidence presented here suggests that the mechanism of inhibition probably involves direct binding. However, our data suggest the AnxA1-AnxA2 interaction differs from the AnxA2-AnxA5 interaction. First, the IC50 values were consistently 5-10-fold lower for AnxA1 than for AnxA5 (table 5), suggesting different types of associations. Second, while both AnxA5 and AnxA1 inhibition could be reversed by purified AnxA2t, only AnxA1 could be abrogated by purified p11 and p36. Third, inhibition was restored when the concentration of AnxA1 was four-fold higher than that of purified AnxA2, but the AnxA5 concentration required was ten-fold higher. Finally, different mAbs to p36 variously abrogated the inhibition of either AnxA5 or AnxA1, but not both, indicating that distinct sites on AnxA2 are involved in the interactions with AnxA5 or AnxA1. These observations suggest the mechanism of inhibition may not be identical for AnxA5 and AnxA1.

The dissociation constant of the AnxA5-AnxA2 complex is approximately 3 nM (69). Since the IC50 values of AnxA5 range from 10-40 nM, inhibition of CMV infection is probably not governed solely by the AnxA2-AnxA5 interaction. AnxA5 interacts with various cellular species in and on the cell (20, 82, 113, 244, 533, 568, 593) and may be further regulated by conditions such as local calcium concentration (114) and membrane voltage (142, 228). Since the evidence presented here indicates that the inhibition mechanism involves an AnxA2-AnxA5 interaction, it is likely an additional AnxA5-mediated interaction may be required for inhibition of CMV infection.

AnxA5-mediated inhibition could be reversed by AnxA2t only, but not by p11 or p36, suggesting that AnxA5 binds only AnxA2t. However, this notion is in contradiction

with results presented here and elsewhere (69) showing that in a purified AnxA5 could bind AnxA2t and p36 equivalently. A possible explanation may be that AnxA5 forms trimers and higher-order oligomers upon membrane binding (113, 379), resulting in altered conformation in the AnxA5 molecule (405, 582). While monomeric AnxA5 binds AnxA2t and p36 equally, it is possible membrane-bound oligomeric AnxA5 may possess a binding site requiring p36 in the conformation induced by AnxA2t complex formation (169). The hypothesis that AnxA5 binds the p36 moiety is consistent with the observation that only anti-p36 mAbs reversed AnxA5 inhibition, while anti-p11 had no effect. Another less likely possibility is AnxA5 might possess a binding site for both p36 and p11 simultaneously, although there is no evidence that AnxA5 binds p11 or any other S100 family member. The hypothesis of membrane-dependent self-association reconciles the observed interaction between AnxA5 and either p36 or AnxA2t by SPR, with the reversal of AnxA5-mediated inhibition of infection of cells by AnxA2t only, but not p36 or p11.

