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PROCESSING AND SECRETION OF HUMAN IMMUNODEFICIENCY VIRUS GLYCOPROTEIN, GP120

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ABSTRACT

The natural signal sequence of HIV-1 gp120 contains an unusually long hydrophobic domain and five positively charged amino acids. When the gp120 gene was cloned into a baculovirus expression vector under the control of the baculovirus polyhedrin gene promoter, it exhibited an extremely low level of secretion. However, deletion of the signal sequence resulted in the production of large quantities of a nonglycosylated form of gp120 and fusion of honeybee melittin or murine interleukin 3 signal sequences, which contain only one or no positively charged residues, respectively, resulted in a high level of expression as well as glycosylation and secretion. Four charge-altered signal mutants were generated by oligonucleotide-directed mutagenesis. Positively charged amino acids in the natural signal sequence were substituted with neutral amino acids. The results of these experiments showed that the expression and secretion of gp120 was progressively increased by decreasing the positive charge in a stepwise fashion from +5 to +3, +2, and +1. However, elimination of all five positive charges (leaving a net negative charge of -1 at the NH_2 terminus) caused accumulation of large amounts of a nonglycosylated form of gp120 but decreased the amounts of glycosylated forms of gp120. These signal peptide mutants clearly demonstrate that the positively charged amino acids in the natural signal sequence of HIV-1 gp120 are key factors determining its poor expression and secretion in insect cells. Analysis of intracellular transport and folding of gp120 further indicates that the highly

charged uncleaved signal peptide rather than disulfide bond formation is an important factor limiting transport of gp120 from the rough endoplasmic reticulum (RER) to the Golgi apparatus; its presence affects gp120 folding and slows its rate of transport to the cell surface.

The requirement for carbohydrate on HIV gp120 in CD4 binding has been the subject of much debate. There have been conflicting reports regarding the role of gp120 glycans in binding to CD4. An important question is whether the carbohydrate itself plays an important role in this interaction. Nonglycosylated and glycosylated forms of gp120 from HIV-1 and HIV-2 were produced using the baculovirus expression system and their CD4 binding properties were determined. The nonglycosylated forms of gp120 generated by either deletion of the signal sequence or synthesized in the presence of tunicamycin failed to bind to CD4. In contrast, highly mannosylated recombinant gp120 bound well to soluble CD4. Enzymatic removal of carbohydrate chains from glycosylated gp120 by endoglycosidase H (endo H) or by a mixture of endoglycosidase F and N-glycanase (endo FNG) in the presence or absence of SDS had little or no effect on the ability of gp120 to bind CD4. The data indicate that carbohydrate chains per se do not play a significant role in interaction between gp120 and CD4 molecules but that N-linked glycosylation is required for correct protein folding that provides the proper conformation for CD4 binding. Analysis of intracellular folding of gp120, using its ability to bind CD4 as a functional assay for overall conformation, further supports the hypothesis that N-linked glycosylation of HIV gp120 plays an essential role in promoting either the correct folding of the protein or in its stabilization.

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DEDICATION

This thesis is dedicated to my wife

Lizhong

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LIST OF ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate toluidine salt
BFA	brefeldin A
Bis	N'N'-bis-methylene-acrylamide
bp	base pair
BSA	bovine serum albumin
EtBr	ethidium bromide
cDNA	complementary deoxyribonucleic acid
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	dideoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol

dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid
Endo FNG	endoglycosidase F/N glycanase mixture
Endo H	endo-β-n-acetylglucosaminidase H
FBS	fetal bovine serum
hr	hour
kb	kilobases
kDa	kilodaltons
L	litre
LB	Luria broth
LTR	long terminal repeats
MAb	monoclonal antibody
mg	milligram
min	minute
mL	millilitre
mM	millimolar
mm	millimetre
MOI	multiplicity of infection
mU	milliunit
NBT	p-nitro blue tetrazolium chloride
ng	nanogram
NP40	Nonidet P40

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
p.i.	Postinfection
PMSF	phenyl methylsulfonylfluoride
RIPA	radioimmunoprecipitation assay
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
TBS	Tris buffered saline
TE	10 mM Tris-HCI pH 8.0 and 1 mM EDTA
Tris	tris (hydroxymethyl) aminomethane
TTBS	TBS containing 0.05% (v/v) Tween-20
μCi	microcuries
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER 1: GENERAL INTRODUCTION

1. RETROVIRUS CLASSIFICATION

Retroviruses are RNA-containing viruses that replicate through a DNA intermediate by virtue of a viral-coded RNA-dependent DNA polymerase, also called reverse transcriptase (Weiss et al. 1982). Distinguishing characteristics of retroviruses include the morphology and composition of virions and a single-stranded, positive-sense RNA genome. While retroviruses represent a relatively homogenous viral family, they have customarily been subdivided into three taxonomic groupings primarily on the basis of in vivo and in vitro consequences of infection (Weiss et al. 1982; Varmus and Brown, 1989). The oncovirus subgroup includes retroviruses able to cause neoplastic disease in the infected host animal but also includes some related, relatively benign viruses. The lentivirus subgroup includes retroviruses that cause slow, chronic disease that generally, but not always, lack a neoplastic component. Recognized members of the lentivirus subgroup include visna-maedi virus, caprine arthritis encephalitis virus (CAEV), equine infectious anaemia virus (EIAV), and the immunodeficiency viruses, including most notably human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) (Haase, 1986). Simian (SIV-1) and feline (FIV) immunodeficiency virus (Franchini et al. 1987a; Pedersen et al. 1987) are recent additions to this expanding taxonomic group. The third subgroup, spumavirus, consists of the foamy viruses, which induce persistent infections without any clinical disease but cause vacuolization of cultured cells.

2. DISCOVERY OF HUMAN RETROVIRUSES

Human retrovirology is a young but rapidly advancing scientific discipline. The earliest description of animal retroviruses came early in the 20th century from the work of Ellerman and Bang (1908) and from Rous (1911), who showed that cell-free filtrates from leukaemic chickens could induce leukaemia and sarcomas in normal animals. Some 40 years later, Ludwik Gross isolated the first mammalian retrovirus from an inbred strain of mice infected with murine leukaemia virus (Ludwik, 1951), which provided the first reproducible experimental system to study viral leukaemogenesis. In the ensuing period, retroviruses were shown to cause lymphomas, leukaemias, sarcomas and various other cancers in species ranging from chicken, mice, and cattle to subhuman primates (Weiss, 1982). The discovery of reverse transcriptase in 1970 by Temin and Mizutani (Temin and Mizutani, 1970) and independently, by Baltimore (Baltimore, 1970) substantiated the "provirus hypothesis" proposed by Temin in 1964 (Temin, 1964) and also established a genetic and biochemical basis for the classification of retroviruses (Weiss et al. 1982).

With the role of retroviruses in carcinogenesis firmly established in animal models, massive efforts were then directed towards the search for human retroviruses in similar diseases (Vaishnav and Wong-Staal, 1991). Unlike most animal models where abundant virus replication and viraemia are usually present, retroviruses could not be easily isolated from tissue or fluids of patients with

malignant disorders. Progress in cell culture techniques, above all, the discovery of interleukin-2 (IL-2; Morgan et al. 1976), made long-term culture of human T-lymphocytes possible and thus paved the way for the discovery of the first human retrovirus, human T-cell leukaemia virus type I (HTLV-I) (Poiesz et al. 1981). HTLV-I was soon identified as the causative agent of adult T-cell leukaemia (ATL). In 1982, a second human retrovirus, HTLV-II, was isolated from a patient with a T cell variant of hairy cell leukaemia (Kalyanaraman et al. 1982a).

In spite of HTLV-1 and HTLV-II, human retroviruses would probably have remained relatively obscure in the United States and Europe had it not been for the sudden emergence of another retroviral disease that reached epidemic proportions. In 1981, several groups reported an outbreak of severe opportunistic infections among previously health male homosexuals (Gottlieb et al. 1981; Masur et al. 1981). This newly described syndrome, subsequently termed acquired immunodeficiency syndrome (AIDS), spread rapidly into several other risk groups, including intravenous drug abusers and recipients of contaminated blood or blood products. The pattern of disease transmission observed for AIDS strongly suggested that the causative agent was a blood-borne viral pathogen. This expectation was fulfilled in 1983 with isolation of a novel retrovirus from the lymphocytes of a patient with lymphadenopathy, a frequent prodrome of AIDS (Barre-Sinoussi et al. 1983). This virus was designated as LAV (lymphadenopathy-associated virus). Repeated isolation of similar viruses, the discovery of a cell line permitting their large-scale production, and serological studies consequently led to

the identification and characterization of the AIDS agent (HTLV-III; Popovic et al. 1984; Gallo et al. 1984; ARV; Levy et al. 1984). Later on, HTLV-III, LAV, and ARV were found to be variants of the same AIDS virus (Ratner et al. 1985). In 1986, an international committee recommended the name human immunodeficiency virus (HIV) for all isolates of the AIDS virus (Coffin et al. 1986).

To date, two related but distinct types, HIV type 1 (HIV-1) and HIV-2 have been identified. HIV-1 refers to retroviruses which cause AIDS in the United States, Europe, Central Africa, and most other countries worldwide (Wong-Staal, 1990). Seroepidemiological studies suggested Central Africa to be the cradle of HIV-1 and the current AIDS epidemic. HIV-2 refers to another AIDS retrovirus identified in West Africa (Clavel et al. 1986). This virus was found to be more closely related to the simian immunodeficiency virus isolated from macaques in captivity (SIV_{mac}) than to HIV-1 (Alizon et al. 1986). Nonetheless, HIV-1 and HIV-2 clearly share a relatively recent progenitor. The major biological and genetic features are entirely parallel. Both are associated with immunodeficiencies, although the degree of virulence may be less for HIV-2 (Wong-Staal, 1990).

Since the bulk of information has come from studies of HIV-1, the following description will pertain to that virus.

3. VIRION MORPHOLOGY

Like all retroviruses, HIV-1 is a spherical particle with virion diameter of slightly larger than 100 nm. It has a dense, cylindrical nucleocapsid containing two

copies of single-stranded genomic RNA encapsidated with core proteins and viral enzymes (reverse transcriptase, integrase and protease) (Fig. 1). The major structural core protein of HIV-1 is the p24 gag protein. This, and the myristylated protein p17 (which forms the outer shell) comprise the major gag structural proteins. Surrounding the viral core is a lipid membrane derived from the outer membrane of the host cell. Studding the outer membrane of the virus are the envelope glycoproteins, gp120 and gp41, which exist in the form of a noncovalent complex. These ultrastructural features of HIV are indistinguishable from those of another lentivirus, visna virus, and quite different from the human type C retroviruses, HTLV-1 and HTLV-II (Gallo et al. 1984).