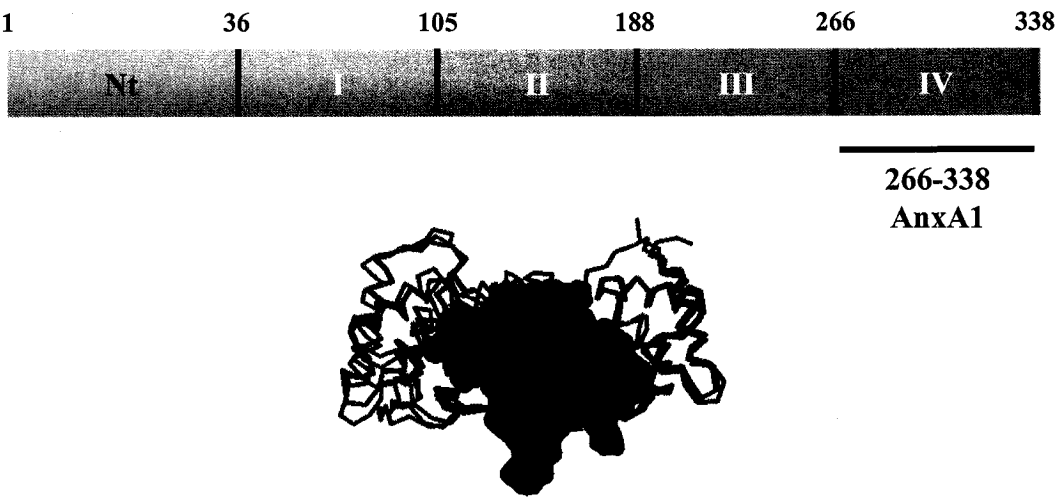
Though the Kd for the AnxA2-AnxA1 interaction is unknown, that of other annexin-annexin interactions have been reported to be in the low nanomolar range (69, 393, 618). The IC50 of AnxA1 of 1-6 nM (table 5) therefore suggests that inhibition of infection may be governed by the AnxA2-AnxA1 interaction. AnxA1 is known to bind the p36 moiety of AnxA2 (323). Since inhibition was abrogated by the addition of purified AnxA2t, p11 or p36, AnxA1 may possess a binding site for p11 as well. This is consistent with the observation that mAbs directed against p11 or p36 (Oncogene) also reversed inhibition. AnxA1 is known to bind the p11-related protein S100C through a hydrophobic cluster in the N-terminus (143, 350, 462, 494), the secondary structure of

which is conserved in S100-binding sites in annexins (531). However, there is no evidence in the literature of an AnxA1-p11 interaction. It is possible that AnxA1 binds the p36 moiety only, and that the anti-p11 mAb may impede access to AnxA2 by simple steric hindrance. Further experiments are required to determine whether AnxA1 may bind both p36 and p11.

The AnxA1-p36 binding site has been previously mapped (323), to the N-terminus plus domain I of AnxA1 and to domain IV of p36 (figure 27). The AnxA5-p36 interaction has not been mapped, but the epitopes targeted by the mAbs that abrogated AnxA5-mediated inhibition provide clues to the binding site. The Translab anti-p36 was raised to peptide 123-328 spanning domains II-IV in the tetrad core, indicating that the binding site for AnxA5 may reside in this region. That the Oncogene anti-p36 mAb abrogated AnxA1-mediated CMV inhibition suggests that the Oncogene mAb epitope resides near the AnxA1 binding site, in domain IV of p36. This may indicate that the AnxA5 binding site also resides in domain IV of p36 (figure 28). Therefore, it is possible that the respective binding sites on p36 overlap. However, since only AnxA5 inhibition was reversed by the Zymed and Translab anti-p36, the binding site for AnxA5 is probably not identical to that of AnxA1.

It is currently unknown whether other factors regulate binding of AnxA2 by AnxA1 or AnxA5. Millimolar levels of calcium were required for interactions with AnxA2 (69, 323), consistent with the physiological plasma concentration expected outside of cells (295). Though AnxA5 and AnxA1 have both been reported to interact with other cell surface species (82, 83, 174, 244, 423, 515), it is as yet unknown whether

A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36
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188
266
338
Nt
I
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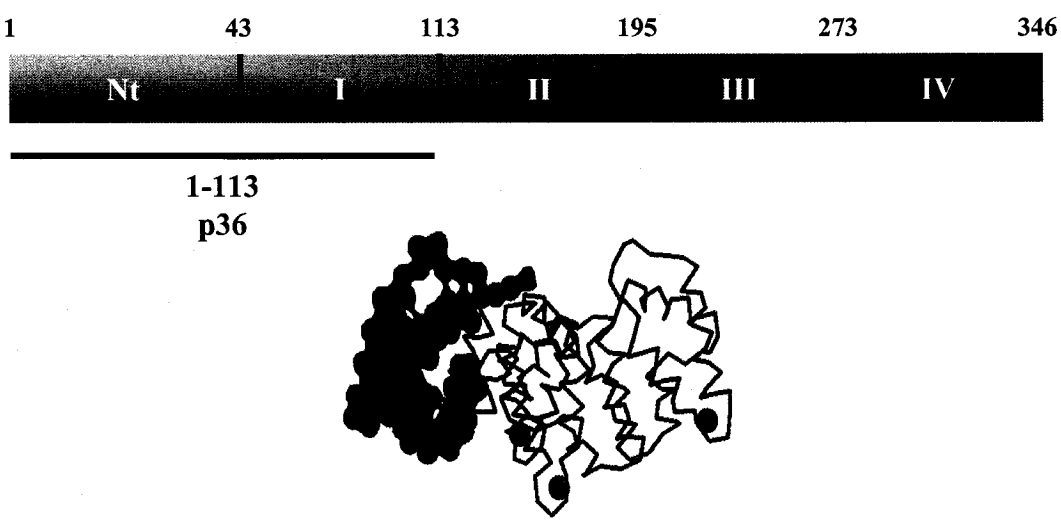
AnxA1

B


1
43
113
195
273
346
Nt
I
II
III
IV
1-113
p36

C

Figure 27. Interaction between AnxA2 and AnxA1. A. The AnxA1 binding site on p36 has been mapped to domain IV of the tetrad core, spanning amino acids 266-338 (323). The calculated available surface of this region is shown in red on a homology model of p36. B. The p36 binding site on AnxA1 has been mapped to the N-terminus plus domain I of the tetrad core, spanning amino acids 1-113 (323). The calculated available surface of this region is shown in red on a crystal structure of AnxA1 (594). C. Interaction between p36 and AnxA1, based on the known binding sites.

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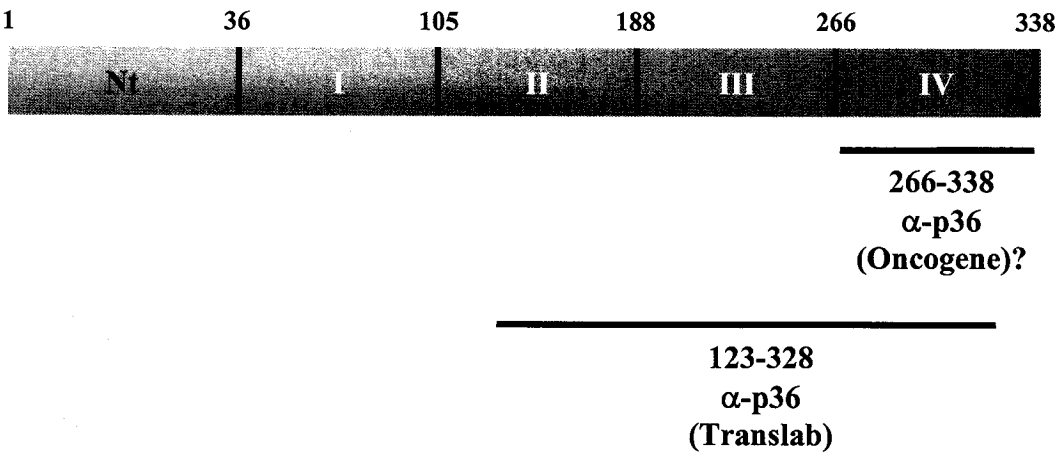
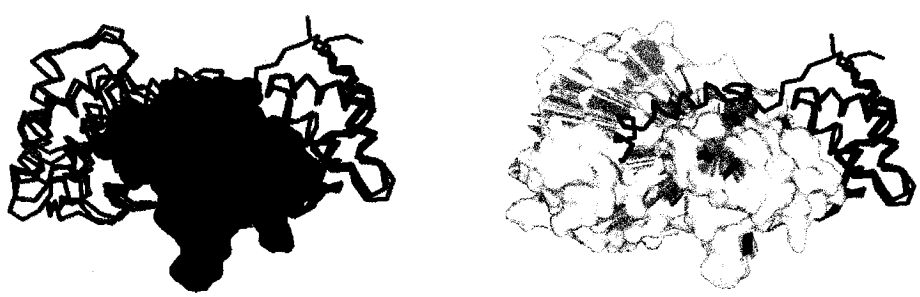