4. GENOMIC ORGANIZATION AND FUNCTION OF VIRUS PROTEINS

The HIV has a single-strand positive sense RNA genome which is reverse transcribed by the virion enzyme to form a proviral DNA. The HIV-1 proviral DNA has been well characterized (Hahn et al. 1984; Ratner et al. 1985; Muesing et al. 1985). It is about 9200 bp in length and is flanked by long terminal repeat (LTR) sequences (Fig. 2). In common with other retroviruses, the 5' LTR contains a series of DNA target sequences for cellular transcription factors and functions to promote transcription of the initial genome length viral mRNA. The 3' LTR is required for the appropriate polyadenylation of this viral transcript (Varmus and Brown, 1989).

Figure 1. Schematic cross-section of a HIV-1 particle. gp120 is the major extracellular envelope glycoprotein, gp41 is the transmembrane envelope glycoprotein, p24 is the major core antigen, p17 is the myristylated gag protein, and p9 is the RNA-binding protein, RT, reverse transcriptase. Taken from Wong-Staal (1990).

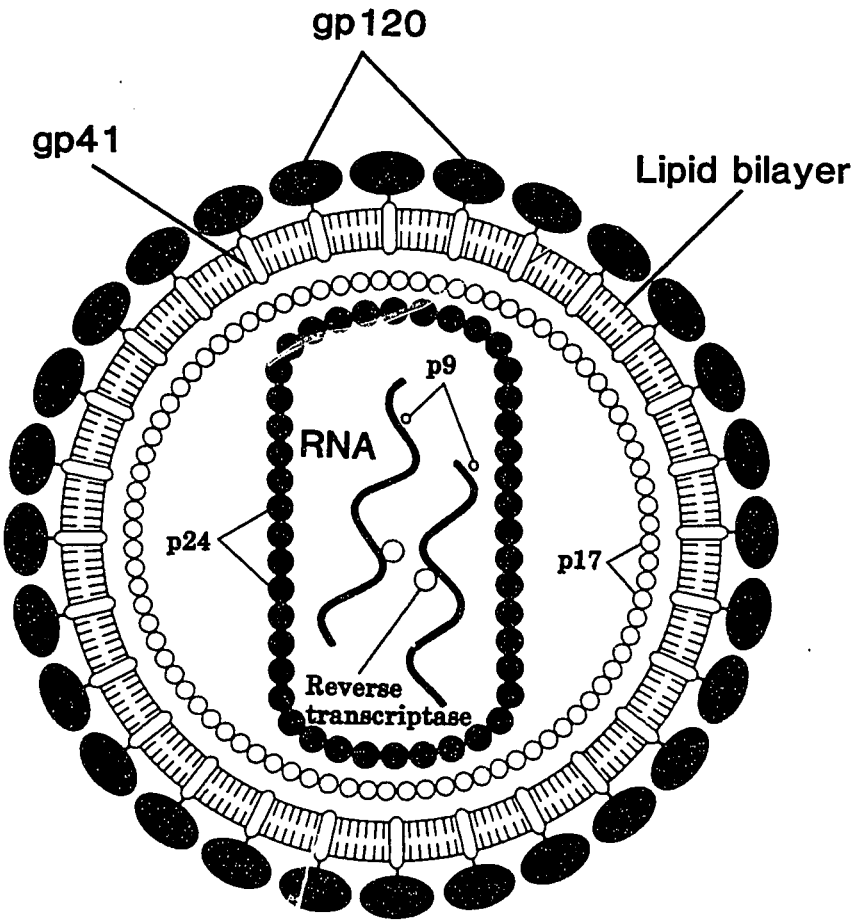
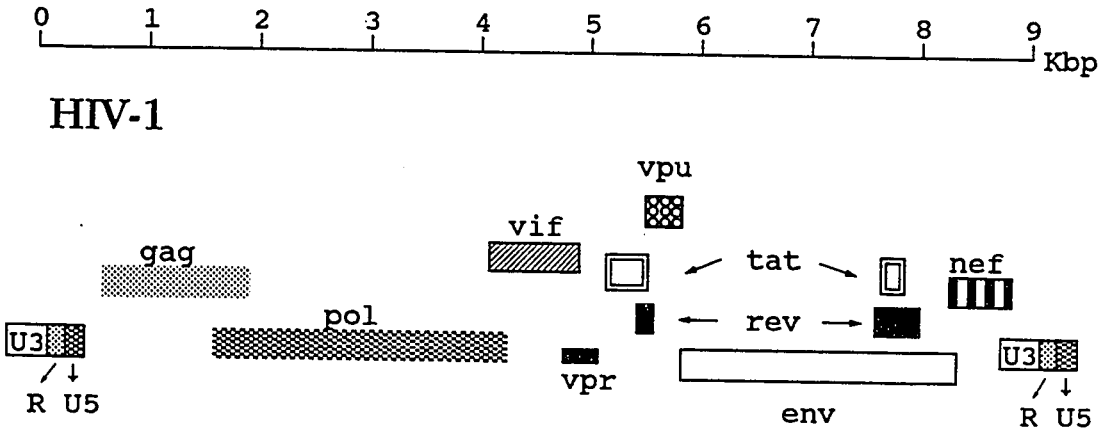

gp120
gp41
Lipid bilayer
p9
RNA
p24
p17
Reverse
transcriptase

Figure 2. Genomic organization of HIV-1. HIV-1 contains nine known genes. The nomenclature and known properties of these genes are descried in the text. Both tat and rev are coded by noncontiguous gene segments.


0
1
2
3
4
5
6
7
8
9
Kbp
HIV-1
vpu
vif
gag
tat
nef
pol
rev
U3
R U5
vpr
env
U3
R U5

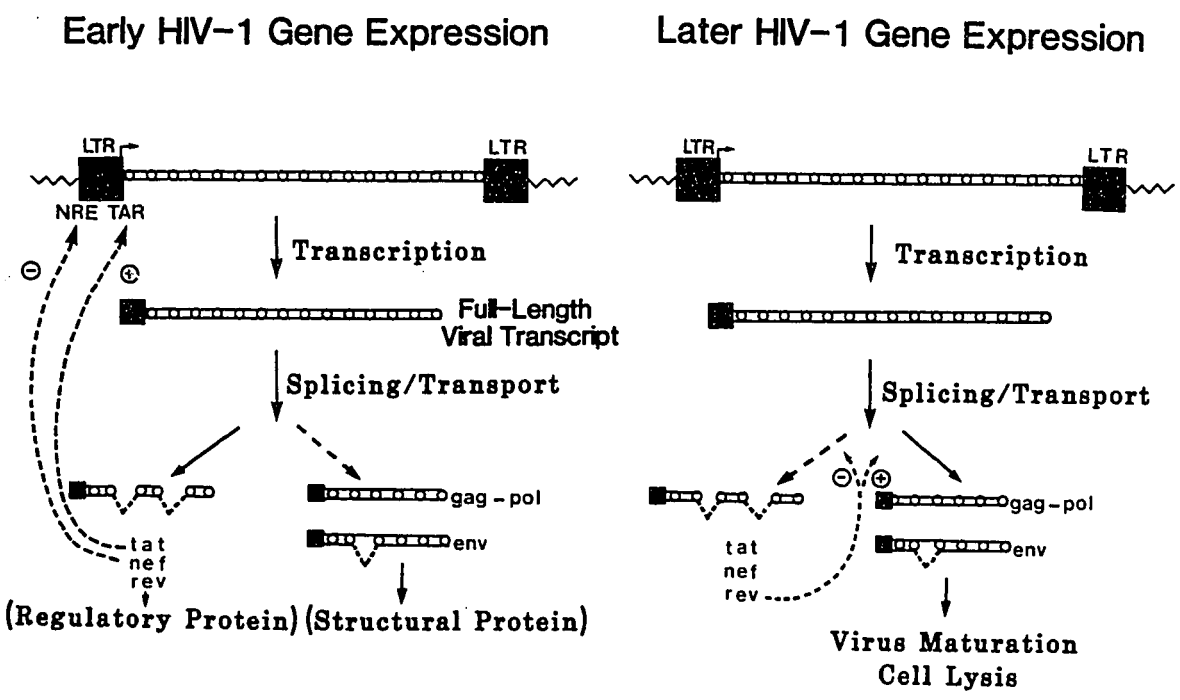
In addition to three essential sets of structural genes, which respectively encode the virus internal structural proteins (gag), the reverse transcriptase (pol), and the envelope glycoproteins (env), HIV-1 carries at least six other genes encoding the proteins that regulate viral gene expression (tat, rev, and nef) and proteins of uncertain function (vif, vpu, and vpr) (Wong-Staal, 1990) (Fig. 2). A schematic representation of what is currently known about their interaction is given in Fig. 3.

In general, the viral regulatory proteins (tat, rev, and nef) are encoded by multiple spliced mRNA species about 2.0 kb in length while the viral structural proteins including viral enzymes are translation products of unspliced (gag, pol) or singly-spliced (env, vif) mRNAs (Greene, 1990).

The structural genes of HIV-1

The gag gene codes for various structural components of virus particles. It is translated from full-length viral mRNA to produce a polyprotein precursor $Pr55^{gag}$ which consist of NH_2-p17-p24-p9-p7-COOH (Veronese et al. 1988). This precursor is then cleaved to produce the p24 (phosphorylated), p17 (myristylated), p9 and p7 gag proteins. These polypeptides form the nucleocapsid of HIV-1. p24 is the quantitatively predominant protein of the virion and the major capsid antigen. The p9 protein contains a "zinc finger" domain that may be involved in direct interaction with the viral RNA (Veronese et al. 1988). Like most myristylated proteins, p17 is associated with membrane structures and may serve to stabilize the exterior and interior components of the virion (Veronese et al. 1988) (Fig.1).

Figure 3. Summary of the early and late phases of HIV-1 gene expression. During early HIV-1 gene expression, transcription of the HIV provirus results predominately in the expression of the multiply spliced 2.0 kb class of viral mRNA encoding the various regulatory proteins tat, nef, and rev. Tat transactivites the expression of all viral genes via its action on the TAR element located in the regulatory region (R) of the LTR of RNA. Nef has been proposed as a negative regulatory factor. Rev promotes the transition from early expression of the regulatory proteins to late expression of the viral structural and enzymatic gene products (e.g., gag, pol, env). Taken from Greene (1990).


Early HIV-1 Gene Expression
Later HIV-1 Gene Expression
LTR
LTR
NRE TAR
Transcription
Full-Length
Viral Transcript
Splicing/Transport
gag-pol
env
tat
nef
rev
(Regulatory Protein)
(Structural Protein)
LTR
LTR
Transcription
Splicing/Transport
gag-pol
env
tat
nef
rev
Virus Maturation
Cell Lysis

The pol gene product of HIV-1 is translated from the full length message as the gag proteins but in a different overlapping reading frame as a result of ribosome frameshifting (Jacks et al. 1988). Pol encodes three proteins that are cleaved from a larger precursor polypeptide. These proteins include NH_2-protease(p10)-reverse transcriptase(p66/p51)-endonuclease (p32)-COOH (Jacks et al. 1988). The p66 contains both reverse transcriptase and RNase H activities (Hu and Kang 1991). The viral protease cleaves gag and pol precursor polypeptides into functionally active proteins. The RNase H activity is required for removal of the RNA template by digesting the RNA template portion of RNA-DNA hybrids during the synthesis of the double-stranded DNA (Varmus. 1988). Because of the lack of proof reading, HIV-1 RT seems to be significantly more error-prone than other RTs, with a frequency of misincorporation ranging from 1:1700 to 1:4000 nucleotides (Preston et al. 1988). This appears to partly account for the high mutation rate of HIV-1 in vivo. The endonuclease or integrase, is important in proviral integration as shown with other retrovirues (Varmus, 1988).