1
36
105
188
266
338
N1
I
II
III
IV
266-338
α-p36
(Oncogene)?
123-328
α-p36
(Translab)

B



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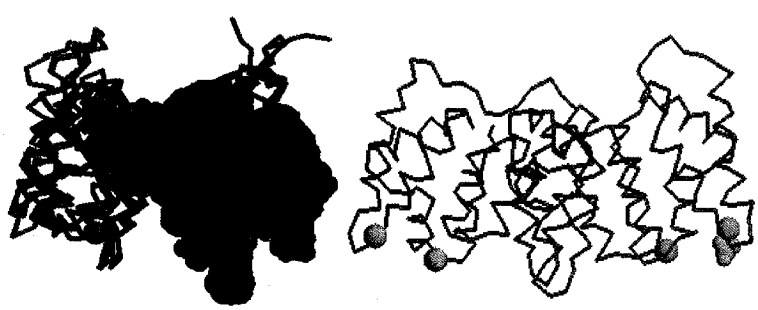

Figure 28. Interaction between AnxA2 and AnxA5. A. The AnxA5 binding site on p36 may reside in domain IV of the tetrad core, spanning amino acids 266-338, based on the regions targeted by anti-AnxA2 mAbs. B. The calculated available surface of domain IV (red) and of domains II-IV (yellow) are shown on a homology model of p36. C. Interaction between p36 and AnxA5 (518), shown in green, based on the potential binding site in p36. The binding site on AnxA5 is unknown.

other components on the cell or virus may regulate either their respective interaction with AnxA2, or their subsequent effect on AnxA2 activity.

4.4.4. Molecular mechanism of inhibition. The molecular mechanism by which AnxA5 and AnxA1 inhibit AnxA2-mediated infection is unknown. Previous work in our laboratory has shown that AnxA5 inhibited AnxA2-dependent fusion, but not binding, of CMV to model phospholipid membranes (458). Characterization of the AnxA5-AnxA2 interaction showed that maximal binding and affinity were unaffected by the presence or amount of phospholipid, suggesting that AnxA5 bridged AnxA2 to the membrane, with concurrent loss of AnxA2-phospholipid contact (69). This model may also apply to the AnxA1-AnxA2 interaction, though more study is required. The mechanism of inhibition may therefore consist of the insertion of AnxA5 or AnxA1 between AnxA2 and the cell membrane, allowing AnxA2 to interact with CMV, but preventing membrane binding and thus potential fusogenic activity and signal transduction. This hypothesis is consistent with the known ability of AnxA5 and AnxA1 to regulate membrane trafficking events. AnxA5 is known to inhibit membrane aggregation (25, 296) and fusion (407, 408, 458, 469). While AnxA1 is generally believed to promote membrane aggregation and endocytosis, AnxA1 trafficking-related properties have been shown to be altered by phosphorylation and proteolysis (53, 172, 251, 513, 590, 591). Infection of host cells by CMV infection, a process known to trigger intracellular signalling including kinase activation (64, 505, 616, 617), could therefore "activate" the inhibitory activity of AnxA1.