The env gene encodes a glycosylated polypeptide precursor (gp160) that is processed to form the external glycoprotein (gp120) and the transmembrane glycoprotein (gp41). It is the viral envelope that possesses important functional domains responsible for CD4 binding, fusion, and epitopes primarily involved in virus-host interaction, which will be discussed in detail in Section 1.6.

The regulatory genes of HIV-1

HIV-1 encodes a powerful transactivator protein termed tat that dramatically increases the expression of all genes linked to the retroviral LTR (Greene, 1990) (Fig.3). The tat gene is absolutely essential for viral replication (Greene, 1990; Vaishnav and Wong-Staal, 1991). It is synthesized from two exons which will code for a protein of 86 amino acids (Fig. 2). The tat protein is primarily localized in the nuclei of HIV-infected cells and contains two important functional domains: a cysteine-rich central portion and a positively charged segment (Greene, 1990). The cysteine rich domain appears to form a metal-linked tat dimer. The positively charged segment acts as a nuclear localization signal. Tat functions in a sequence-specific manner. The target sequence for tat, the trans-activation response (TAR) element, is a 59 nucleotide RNA stem-loop located at the 5' end of all viral mRNA species (Feng and Holland, 1988) (Fig. 3). At present, the precise mechanism of tat action remains unsolved. However, experimental evidence supports that tat may act at the transcriptional and/or posttranscriptional level as an antiterminator of transcription (Cullen and Greene, 1990).

Rev is the second regulatory protein of HIV-1 that is essential for viral replication (Cullen and Greene. 1990). It is a 19-kDa protein (116 amino acids) predominantly located in the nucleolus of HIV infected cells. Recent studies suggest that rev acts, at least in part, by activating the transport of unspliced or partially spliced viral mRNAs from the cell nucleus to the cytoplasm where they are then translated (Malim et al. 1988), and consequently, down-regulates expression

of the 2-kb multiply spliced mRNAs (Fig. 3). Thus, rev inhibits its own production as well as the expression of the tat and nef mRNAs. Rev also functions in a sequence-specific manner acting through a rev response element (RRE) located within the env gene (Cullen and Greene. 1990). It is now clear that a 66 nucleotide stem-loop subdomain of the RRE is both necessary and sufficient for high affinity binding of rev (Malim, 1990). Two distinct domains have been identified in Rev; a stretch of arginine-rich sequence and a leucine-rich sequence. Thus, rev appears to have a modular structure with a basic region acting as a "specificity" domain responsible for interaction with RRE-containing transcripts and an "activation" domain responsible for inducing nuclear export of such transcripts as a consequence of the binding event.

The nef gene is contained in a single open reading frame at the 3' end of the genome overlapping the env gene and the 3' LTR (Fig. 2). Nef is a 27-kDa myristylated protein that is associated with cytoplasmic membrane structures in cells (Guy et al. 1987). In contrast to tat and rev, nef may act as a transcriptional silencer to inhibit activation of the HIV-1 LTR (Fig. 3) and viral replication by binding to a negative regulatory element (NRE) (Ahmad and Venkatesan, 1988). However, these negative effects of nef remain controversial as others have observed no effect of the nef protein on either viral replication or gene expression (Kim et al. 1989b).

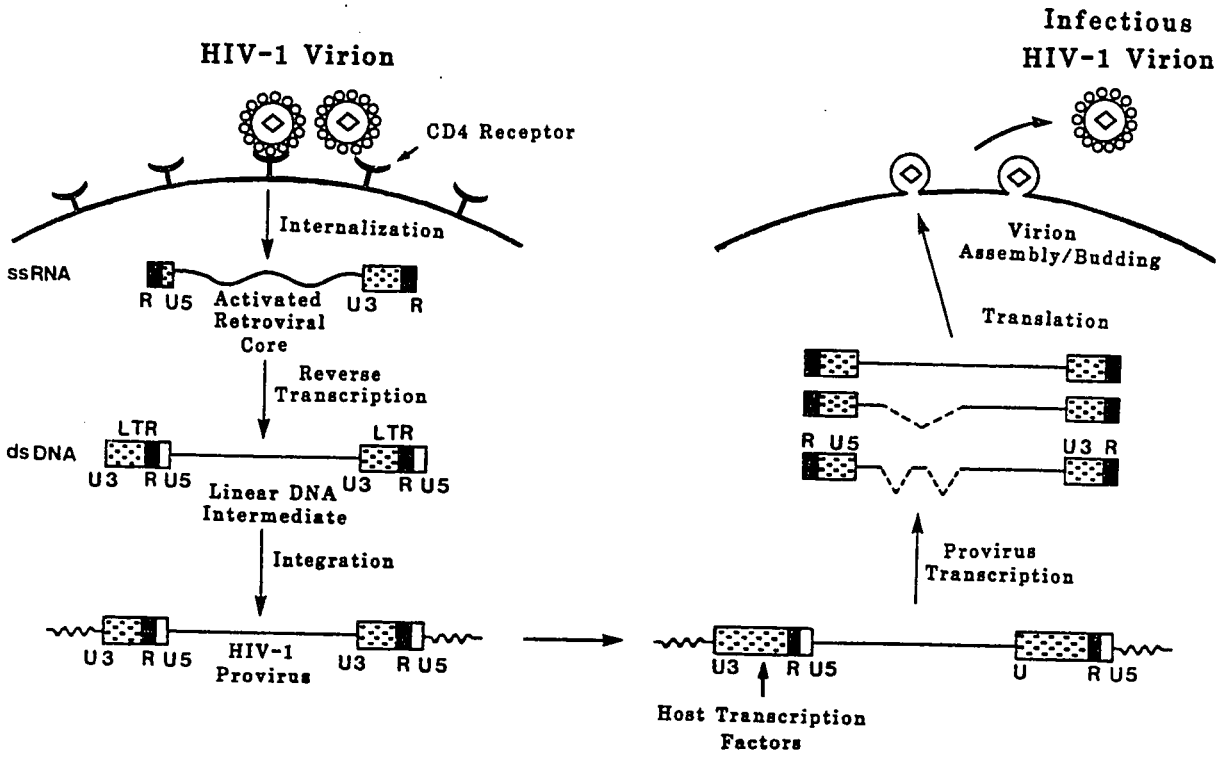
Viral genes with uncertain function

The vif (viral infectivity factor) gene appears to play a role in virion morphogenesis but is not absolutely required for viral replication (Greene, 1990). Proviral clones containing mutations in the vif gene yield only low levels of infectious particles; however, cell-to-cell transmission of virus may be unperturbed. The vpu gene also appears to be involved in virion morphogenesis by promoting efficient assembly and release of the virus (Cullen and Greene. 1990). Although no clear function has yet been elucidated for the vpr gene, it may play a role in accelerating virion assembly (Greene, 1990).

5. REPLICATION CYCLE OF HIV-1

The first step in infection is the binding of a virus particle to a specific receptor on the surface of the target cell. In the case of retroviruses and other enveloped viruses, the interaction with the cell receptor is mediated by the envelope glycoprotein. The interaction between HIV-1 and a cell has been shown to be due to specific high affinity binding of the viral gp120 molecule and the target cell CD4 molecule (Dalgleish et al. 1984; Klatzmann et al. 1984; McDougal et al. 1986; Lasky et al. 1987). CD4 is not only the first retrovirus receptor to be identified, but also has been extensively studied. After HIV-1 binds to the CD4 molecule, the virus is internalized and uncoated (Fig. 4). The precise mechanism of virus entry into target cells is unclear. It has been suggested that receptor-mediated endocytosis plays a role in this process (Maddon et al. 1986). However,

Figure 4. Schematic summary of the life cycle of HIV-1. Events depicted include HIV-1 binding to the CD4 receptor, virion internalization, reverse transcription of the RNA genome, integration and establishment of the HIV-1 provirus, proviral transcription involving inducible host factors and viral regulatory proteins, virion assembly, and the budding of infectious particles. Taken from Greene (1990).


HIV-1 Virion
CD4 Receptor
Internalization
ssRNA
R U5
Activated Retroviral Core
U3 R
Reverse Transcription
dsDNA
LTR
LTR
U3 R U5
Linear DNA Intermediate
U3 R U5
Integration
U3 R U5
HIV-1 Provirus
U3 R U5
Host Transcription Factors
U R U5
Provirus Transcription
R U5
U3 R
Translation
Virion Assembly/Budding
Infectious HIV-1 Virion

it has recently been demonstrated that pH-independent fusion of the transmembrane portion (gp41) of the virus envelope with the cell membrane is required for virus entry (Stein et al. 1987). In addition, the inability of mouse cells transfected with the CD4 gene and expressing the human CD4 protein on the cell surface to be productively infected with HIV (Maddon et al. 1986) suggested that other proteins expressed on human $T4^+$ cells may be required for virus internalization.

Once internalized, HIV begins its life cycle by conversion of the RNA genome into a duplex proviral DNA. The regulatory sequences flanking the viral RNA have to be duplicated, leading to the formation of complete LTRs (U3-R-U5) at either end of the viral DNA (Fig. 4). This process is dependent upon the action of the retrovirus reverse transcriptase as well as on various ill-defined cellular factors (Varmus, 1988). The proviral DNA, which can exist in a linear or circularized form, is integrated into the host DNA in a process dependent on an integrase encoded by the viral pol gene (Varmus 1988), thus establishing the proviral form of HIV-1 (Fig. 4). An unusual feature of HIV-1 infection compared to most other retroviruses is the accumulation of large amounts of unintegrated viral DNA in the infected cells, which may be an important factor in the cytopathogenicity of HIV-1 (Shaw et al. 1984).

Once integrated, the provirus behaves as a resident cellular gene. The infection may assume a latent phase with restriction of the cycle until the infected cell is activated.

For most retroviruses, the expression of integrated provirus results from the

action of host cell transcription factors (Varmus and Brown. 1989). The expression of HIV-1, however, is additionally controlled by viral regulatory proteins. Transcription is a complex process involving interplay between cis-acting regulatory sequences present in the viral LTR and trans-acting cellular transcription factors as well as viral transactivators (e.g. tat and rev) (Fig. 3). This interaction results in a rather low but significant level of viral mRNA synthesis. It has been demonstrated that this initial population of genome length RNA transcripts reaches the cytoplasm exclusively as a class of multiply spliced 2.0 kb viral mRNAs that encode the HIV-1 regulatory proteins tat and rev (Cullen and Greene. 1989). The tat protein acts as a potent activator of HIV-1 LTR-specific transcription and therefore establishes a strong positive feedback loop. This action of tat results in the accumulation of a critical level of the rev protein which then inhibits further synthesis of the multiply spliced mRNAs and instead activates the expression of unspliced (gag-pol) and single spliced (env) mRNAs that encode the virus structural proteins. The action of rev divides HIV-1 gene expression into two temporal phases, an early, regulatory phase and a later, structural phase (Cullen and Greene, 1989) (Fig. 3).