AnxA5 and AnxA1 may also act indirectly to inhibit AnxA2 activity. AnxA5 self-association and lattice formation on membranes has been shown to diminish local membrane fluidity (87, 362, 367, 369), which could impede the fusogenic ability of AnxA2 if the lattice were located nearby on the membrane. AnxA5 is well-documented to inhibit PKC activity (246, 455, 474, 491, 500). Specifically, AnxA5 has been shown to reduce PKC-mediated phosphorylation of AnxA2 (149) which could affect such activities as aggregation (29, 250). AnxA5 might also inhibit CMV infection through mechanisms not directly related to AnxA2. For example, AnxA5 has been shown to bind to HSPs (82, 83, 244), potentially rendering them unavailable to CMV for intracellular priming prior to infection (112). Since heparin inhibits the AnxA2-AnxA5 interaction (69), AnxA5 might interfere with AnxA2-HSP binding, potentially important in optimizing infection. AnxA5 also prevents thrombin formation (89, 452, 572), which is generated on the surface of Herpesviruses (535) and which has been shown to stimulate cells in such a way that could result in enhanced infection (426).

Similar to AnxA5, AnxA1 possesses certain biochemical properties and activities that might inhibit CMV infection independently from AnxA2. AnxA1 has been implicated as a mediator of anti-inflammatory events, including the inhibition of cytosolic phospholipase A2 activity (123, 374, 415) and consequently of the release of arachidonic acid and its metabolites (17, 124, 161, 376, 529, 610). AnxA1 also antagonizes various pro-inflammatory effects of elevated intracellular calcium (354, 541) and cAMP, including production of arachidonic acid (117, 541, 542, 543, 545). The inhibitory effect on arachidonic acid release has two potential consequences: first, CMV cell infection has been shown to result in elevated release of arachidonic acid and its

derivatives, resulting in elevated intracellular cAMP (3, 293, 397, 502), response elements for which reside in the IE72 gene promoter (242, 284). Arachidonic acid has also been shown to decrease the calcium requirement for AnxA2t-mediated exocytosis (93). In addition to its effect on arachidonic acid release, AnxA1 also counters the pro-inflammatory effects of TNFα, IL-1β, IL-6, and IL-8 (161, 420, 425, 481, 544). Stimulation by TNF-α, IL-1β, IL-6, and IL-8 has been shown to increase intracellular cAMP and promote binding of its ligand, cAMP-response element binding protein, to response elements in the IE72 promoter (293, 397). AnxA1 interference in pro-inflammatory signalling could therefore down-regulate intracellular events favorable to CMV infection. The data presented here suggest that CMV is attenuated in part by binding to AnxA2. However, other properties of AnxA5 and AnxA1 may also play a part in the inhibition mechanism.

4.4.5. Potential therapeutic applications. There are no CMV vaccines currently available for clinical use. CMV infections are treated with antiviral drugs, which can cause serious side effects and can lead to viral resistance (581). In addition to infection, CMV may be damaging to vascular tissues in apparently healthy individuals by various mechanisms including alteration of lipid metabolism, induction of cytokines and growth factors, and thrombosis, all of which are postulated to contribute to atherosclerosis (395). The development of anti-CMV therapies that target the interaction with the host cell would therefore be beneficial. AnxA2 augments infection by both extracellular and intracellular pathways, making it a candidate mediator of entry and pro-atherogenic signalling of CMV. The inhibition of CMV by AnxA5 and AnxA1, presumably by interfering with

AnxA2-mediated infection, provides a therapeutic strategy for targetting early CMV events.

The administration of either AnxA5 or AnxA1 would have to be carefully considered, since each affects a number of physiological processes. In animal models, administration of AnxA5 has been reported to inhibit fibrin generation (101, 573), fibrin accumulation and platelet adhesion (549), and thrombosis (468, 549). Administration of AnxA1 in animal models has had various anti-inflammatory effects, inhibiting production of pro-inflammatory molecules (72, 81, 610, 611), cell recruitment to sites of inflammation (14, 107, 128, 183, 202, 218, 330, 353, 402, 417, 420, 422, 424, 547, 611), and swelling of skin and joints (5, 150, 202, 241, 339, 612). The use of AnxA5 or AnxA1 peptides possessing AnxA2-attenuating activity may therefore provide a way to maximize CMV inhibition while minimizing unwanted physiological side-effects. Future experiments should first be aimed at determining whether the interaction of AnxA2 with either AnxA5 or AnxA1 is causative of inhibition, then should more precisely map the p36-binding site and/or the CMV-inhibiting domain, on AnxA5 or AnxA1.

The ability of AnxA5 and AnxA1 to inhibit CMV infection may confer to the host cell natural resistance to CMV infection. It is tempting to speculate that molar ratios of either AnxA5 or AnxA1 to AnxA2 may determine host cell permissiveness to CMV infection. This notion is particularly interesting in light of findings from our laboratory detailing how CMV, and other Herpesviruses, may alter cell-surface expression of annexins to optimize infection conditions (figure 29). Our laboratory has demonstrated that Herpesviruses are capable of assembling prothrombinase on the envelope, leading to the production of thrombin (535). Thrombin binds cell-surface receptors, including

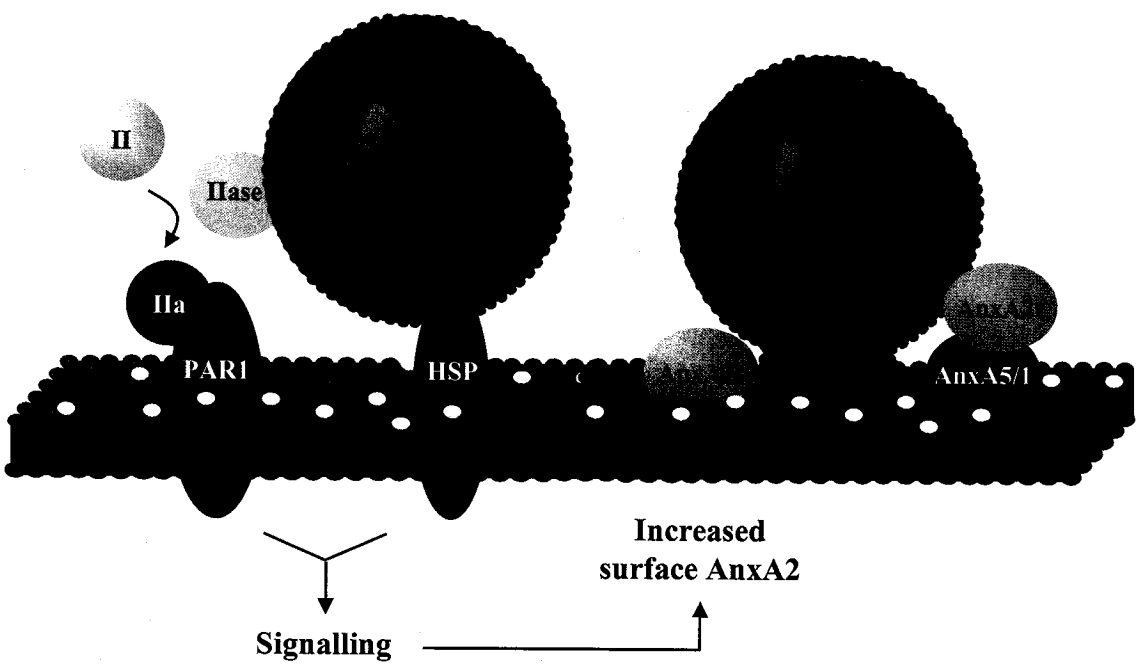
II
IIase
IIa
PAR1
HSP
AnxA5/1
Increased
surface AnxA2
Signalling

Figure 29. AnxA2t induction, CMV enhancement, and inhibition by AnxA5 or AnxA1. CMV may signal to the cell to increase surface AnxA2t expression by two mechanisms. First, prothrombin (II) is activated to thrombin (IIa) by prothrombinase (IIase), which assembles on the virus envelope. Thrombin binds to cell-surface receptors, including protease-activated receptor 1 (PAR1), resulting in induction of surface AnxA2t. Second, CMV may also directly signal to the cell, possibly through HSP binding, to upregulate surface AnxA2t. AnxA2t augments CMV infection. AnxA5 or AnxA1 inhibit AnxA2t-mediated enhancement of infection in a mechanism involving an interaction with AnxA2, possibly by preventing AnxA2t-phospholipid contact.