Transcribed viral mRNAs are subsequently translated into virus-specific proteins, and new virions are assembled at the cell surface where viral RNA, reverse transcriptase, structural and regulatory proteins, and envelope proteins are assembled in a highly organized fashion to produce infectious progeny (Fig. 4).

6. THE ROLE OF HIV-1 ENVELOPE GLYCOPROTEIN IN INTERACTION BETWEEN VIRUS AND HOST CELLS

Structure of HIV-1 env protein

The HIV-1 envelope consists of two glycoprotein subunits: the external gp120 and the membrane-associated gp41 (Allan et al. 1985). These proteins are synthesized from an 856-amino-acid precursor, gp160, which is cleaved intracellularly to produce the mature gp120-gp41 complex (Allan et al. 1985, Veronese et al. 1985). The envelope glycoprotein complex is anchored to the virion envelope and infected cell membranes through gp41, while gp120 is attached to gp41 through noncovalent interactions (Allan et al. 1985; Veronese et al. 1985). The envelope glycoprotein complex of HIV-1 mediates three functions critical for virus infectivity and pathogenesis: (1) attachment of virions to target cells by its binding to CD4 (Dalgliesh et al. 1984; Klatzman et al. 1984; McDougal et al. 1986), (2) fusion of the HIV-1 envelope to the plasma membrane of CD4+ target cells to transfer the viral genome into cells (Kowalski et al. 1987; McCune et al. 1988; Stein et al. 1987), and (3) fusion of the plasma membrane of HIV-1-infected cells with those of uninfected CD4+ cells to form multinucleated syncytia (Kowalski et al. 1987; Lifson et al. 1986; Popovic et al. 1984; Sodorski et al. 1986).

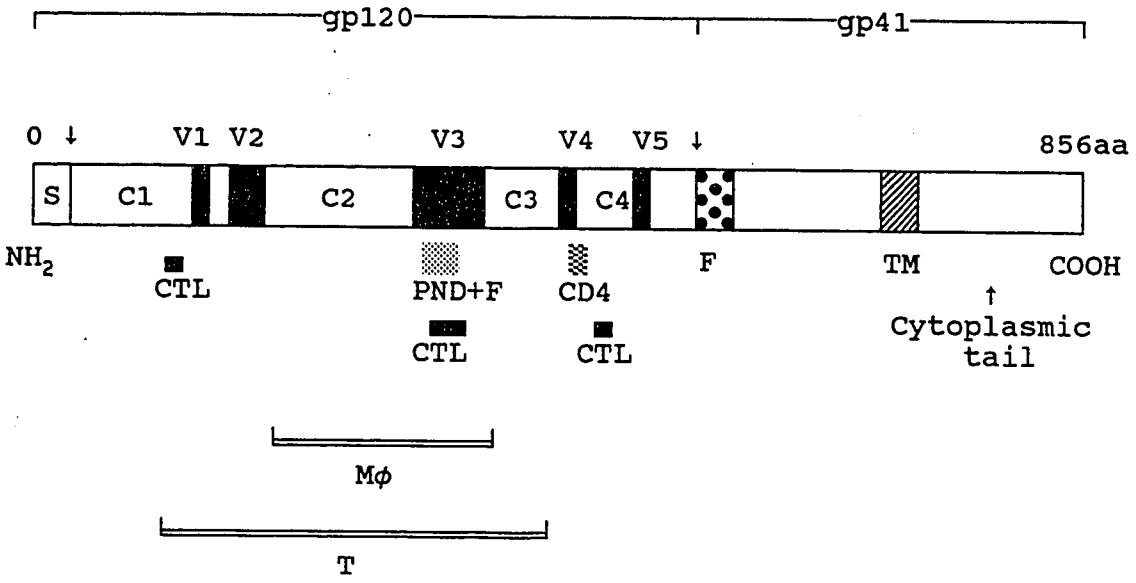
The HIV-1 env protein contains as many as 30 potential glycosylation sites and 23 cysteine residues, with the majority (21 and 18, respectively) located in the gp120 (Myers 1988); carbohydrate and cysteines are expected to play roles in the

formation and accessibility of both binding sites and neutralizing epitopes as well as in maintaining a proper three-dimensional structure of the envelope protein. Computer analysis shows that there are several highly conserved regions (C1-C4) that are interspersed with regions of high variability (V1-V5) within gp120 (Mordrow et al. 1987) (Fig. 5). Despite the considerable variability of the HIV-1 envelope glycoprotein, hydropathic analysis (Muesing et al. 1985) and functional studies (Kowalski et al. 1987; Lasky et al. 1987; Berman et al. 1988) have revealed that HIV-1 envelope glycoprotein (gp160) possesses the following functional domains (Fig. 5): (i) an amino-terminal signal sequence (residues 1 to 30) that directs the nascent env gene product into the secretory pathway of the infected cell, (ii) a dibasic amino acid protease-processing site (residues 510 and 511) that is cleaved to generate gp120 and gp41 from the 160-KDa precursor, (iii) a domain (residues 397 to 439) capable of binding to the CD4 receptor, (iv) a fusogenic domain (residues 512 to 541) which mediates syncytium formation, (v) a stop transfer sequence (e.g. transmembrane domain; residues 687 to 706) that binds the protein to the surface of virions and virus-infected cells, and (vi) a cytoplasmic tail (residues 707 to 856) of unknown function which might interact with other viral proteins (e.g. p17) during virion assembly.

Biosynthesis of HIV-1 env protein

The HIV-1 env gene is transcribed as a singly spliced viral mRNA species that is translated, as are most membrane-spanning or secreted proteins, on membrane-

Figure 5. Functional organization of HIV-1 envelope glycoprotein. Conserved (open) and variable (shaded) regions within the gp120 and gp41 portions of the env reading frame are indicated. S, signal sequence; CTL, T cell epitope; F, fusogenic domain; TM, transmembrane domain; CD4, CD4 binding site; Mø, macrophage tropism determinant; T, T cell tropism determinant; and PND+F, principal neutralizing determinant (V3 loop) and fusogenic domain. Small arrows indicate the sites of protease cleavage.


gp120
gp41
0
V1
V2
V3
V4
V5
856aa
S
C1
C2
C3
C4
F
TM
NH2
COOH
CTL
PND+F
CD4
CTL
CTL
Cytoplasmic tail
Mϕ
T

bound polyribosomes. The initial step that introduces the nascent polypeptide into the secretory pathway is mediated by the interaction of a sequence of amino acids (the signal sequence) located at the N-terminus of the nascent polypeptide with a component of the cellular transport machinery-the signal recognition particle (SRP)/docking protein complex (Walter and Lingappa, 1986). After docking, cotranslational translocation of the polypeptide across the membrane of the endoplasmic reticulum is accomplished. The signal sequence is removed cotranslationally by a host protease-mediated cleavage event and is not present in the mature protein (Walter and Lingappa, 1986).

Although the exact nature of the translocation complex through which membrane and secreted proteins are moved into the lumen of the rough endoplasmic reticulum (RER) is not known, it was demonstrated that deletion of signal peptides led to the synthesis of cytoplasmically located and nonglycosylated proteins that were rapidly degraded (Gething and Sambrook, 1982; Sekikawa and Lai. 1983). These experiments demonstrated the importance of the signal peptide for the translocation of viral membrane proteins. Furthermore, the driving force for translocation also remains obscure. Studies on the paramyxovirus fusion protein and Rous sarcoma virus (RSV) env gene product have suggested that the forces involved in folding the molecule into its final tertiary structure might be responsible for pulling the nascent molecule through the membrane (Hunter, 1988, Paterson and Lamb, 1987).

After insertion into the RER, HIV env protein is transported from the RER

through the Golgi complex to the plasma membrane. During transport, co- and post-translational modification occurs including glycosylation, disulfide bond formation, and proteolytic cleavage.

Initially, the envelope glycoprotein of HIV-1 is synthesized as an 88-kDa precursor inserted into the RER, where the addition of high-mannose N-linked carbohydrate chains as well as folding take place (Allan et al., 1985; Fennie and Lasky, 1989; Stein and Engleman, 1990). After transport of the oligomeric gp160 precursor to the Golgi complex, two major modifications occur (Willey et al., 1988b; Stein and Engleman 1990): (a) the attached mannose-rich carbohydrate side chains are "trimmed" by specific carbohydrate-modifying enzymes. Some of the associated carbohydrate is processed to more complex type by acquiring fucose and sialic acid residues in the medial and trans-Golgi cisternae, but only after gp160 is cleaved to produce gp120 and gp41 (Stein and Engleman, 1990). (b) precursor molecules are cleaved by a host-mediated protease into gp120 and gp41 (McCune et al. 1988; Geyer et al. 1988). This processing of gp160 appears to be essential for HIV-1 infection and syncytium formation (McCune et al. 1988).

The extensive oligosaccharide processing leads to the production of a mature gp120 which contains high-mannose and complex carbohydrate structures (Geyer et al. 1988). Following this complex series of modifications, the mature gp120 and gp41 are transported from the trans-Golgi reticulum to the plasma membrane where they can be incorporated into budding progeny virions.

Cell tropism

One of the chief clinical hallmarks of AIDS is a selective depletion of CD4-bearing (helper/inducer) T-lymphocytes (Gottlieb et al. 1981). This observation first suggested that the CD4 molecule might be the receptor for the virus. This suggestion was supported by in vitro demonstration that purified CD4+ T-cell lines preferentially support the replication of HIV (Klatzman et al. 1984) and that syncytia and cell death are frequently associated with infection. Furthermore, it was shown that both infection and syncytium formation could be blocked by monoclonal antibodies directed against certain epitopes of the CD4 molecule (Dalgleish et al. 1984, Klatzman et al. 1984; McDougal et al. 1985). The HIV-1 gp120 binds to CD4 as indicated by their coprecipitation by antibodies directed against either of the two proteins (McDougal et al.1986). Measurements of the affinity constant for this reaction have been shown that gp120 and CD4 form a high affinity complex with a dissociation constant of approximately 4×10^{-9} M/l (Lasky et al. 1987). The definitive proof that CD4 is a receptor for HIV was provided by Maddon et al. (1986), who demonstrated that transfection and expression of the cDNA for human CD4 rendered nonpermissive CD4- HeLa cells, permissive for HIV infection. Interestingly, expression of human CD4 is not sufficient to confer susceptibility to murine cells, suggesting a second component present in human cells but not in murine cells is required for postbinding events in the infection process (Maddon et al. 1986).

In addition to helper T-cells and monocyte-macrophages, other cells such as

Langerhans cells (Tschachler et al. 1988), follicular dendritic cells (Rappersberger et al. 1988), glial cells (Cheng-Mayer et al. 1987, Tschachler et al. 1987), and certain tumour cell lines (Adachi et al. 1987) are susceptible to HIV infection. These cells express low levels of CD4 on their surface. Similarly, five hepatoma cell lines have been shown to be infected productively by HIV-1 despite the absence of detectable CD4 expression (Cao et al. 1990). Soluble CD4 and monoclonal antibodies against CD4 failed to block HIV-1 infection in these cell lines. However, the nature of an alternative receptor suggested by these studies remains to be determined.

A CD4-binding region within gp120 was defined by linker-insertion mutagenesis (Kowalski et al. 1987) and by blocking monoclonal antibodies (Lasky et al. 1987). These findings suggest that a conformational epitope composed of domains within the carboxyl-terminal third of gp120 (residues 397 to 439) is involved in CD4 binding (Fig. 5).

Antigenicity

The envelope glycoprotein of HIV-1 represents a major target of the humoral immune response (Barin et al. 1985), and paradoxically, high titered antibodies to this surface component of the virus (as assessed by membrane fluorescence or RIA) are found even in patients with advanced AIDS (Weiss et al. 1985). However, the titer of neutralizing antibody in the sera of these patients is generally quite low (Weiss et al. 1985), may decline as disease progresses, and the presence of neutralizing antibody does not result in the abrogation of viral persistence.

A major strain-specific, immunodominant neutralizing epitope (which is also named the principal neutralizing determinant, PND) has been located near the middle of gp120 (residues 303-330) (Lasky 1986; Rusche et al. 1988; Putney et al. 1986; Matsusbita et al. 1988; Palker et al. 1988) (Fig. 5). Antibodies directed against the complete envelope predominantly recognize this particular epitope and block both infectivity and virus-induced cell fusion. In contrast to the CD4-binding domain that forms a pocket that is not accessible to antibodies (Wong-Staal, 1990), this neutralizing epitope resides in hypervariable region 3 (V3) and putatively exists as a loop (V3 loop) between two cysteine residues (amino acids 296-331) connected by a disulfide bond (Putuey et al. 1986, Rusche et al. 1988) (Fig. 5). Although most of the amino acids in the V3 loop are highly variable among different strains of HIV-1, a Gly-Pro-Gly-Arg sequence at the tip of the loop is highly conserved. The region encompassing this highly conserved tip forms the binding site for antibodies that block HIV-1 infection and inhibit the fusion of HIV-1-infected cells (Matsusbita et al. 1988; Palker et al. 1988; Rusche et al. 1988). A significant percentage of sera from HIV-infected individuals react with peptides from this region (Palker et al. 1988) (Fig. 5).

Less information is available on the cellular immune response to the HIV envelope proteins. Studies from human volunteers vaccinated with a recombinant vaccinia virus expressing gp160 of HIV-1 indicated that cytotoxic T lymphocytes (CTL) capable of recognizing two regions of gp120 (residues 112-114 and 428-443) predicted to be T-cell epitopes, were present (Berzofky et al. 1988) (Fig. 5). Similar

studies in mice have suggested that, at least in this host, a portion of the immunodominant humoral epitope, the V3 loop (residues 307-322), is also a major CTL epitope (Takahashi et al. 1988) (Fig. 5).

Recently, the V3 loop sequence was determined for a large numbers of HIV field isolates using PCR amplification and sequencing (Palker et al. 1988). Results from this study revealed that variation in this region is much less than previously thought (Palker et al. 1988). Of practical importance is the finding that antibodies that bind the consensus peptide sequence Gly-Pro-Gly-Arg-Ala-Phe, the central portion of the V3 loop, were able to neutralize 60% of random HIV isolates (LaRosa et al. 1990). Furthermore, recent studies have identified the V3 loop as a fusion domain in which single-amino-acid changes completely abolish or greatly reduce the ability of the HIV-1 envelope protein to induce cell fusion (Freed et al. 1991). This finding suggests that the V3 loop represents a region against which anti-HIV therapeutic agents could be targeted.

Very recent studies (Shioda et al. 1991) have shown that a specific region of gp120, amino acids 174 to 332, which also encompasses the V3 hypervariable region, seems to determine macrophage tropism, whereas an over-lapping region, amino acids 96 to 416 determines T-cell tropism (Fig. 5). These results provide a basis for relating functional domains of the HIV-1 env gene to pathogenic potential (Shioda et al. 1991).

Pathogenicity

Although the clinical hallmark of AIDS is a progressive deterioration of

immune competence due to a progressive loss of CD4+ helper/inducer lymphocytes (Fauci, 1988), the precise mechanisms involved in both cell cytopathogenicity and immune cell depletion remain unclear. However, the viral glycoproteins clearly play critical and probably multiple roles in these processes. One possible mechanism is through syncytium formation, which involves interaction of HIV-infected cells, expressing viral glycoproteins (gp120 and gp41) on the cell surface, and uninfected cells expressing CD4 (Sodroski et al. 1986, Lifson et al. 1986). The mutinucleated giant cells are unstable in culture, do not proliferate, and usually die within 48 hours. In this way, a few infected cells may be responsible for the death of many cells. However, syncytium formation does not appear to be the main basis of cell killing since mutations within the env gene can abrogate cell killing without affecting cell fusion (Fisher et al. 1986a) and HIV is cytopathic in some cells in the absence of cell fusion (Somasundaran and Robinson, 1987). Intracellular complexing of CD4 and gp120 or destruction of the permeability of the membrane owing to profuse virus budding have also been proposed to play a role in cell killing by HIV (Hoxie et al. 1986).

In vivo, additional mechanisms may be responsible for T helper/inducer cell loss. Paradoxically, while only a very small portion of the circulating CD4+ lymphocytes perhaps (less than 1 in 10,000) express HIV mRNA or proteins at any time during the course of the disease, there is progressive and eventually total destruction of this cell type (Shaw et al. 1984). It has been suggested that progressive recruitment of uninfected T cells into env expressing syncytia might be

one of the mechanisms by which this could occur. In addition, it has been shown that free gp120 (shed by infected cells) can bind to the CD4 molecule on cells expressing this marker, making them targets for an antibody-dependent cell-mediated cytotoxic (ADCC) response (Lyerly et al. 1987) or class II restricted T cell-mediated cytolysis (Lanzavecchia et al. 1988).

A research team at the University of Brescia, Italy, has provided a new clue to immune cell depletion (Imberti et al. 1991). A "superantigen" encoded by HIV may cause progressive T cell depletion that leads to the collapse of the immune system. One possibility that has been suggested is that gp120 might be involved in the T cell depletion (Imberti et al. 1991). However, definitive proof will require identification of the superantigen.

In view of these findings, the envelope glycoprotein of HIV continues to be a focus of AIDS research. Understanding how these highly variable molecules modulate cell surface marker expression, induce a cytopathic effect, and target both beneficial and deleterious immune responses to HIV infection will clearly play a major role in the development of therapeutic and preventative approaches for this disease.

7. STATEMENT OF OBJECTIVES

However, because the amount of HIV-1 envelope glycoprotein produced by virus-infected cells is low (Robey et al. 1986) and gp120 is readily shed from the surface of purified virions (Robey et al. 1986), it is not practical to purify sufficient

quantities of gp160 or the gp120-gp41 complex for detailed biochemical, biophysical, and biological analysis. Recombinant DNA technology offers attractive alternatives to virion-derived materials. Several expression systems have been used to produce the envelope glycoproteins of HIV-1 and to study their biosynthesis, processing, and biological activity. These include E. coli (Crowl et al. 1985), yeast (Barr et al. 1987), and mammalian cells (Hu et al. 1986; Lasky et al. 1986). While mammalian cell expression results in the production of fully modified, functional protein, yields are often low. Proteins expressed in yeast are either in low yield or inefficiently glycosylated so that their proper antigenicity or immunogenicity may be affected. *E. coli* results in high yields of recombinant protein but the protein is not modified and difficult to purify in a nondenatured state.

To circumvent these problems, the baculovirus expression system has been developed to express a variety of proteins in insect cells (Luckow and Summers, 1988). The system takes advantage of the strong polyhedrin promoter, which drives expression of the polyhedrin gene or foreign genes to high levels (Luckow and Summers, 1988). Besides the high level of expression, this system offers the opportunity to study the processing and the biological properties of proteins of mammalian cells or viruses in insect cells. Recently, several groups have expressed the HIV-1 glycoprotein using the baculovirus expression system and studied its biosynthesis and biological activity. However, the processing and secretory pathways of the HIV-1 protein in insect cells are still not well understood.

Therefore, the objectives of my research have been:

1). To study the role of the signal sequence of HIV-1 gp120 in expression and secretion of gp120 in SF9 cells.

2). To test the ability of heterologous signal sequences to increase expression and secretion of gp120 in SF9 cells.

3). To examine the role of positively charged amino acids within the natural signal sequence in the secretion of gp120.

4). To investigate the role of glycosylation of gp120 in the interaction between gp120 and CD4 by comparing the ability of glycosylated, nonglycosylated, and deglycosylated forms of gp120 to bind CD4 molecules.

CHAPTER 2: MATERIALS AND METHODS

1. CLONES, CELLS, AND VIRUSES

Clones

The full length clones pHXB-2D and p1BM representing HIV-1_{HXB2D} and HIV-2_{NIHZ} genomes, respectively, were kindly provided by Dr. R. Gallo (National Institutes of Health, Bethesda, MD) and were used as the sources of the HIV-1 and HIV-2 envelope glycoprotein coding sequences (Fisher et al. 1986b; Franchini et al. 1987b). Convenient restriction sites flanking the envelope genes were not available to delete the upstream sequence containing alternative initiation codons and premature downstream translational termination codons. Therefore, multiple steps were used to subclone the envelope genes with deletion of the non-coding flanking sequences at both termini. A description of the subcloning and specific deletions will be discussed in the results section.

Cells

Spodoptera frugiperda (SF9) cells were provided by Dr. M. D. Summers (Texas A & M University, College Station, TX) and maintained as monolayer cultures in TNM-FH medium (Summers and Smith, 1987) containing antibiotics (penicillin 100 u/ml, streptomycin 100 mg/ml, kanamycin 100 mg/ml) at 27°C. The medium was supplemented with 10% heated-inactivated FBS and 2 mM glutamine (Gibco).

Virus

Autographa californica nuclear polyhedrosis virus (AcNPV) was obtained from Dr. D. H. L. Bishop (NERC Institute of Virology, Oxford, U. K.).

Virus infection and production

A virus plaque was picked and resuspended in 1 ml of TNM-FH medium. Approximately 3 x 10^6 SF9 cells, in a T-25 flask (25 cm^2, Corning Co. Corning, NY), were infected with the plaque-suspension at a moi of 0.01 pfu/cell and maintained for 4 days at 27°C. The culture fluid was used to infect 1.5 x 10^7 SF9 cells in a T-75 flask at a moi of 0.1 pfu/cell. The virus supernatant was harvested 5 days post-infection and stored at 4°C. The titer of the virus stock was 3-5 x 10^8 pfu/ml.