protease-activated receptor 1 (PAR1), which stimulates the induction of cell surface AnxA2t (426). CMV may also directly signal to the cell to upregulate surface AnxA2t (M. Sutherland and E. Pryzdial, unpublished), leading to increased infection. These results, combined with the data presented in the current study, may suggest that CMV and other Herpesviruses may alter molar ratios of annexins in order to circumvent the host cell's natural resistance strategies.

4.5. GENERAL CONCLUSIONS.

In summary, the data presented in this study indicate that: 1) AnxA2 enhances CMV infection by at least two pathways, by a post-attachment, pre-entry pathway, and a non-entry pathway, possibly through its interaction with gB. 2) Both AnxA5 and AnxA1 inhibit CMV infection by interfering with AnxA2-dependent infection events through a mechanism that involves direct binding. Various annexin-annexin interactions have been reported, including self-association of AnxA2 (333), AnxA1 (7, 528), and AnxA5 (113, 383, 518, 582), as well AnxA4 (259, 619), AnxA6 (37, 619), AnxA7 (48, 119, 329, 619), and AnxA12 (84, 319, 320, 341, 351). Associations between different annexins have also been reported, including AnxA4-AnxA7 and AnxA6-AnxA7 (618). AnxA2 has also been shown to interact with two other annexins, AnxA4 and AnxA6 (338), though their possible effects on CMV infection have not yet been investigated. Almost all annexin-annexin interactions regulate biological properties or otherwise have functional consequences (333, 367, 369, 458, 528, 618, 619), and may therefore constitute a general mechanism of functional regulation.

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CURRICULUM VITAE

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EDUCATION

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RELEVANT WORK EXPERIENCE

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Honours student
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Project title: The effect of low-level radiation on immune suppression in mice
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PUBLICATIONS

Derry, M.C., St-Pierre, S., Restall, C.M., Waisman, D.M., and Pryzdial, E.L.G. Annexins 5 and 1 inhibit annexin 2-mediated enhancement of cytomegalovirus infection. Article in preparation.

Derry, M.C., Restall, C.M., Waisman, D.M., and Pryzdial, E.L.G. Annexin 2 tetramer ($p36_2p11_2$), not monomer (p36), enhances cytomegalovirus production. Submitted to J. Virol., 2003.

Brooks, N.D., Grundy, J.E., Lavigne, N., Derry, M.C., Restall, C.M., MacKenzie, C.R., Waisman, D.M., and Pryzdial, E.L.G. **(2002).** Ca^{2+}-dependent and phospholipid-independent binding of annexin 2 and annexin 5. Biochem. J. 367: 895-900.

CONFERENCE PRESENTATIONS

Derry, M.C., Restall, C.M., Waisman, D.M., and Pryzdial, E.L.G. **(2003).** Annexin 2 tetramer enhances cytomegalovirus infection. Oral presentation at Annexins, Banff, Alberta, Canada.

Derry, M.C., Restall, C.M., Waisman, D.M., and Pryzdial, E.L.G. **(2003).** Annexin 2 tetramer enhances cytomegalovirus infection. Oral presentation at Frontiers in Cardiovascular Sciences, Vancouver, British Columbia, Canada.

Derry, M.C., Brooks, N.D., Grundy, J.E., Lavigne, N., Restall, C.M., St-Pierre, S., MacKenzie, C.R., Waisman, D.M., and Pryzdial, E.L.G. **(2002).** Annexin 2-annexin 5 interaction: implications for cytomegalovirus infection. Poster presentation at the International Society for Blood Transfusion, Vancouver, British Columbia, Canada.