Preparation of AcNPV DNA

AcNPV DNA was prepared according to the procedure described by Summers and Smith (1987). Briefly, culture fluids from AcNP virus-infected SF9 cells were clarified by centrifugation for 30 min at 3,000 x g at room temperature (Damon/IEC) and then centrifuged in a Beckman SW28 rotor for 90 min at 81,000 x g at 4°C. The virus pellet was resuspended in TE buffer and banded on a 10-50% (w/v) sucrose step gradient in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA for 90 min at 120,000 x g using a Beckman SW41 rotor at 4°C. The viral band was collected and pelleted at 100,000 x g for 30 min. Virus was disrupted with 1% sodium N-lauryl sarcosinate and 200 μg/ml proteinase K. The DNA was banded in 54% CsCl (w/v)

gradients using a Beckman SW50 rotor at 195,000 x g for 16 hrs at 25°C. The DNA band was extracted with water-saturated butanol, dialysed in TE buffer and precipitated with ethanol.

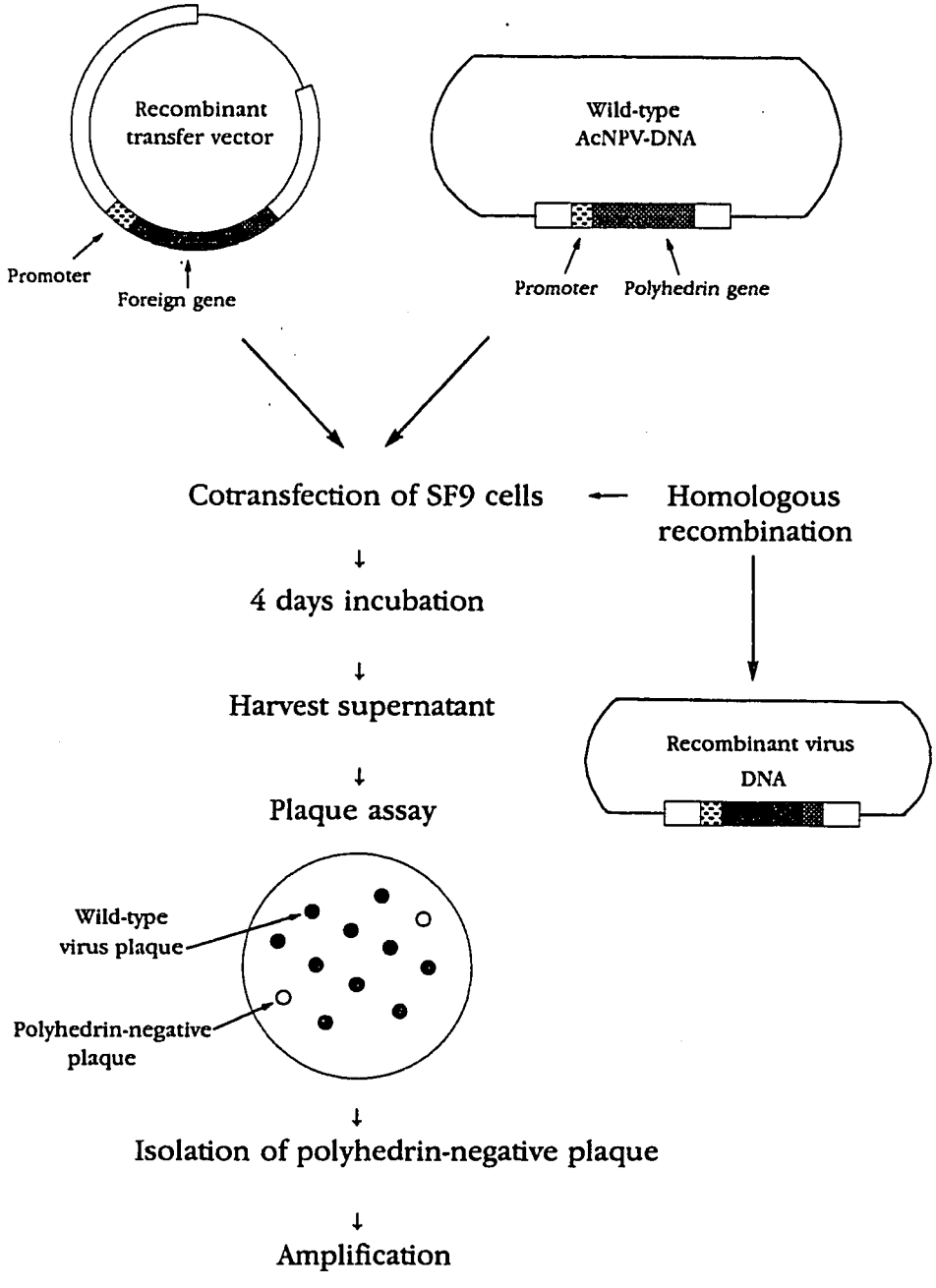
Insertion of the HIV-1 gp120 sequences into pAcYM1

The transfer vector, pAcYM1 (Matsuura et al. 1987), was digested with BamHI and dephosphorylated. A 1.5 kb BamHI fragment recovered from plasmid pUC19gp120-NSS, which will be discussed in details in the results section, was ligated to the BamHI site of pAcYM1. Following transformation of E. coli strain RR1 with the ligation mix, plasmids were prepared. With similar procedures, other fragments of the gp120 gene were inserted into pAcYM1. The orientation of the glycoprotein gene inserts were characterized by restriction endonuclease EcoRV and StuI (Matsuura et al. 1987).

Transfection of *Spodoptera frugiperda* cells

SF9 cells were cotransfected with a mixture of purified wild type AcNPV DNA and pAcYM1 containing the gp120 coding region by the procedure described by Summers and Smith (1987). Figure 6 shows the strategy for cotransfection, production, and selection of recombinant baculovirus. 1 μg of AcNPV DNA was mixed with various concentrations of plasmid DNA (3 to 10 μg) and the volume was adjusted to 950 μl with Hepes-buffered saline (20 mM Hepes, 1 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 10 mM glucose, pH 7.05). A fine precipitate was formed

Figure 6. Strategy for the production of recombinant baculovirus. Open areas represent baculovirus sequences. The dotted and shaded boxes correspond to the baculovirus polyhedrin promoter and the polyhedrin gene. The black area represents the foreign gene to be expressed. Taken from Kang (1988).


Recombinant
transfer vector
Promoter
Foreign gene
Wild-type
AcNPV-DNA
Promoter
Polyhedrin gene
Cotransfection of SF9 cells
Homologous
recombination
4 days incubation
Harvest supernatant
Plaque assay
Recombinant virus
DNA
Wild-type
virus plaque
Polyhedrin-negative
plaque
Isolation of polyhedrin-negative plaque
Amplification

by adding 50 μl of 2.5 M $CaCl_2$, and the DNA mixture was added to a monolayer of 1.2 x 10^6 SF9 cells in 35 mm tissue culture dishes (Corning) and incubated at 27°C. After 3 hr, the DNA was removed and 2 ml of TNM-FH medium was added. The transfected cells were incubated for 4 days at 27°C. The supernatant was harvested and titrated on SF9 cell monolayers.

Selection and purification of recombinant baculovirus

SF9 cells were plated into 35 mm tissue culture dishes (Corning) in TNM-FH serum-free medium at a density of 1.2 x 10^6 viable cells. Serial 10 fold dilutions of virus were prepared. Typically, transfection supernatants were diluted 10^{-2} to 10^{-4} fold, and viruses picked from a plaque were diluted 10^{-1} to 10^{-3} fold. After medium was removed, 200 μl of diluted virus was inoculated into each dish. After 1 hr of incubation, 2 ml of overlay (1.5% low melting agarose in TNM-FH medium) was added. When the overlay was solidified, 1 ml of medium was added and plates were incubated at 27°C for 5-6 days in a humid environment. The plaques were visually examined by looking against a black background with a fluorescent light source. Clear plaques were differentiated from opaque plaques. The clear plaques were further examined by a light microscope, and those exhibiting no evidence of occlusion bodies were picked and titrated on SF9 cells to obtain pure polyhedrin-negative recombinant viruses (Fig. 6).

2. MOLECULAR CLONING AND RELATED TECHNIQUES

Maintenance of bacterial strains, general techniques for the handling and manipulation of DNA and for the preparation of necessary media and reagents are thoroughly discussed by Maniatis et al. (1982). These methods were used unless otherwise specified.

Preparation of competent *E. coli*

Bacteria were rendered competent for the uptake of plasmid DNA by treatment with $CaCl_2$. First, 10 ml of an overnight culture of bacteria grown in 2YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) without shaking were inoculated into 100 ml of P-medium (15.9 mM K_2HPO_4, 6.3 mM KH_2PO_4, 10 mM $MgSO_4$, 1.8 μM $FeSO_4$, 1% Casamino acids and 0.25% glucose) and cells were grown to an A660 of 0.4 as measured in a Beckman Du-8B spectrophotometer. Bacteria were pelleted at 6,000 x g for 10 min in a Beckman JA-10 rotor. After washing in 100 ml of 10 mM NaCl at 4°C, cells were repelleted. Bacteria were resuspended in 100 ml of 50 mM $CaCl_2$ and incubated at 4°C for 15 min. Finally, bacteria were pelleted and resuspended in 10 ml of 50 mM $CaCl_2$, 16% (v/v) glycerol, aliquoted and quickly frozen in a dry ice/ethanol bath before being stored at -80°C. The transformation efficiency of the cells was 5×10^6 to 5×10^7 colonies/μg of plasmid DNA.

Transformation of *E. coli*

Transformations were performed according to the method of Maniatis et al.

(1982) with several modifications. Competent *E. coli* were thawed slowly on ice. DNA in no more than 10 μl was mixed with 200 μl of competent cells in 17 mm x 100 mm polypropylene tubes and incubated at 4°C for 30 min. The mixture was heated in a 42°C water bath for 90 seconds and then returned to ice for 2 min. 800 μl of LB (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl) broth was added and tubes were agitated (200 rpm) at 37°C for 1 hr. Cells were then spread onto LB plates containing ampicillin (100 μg/ml) and incubated overnight at 37°C.

Electrophoresis of DNA

DNA was separated by horizontal gel electrophoresis using 0.8 to 1.5% agarose and Tris-acetate buffer (TAE, 40 mM Tris-acetate, 2 mM EDTA). Electrophoresis was usually performed with a HE 33 Minnie submarine gel apparatus (Hoefer Scientific Instruments). Samples were mixed with 2.5 ul of 5x loading buffer (25% glycerol, 5X TAE, 0.06% BPB) and electrophoresed at 100 volts. The DNA was visualized under ultraviolet (UV) illumination after staining with EtBr (0.5 μg/ml in TAE). Gels were photographed using Polaroid type 667 film.

Purification of plasmid DNA

Large scale purification of plasmid DNA (maxipreparation) was done essentially according to the method of Maniatis et al. (1982). Several modifications were made to adapt the protocol to available rotors. Bacteria were pelleted at 6,000 x g in a Beckman JA-10 rotor. The bacterial pellet from 500 ml of culture

was resuspended in 7.5 ml of solution 1 (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme). 15 ml of solution 2 (0.2 M NaOH, 1% SDS) and finally 12 ml of 5 M potassium acetate (pH 4.8) were added before centrifugation at 12,100 x g for 20 min in a Beckman JA-20 rotor. Pelleting of DNA following isopropanol precipitation was at 12,100 x g in a Beckman JA-20 rotor for 30 min. Samples prepared for cesium chloride gradients were centrifuged at 12,100 x g for 10 min prior to centrifugation to equilibrium in a Beckman VTi 65 rotor at 290,000 x g for 16 hr.

Small scale (minipreparations) purification of plasmid DNA for screening of recombinant plasmid was an adaptation of the above method as described by Maniatis et al (1982).

Isolation of DNA from gels

DNA gels were stained briefly with EtBr (0.5 μg/ml) and observed with long wave ultraviolet light to permit precise excision of DNA fragments for purification. Gel slices were electroeluted using the IBI Model UFA Electro-Eluter (IBI) at 100 volts for 45 min. Eluted DNA was extracted with butanol saturated with H_20 to remove EtBr and was then precipitated with 2.5 volumes of ethanol.

Restriction and modification of DNA

All DNA modifying enzymes were purchased from Pharmacia, New England Biolabs or Boehringer Mannheim. All methods were performed essentially

according to techniques described by Maniatis et al. (1982). Only those techniques which have been modified are discussed below.

Generally, DNA was precipitated at -70°C for 15 min prior to microcentrifugation for 10 min at 4°C.

Restriction enzyme digestions were performed at 37°C for 2 hr using buffers recommended by the manufacturer. Between 0.5 and 10 μg of DNA was incubated with approximately 4 units of enzyme per μg of DNA in a final volume of 20 μl.

Dephosphorylation of DNA was performed in restriction digestion buffer containing calf intestinal phosphatase (CIP). 1 μg of DNA in digestion buffer was treated with 2-5 units of CIP for 15 min at 37°C and then for 15 min at 56°C. Fresh enzyme was added and the treatment repeated. CIP was inactivated by heating at 65°C for 10 min after adjusting the reaction buffer to 20 mM EDTA, 0.5% SDS.

Repair of DNA fragments with recessed 3' ends was performed in nick translation buffer (50 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.1 mM DTT and 50 μg/ml BSA) containing 80 μM of dNTPs and 1-5 units of the large fragment of DNA polymerase I (Klenow fragment) at room temperature for 30 min in a final volume of 20 μl.

For conversion of protruding 3' ends to blunt ends, 0.1-1.0 μg of DNA in 33 mM Tris acetate, pH 7.9, 66 mM KAc, 10 mM MgAc, 0.5 mM DTT and 100 μg/ml BSA was incubated with 100 μM of each dNTP and 2.5 units of T4 DNA polymerase at 37°C for 5 min in a final volume of 20 μl.

Generally, ligation reactions containing up to 200 ng of vector DNA and up

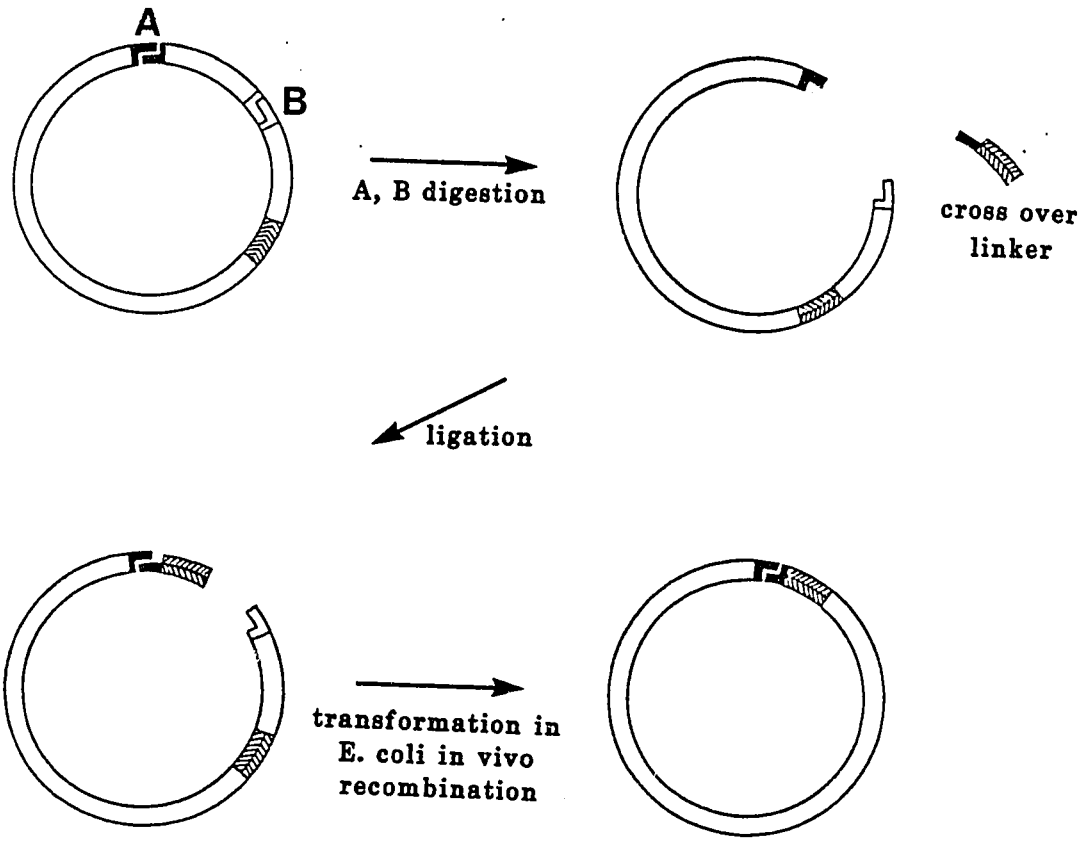
to a 10 fold molar excess of insert fragment were performed in 20 μl of ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM DTT an 1 mM ATP) at 16°C overnight with 5 units of T4 DNA ligase (Biolab).

Crossover linker mutagenesis

Crossover linker mutagenesis (Sung et al. 1986) was performed using double-stranded oligonucleotides. Presented below are the general techniques used for crossover linker mutagenesis, while a description of the specific deletions will be discussed in the results section.

Figure 7 shows the principle of the crossover linker mutagenesis method. Sites a and b are restriction sites. The region contained between site a and the hatched area is the non-translated region or the sequence desired to be deleted. After digestion with restriction endonucleases at sites a and b, the plasmid was purified and ligated to an oligonucleotide complementary to the 3' overhang of site a and containing several nucleotides corresponding to the beginning of the region to be conserved (the hatched area). After ligation, linear plasmid DNA was purified following agarose gel electrophoresis and used to transform *E. coli*. The region not included in the oligonucleotide is eliminated by homologous recombination in vivo. The synthetic oligonucleotide may also contain extra sequences such as restriction sites or regulatory sequences.

Figure 7. Strategy for crossover linker mutagenesis. The black box represents restriction site a and the open box represents restriction site b. The hatched areas are homologous sequences.


A
B
A, B digestion
cross over
linker
ligation
transformation in
E. coli in vivo
recombination

Polymerase chain reaction (PCR)

This technique (Saiki, 1988) consists of adding two oligonucleotides, each complementary to opposite strands of DNA, positioned in such a manner that the action of DNA polymerase copies the DNA back and forth between the two oligonucleotides. Presented below are the general techniques used for amplification, while a description of oligonucleotide-specific mutagenesis by PCR will be discussed in the results section.

DNA was amplified by PCR using the Perkin-Elmer Cetus (Norwalk, CT) GeneAmp DNA Amplification Reagent Kit and DNA thermal cycler. The reaction mixtures consisted of 20 ng of template DNA in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin, 1.0 μM of each of the two primers, 200 μM of each of the four dNTPs and 5 units of Taq DNA polymerase (5 units/μl) in a total volume of 100 μl. The synthetic oligonucleotide primers used to amplify the DNA segment from the template were constructed with convenient restriction sites at their 5'-ends. Samples were overlaid with 100 μl of mineral oil. The DNA was amplified using the Step-Cycle file. The initial template denaturation step was 5 min at 94°C. Afterwards the cycle profile was 1 min at 94°C (denaturation), 2 min at 45°C (annealing) and 3 min at 72°C (extension). Thirty cycles were performed. At the end of the 30th cycle, the 72°C extension step was prolonged for an additional 7 min. Samples were then cooled to 4°C. The bottom aqueous phase was removed and extracted once with chloroform/isoamyl alcohol. One-tenth of the sample was analyzed by agarose gel electrophoresis.

DNA sequencing

Dideoxynucleotide sequence analysis (Sanger et al. 1977) was performed using the modified T7 DNA polymerase sequencing system (Sequenase, United States Biochemical Corporation, Cleveland, OH) according to the manufacturer's instructions. 4-10 μg of plasmid DNA was denatured with 2 M NaOH, 2 mM EDTA at room temperature for 5 to 10 min. The DNA mixture was neutralized with 300 mM NaAc, pH 5.2 and DNA was precipitated with 2 volumes of ethanol. Afterwards the denatured plasmid DNA was annealed with 30 ng of specific primer in 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ and 50 mM NaCl in a volume of 9 μl, by incubating 2 min at 65°C and slowly cooling the sample to 30°C. The DNA/primer hybrid preparation was extended and labelled by incubation at room temperature for 10 min in the presence of 1.0 μl of [α-^{35}S]dATP (600 Ci/mmol, Amersham), 2.0 μl of Sequenase (1.5 units/μl), 1.0 μl of 0.1 M DTT and 2.0 μl of 1:5 diluted dGTP labelling mix (5x concentration = 7.5 μM each of dGTP, dCTP, and dTTP). 3.5 μl of the labelled, annealed primer-template mixture was removed and immediately mixed with a different dideoxynucleotide termination solution (80 μM each of all four dNTPs, 50 mM NaCl) and 8 μM of the specific dideoxynucleotide). Following 5 min of incubation at 37°C, the sequencing reactions were terminated by the addition of 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue) and were incubated at 95°C for 3 min prior to loading.

Sequencing reactions were electrophoresed using the IBI model STS 45

Thermoplate Sequencing Apparatus on 0.2 mm 6% polyacrylamide gels (5.7% acrylamide, 0.3% bis, 8 M urea in 1x TBE). Electrophoresis was at 50 watts constant power until the xylene cyanol reached the bottom of the gel (for sequences greater than 180 nucleotides from the primer) or until the bromophenol blue reached the bottom of the gel (sequences less than 180 nucleotides from the primer). After electrophoresis, gels were lifted onto Whatman 3 MM paper and dried in a Bio-Rad model 583 gel dryer. Gels were exposed to X-ray film at -80°C.

All synthetic oligonucleotides were prepared with an Applied Biosystems 380B DNA synthesizer by the University of Ottawa Biotechnology Research Institute.