Derry, M.C., Restall, C.M., Waisman, D.M., and Pryzdial, E.L.G. **(2001).** Cytomegalovirus uses host cell annexin 2 to enhance infection. Oral presentation by E.L.G. Pryzdial at the 54th Annual Meeting of the American Association of Blood Bankers, San Antonio, Texas, USA.

Derry, M.C., Restall, C.M., Waisman, D.M., and Pryzdial, E.L.G. **(2001).** Annexin 2 tetramer is a novel cytomegalovirus infection cofactor. Poster presentation at the 26th International Herpesvirus Workshop (IHW 2001), Regensburg, Bavaria, Germany.

Derry, M.C., Restall, C.M., Waisman, D.M., and Pryzdial, E.L.G. **(2001).** Annexin 2 tetramer is a novel cytomegalovirus infection cofactor. Poster presentation at the Annual Meeting of the Canadian Federation of Biological Sciences, Ottawa, Ontario, Canada.

Derry, M.C., St-Pierre, S., Raynor, C.M., Waisman, D.M., and Pryzdial, E.L.G. **(2000).** Annexins in cytomegalovirus infection. Poster presentation at the Annual Meeting of the Canadian Federation of Biological Sciences, Ottawa, Ontario, Canada.

Derry, M.C., St-Pierre, S., Raynor, C.M., Waisman, D.M., and Pryzdial, E.L.G. **(2000).** Annexins in cytomegalovirus infection. Oral presentation by E.L.G. Pryzdial at the Annual Meeting of the Canadian Society of Transfusion Medicine/Canadian Blood Services/Héma-Québec, Québec, Québec, Canada.

Raynor, C.M., Brooks, N., Derry, M.C., Lavigne, N., Wright, J.F., Waisman, D.M., and Pryzdial, E.L.G. **(1999).** Annexin II enhances binding and fusion of cytomegalovirus to phospholipid. Oral presentation by E.L.G. Pryzdial at the Meeting of the American Society for Virology, Amherst, Massachussetts, USA.

Filion, L., Derry, M.C., Chambers, K., and Ross, W. **(1997).** Radiation damage to the immune system. Oral presentation by L. Filion at the International Scanning Microscopy Meeting, Chicago, Illinois, USA.

Ross, W., Filion, L., Birnboim, H., Raaphorst, G., Chambers, K., and Derry, M.C. **(1997).** Biodosimetry and mitigation effects after low-Level radiation. Oral presentation by W. Ross at Current Canadian Initiatives: RSG-23 (NATO Panels) on Assessment, Prophylaxis, and Treatment of Ionizing Radiation Injury in Nuclear Environments, Grenoble, France.

OTHER SCHOLARLY ACTIVITIES

Derry, M.C. **(1997).** The effect of low-level radiation on immunosuppression. Unpublished Honours Thesis, Department of Physiology, Faculty of Medicine, University of Ottawa.

HONOURS AND AWARDS

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2001-2003 University of Ottawa 3rd-4th Year Doctoral Research Scholarship
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CONTRIBUTIONS OF COLLABORATORS

All procedures were carried out by M. Derry, with the following exceptions:

- Phospholipid vesicles were prepared by Nicole Brooks, Canadian Blood Services.
- Purified placental AnxA2, *E. coli* lipid substrate, and one lot of purified placental AnxA1 were prepared by Christina Restall, Canadian Blood Services.
- Surface plasmon resonance experiments were carried out with the assistance of Tomoko Hirama and Dr. Roger MacKenzie, National Research Council.
- Some titrations of the effects of AnxA5 and AnxA1 in 1-step IE72 were performed by Simon St-Pierre, Canadian Blood Services, under the supervision of M. Derry.
- The titration of the effect of AnxA5 and AnxA1 in ^{35}S-CMV-cell attachment was performed by S. St-Pierre, under the supervision of M. Derry.