3. PREPARATION OF ANTI-GP120 ANTIBODIES

Expression and purification of recombinant HIV-1 and HIV-2 gp120 proteins

Approximately 3 x 10^7 SF9 cells, in a T-75 flask (Corning), were infected with recombinant baculovirus at a moi of 5 pfu/cell and incubated for 72 hr at 27°C. Cells were harvested and washed once with cold PBS. The cell pellets were resuspended in 2 ml of H_2O and lysed by addition of an equal volume of 2x SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 10% SDS, 25% glycerol, and 0.04% BPB). Prior to electrophoresis samples were heated at 100°C for 5 min. Preparative electrophoresis was performed using the Bio-Rad Protean 2 Dual Slab system. Samples were separated by SDS-PAGE using 1.5 mm thick 10% polyacrylamide gels and preparative wells. Electrophoresis was carried

out at a maximum of 40 mA per gel. After electrophoresis, gels were rinsed briefly in distilled water at 4°C and then stained in 0.25 M KCl at 4°C for 10 min (Hager and Burgess, 1980). Appropriate protein bands were excised from the gel, and soaked in distilled water at room temperature for 15-30 min or until no further staining could be seen. Protein was electroeluted from the gel using the Bio-Rad model 422 Electro-Eluter following the manufacturer's directions. Electroelution was done at 32 mA for 4 hr. The electroeluted protein was diluted to 2 ml with PBS and then concentrated using microconcentrators (Centricon-30, Amicon) at 4,500 x g for 1 hr in a JA-21 rotor. Quantities of proteins were approximated by visual comparison of the intensity of Coomassie blue staining of the gp120 protein with known quantities of molecular weight standard proteins after SDS-PAGE.

Immunization with HIV gp120 proteins

Gel purified gp120 protein (10 μg) in PBS was emulsified in an equal volume of Freund's complete adjuvant for primary immunization or Freund's incomplete adjuvant for the secondary immunization. Antigen was administered intramuscularly in the leg and boosts were performed at three week intervals using alternating legs for immunization. Sera were collected 10 days following the last injection. Each serum sample was monitored for its ability to react with recombinant gp120 in Western blot assays and the serum with the highest titer was used in the present studies.

Collection of sera

All rabbits were bled from the ear immediately prior to immunization and on the tenth day following primary or subsequent immunization. At the completion of immunization rabbits were bled by heart puncture after sedation with Innovar-Vet (0.22 ml/kg). Sacrifice of the rabbit was ensured by cervical dislocation after collection of blood. Sedation, heart puncture and cervical dislocation were performed by trained animal care technicians.

Whole blood was incubated at 37°C to allow complete clotting and spun at 400 x g for 10 min to remove cells and clots from the serum. All sera were aliquoted and stored at -80°C.

4. PROTEIN ANALYSIS

Preparation of lysates of recombinant virus-infected cells

For total protein analysis, monolayers of SF9 cells were infected with recombinant or wild-type baculovirus at a moi of 5 pfu/cell and incubated for 1 hr at 27°C. The inoculum was removed and replaced with fresh TNM-FH medium. After an appropriate incubation time, cells were harvested and washed twice with cold PBS. Whole cell lysates were prepared by resuspending the cell pellet in H_20, adding an equal volume of 2x SDS sample buffer (100 mM Tris-HCl, pH 6.8, 10% β-mercaptoenthanol, 10% SDS, 25% glycerol, and 0.04% bromophenol blue) and

heating for 5 min at 100° C. Cell lysates were stored at -20°C.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE (Laemmli, 1970) on 10% slab gels using the Bio-Rad Mini-Protean II system (Bio-Rad). Gels were made with a 10% resolving gel (10% acrylamide, 0.13% bis, 0.1% SDS, 0.75 M Tris pH 8.8) and a 5% stacking gel (5% acrylamide, 0.07% bis, 0.1% SDS, 63 mM Tris pH 6.8) and were electrophoresed in 200 mM glycine, 25 mM Tris and 0.1% SDS at 150 volts until the bromophenol blue reached the bottom of the gel.

Immunoblot analysis

Proteins were separated by SDS-PAGE in the Bio-Rad Mini Protean II gel system. Following electrophoresis, proteins were transferred to nitrocellulose membranes (0.45 μM, Bio-Rad) using the Bio-Rad Mini Trans-blot assembly. Transfer was carried out at 100 volts for 1 hr in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3 (Towbin et al. 1979). The nitrocellulose membranes were then incubated with buffered gelatin (20 mM Tris, 3% gelatin, 500 mM NaCl, pH 7.5) for 1 hr at 27°C and incubated, first with rabbit anti-gp120 antiserum (1:500) for 2 hr and then with ^{125}I-labelled Staphylococcus protein A for 1 hr (specific activity, 33 mCi/mg; 0.1 μCi/ml, Amersham) in 50 mM Tris, pH 7.5, 10 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% NP40). After extensive washing with buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin,

0.5% Triton, and 0.1% SDS, nitrocellulose membranes were air-dried and exposed to Cronex 4L film at -70°C.

Radiolabelling and immunoprecipitation

SF9 cells were infected with recombinant baculoviruses as described above. At 24 hr postinfection, virus-infected cells were placed in methionine-free Grace medium for 30-60 min, after which they were pulse-labelled for 1 hr with 250 μCi/ml of L-[^{35}S] methionine (Amersham). For pulse-chase experiments, the labelling medium was removed after the appropriate pulse, and replaced with complete TNM-FH medium containing 10% FBS.

Prior to immunoprecipitation, cells were washed once with cold PBS, lysed by addition of 1 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1% NP40) and incubated on ice for 15 min. The extract was then centrifuged at 12, 000 x g for 3 min in a microcentrifuge to separate the NP40 soluble and insoluble proteins. The NP40 insoluble pellet was boiled for 5 min in protein sample buffer (50 mM Tris-HCl, pH 6.8 containing 4% SDS and 4% β-mercaptoethanol) diluted 1:100 in extraction buffer, microcentrifuged for 15 min, and the supernatant was used for further analysis (Jarvis and Summers, 1989). Extracellular medium was prepared for immunoprecipitation by first removing particulate material by centrifugation at 12,000 x g for 3 min, and supplementing with PMSF to a final concentration of 1 mM. 3 μl of human HIV-1 immune globulin (obtained through AIDS Research and Reference Reagent Program,

Division of AIDS, NIAID, NIH, Prince et al. 1988) or 5 μl of rabbit polyclonal monospecific anti-gp120 serum (see section 2.3) was added to the intracellular, extracellular or insoluble fraction, followed by incubation overnight on a rotator at 4°C. Immune complexes were collected by the addition of 5 mg of protein A-Sepharose CL-4B (Pharmacia) and incubation at 4°C for 1 hr. The beads were washed three times with cold RIPA buffer (1% NP40, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5), and resuspended in 30 μl of 1x SDS sample buffer by heating at 100°C for 5 min. The Sepharose CL-4B beads were spun down and samples were analysed by SDS-PAGE. After electrophoresis, gels were fixed in 30% methanol, 10% acetic acid for 30 min and then prepared for fluorography with Amplify (Amersham). Gels were dried using a Bio-Rad model 583 gel dryer and exposed to Cronex 4L film at -80°C.

Endoglycosidase analysis

Endoglycosidase H (endo H) or endoglycosidase F/N glycanase (endo FNG) (Boehringer-Mannheim) digestions were performed on immunoprecipitates. The precipitates prepared as described above were resuspended by boiling in 20 μl of 1% SDS, 50 mM Tris-HCl (pH 6.8) for 3 min and then divided into two equal aliquots after removal of Sepharose CL-4B beads (Rose and Bergmann, 1983). An equal volume of 2x digestion buffer (150 mM Na citrate, pH 5.3, and 2 mM PMSF) without SDS was then added. One sample received 0.6 mU of endo H (2 mU/μl) or 2U of endo FNG, while the other served as a control. Digestion was performed

at 37°C for 16 hr, after which the reactions were stopped by the addition of 2x SDS sample buffer and the samples were incubated for 5 min at 100°C. Samples were analyzed by SDS-PAGE and visualized by fluorography.

Tunicamycin and brefeldin A treatment

For tunicamycin (TM) treatment, SF9 cells were infected with recombinant baculovirus at a moi of 5 pfu/cell. At 20 hr p.i., tunicamycin (Sigma) was added to a final concentration of 15 μg/ml and maintained throughout the experiment. The cells were labelled with 250 uCi/ml of L-[^{35}S] methionine for 2 hr at 24 hr p.i. Cell lysates were prepared and proteins were immunoprecipitated either with rabbit anti-gp120 serum or with monoclonal antibody (MAb) OKT4 after complexing with CD4.

For brefeldin A (BFA) treatment, BFA (Epicentre Technologies) was added to a concentration of 15 μg per ml at 20 hr after infection with recombinant virus. Cells were pulse-labelled with 250 μCi/ml of L-[^{35}S]-methionine at 23 hr p.i. for 1 hr and chased for appropriate times in fresh TNM-FH medium containing 15 μg/ml of BFA. For longer periods of chase (more than 4 hr), fresh BFA was added to the medium. Cells were lysed and proteins were immunoprecipitated with serum from a patient positive for HIV-1. Some samples were treated with endo H before analysis by SDS-PAGE as described above.

CD4-binding assays

CD4-binding assays were performed according to the procedure of Laskey et al. (1987), with the exception that a highly purified, soluble CD4 (American Biotechnologies Inc.) was used for the binding studies. SF9 cells were infected with recombinant baculovirus at a moi of 5 pfu/cell. After 36 hr p.i., the infected cells were pulse-labelled for 1 hr with L-[^{35}S]-methionine (250 μCi/ml) and lysed by addition of 1 ml of extraction buffer (1% NP40, 150 mM NaCl, 1 mM PMSF, and 50 mM Tris-HCl, pH 7.5). One half of the pulse-labelled cell lysate was immunoprecipitated with 5 μl of rabbit anti-gp120 serum, while the remaining half was incubated with 0.4 μg of soluble CD4, and the complex was coprecipitated with 30 μl (1 μg) of OKT4 monoclonal antibody (Ortho Diagnostics) and 5 mg of protein A-Sepharose CL-4B (Pharmacia). The immunoprecipitated material was washed three times with RIPA buffer and resuspended in 30 μl of 1x SDS sample buffer. Samples were analyzed by SDS-PAGE and bands were visualized by fluorography.

CD4-binding assay of deglycosylated forms of recombinant gp120

For intracellular CD4-binding assay, the pulse-labelled cell lysates were first incubated with 1 μg of soluble CD4 at 4°C for 2 hr and then 60 μl of OKT4 MAb were added and the incubation was continued at 4°C for 14 hrs. Immune complexes were harvested by addition of 5 mg of Protein A-Sepharose CL-4B and washed three times with RIPA buffer. The immunoprecipitates were suspended by

boiling in 100 μl of 1% SDS, 50 mM Tris-HCl (pH 6.8) for 3 min and following centrifugation the supernatants was divided into two equal aliquots. One aliquot received an equivalent volume of digestion buffer (150 mM Na citrate, pH 5.3, 2 mM PMSF) containing 3 mU endo H, while the other served as a control. Digestions were performed at 37°C for at least 16 hr, after which samples with endo H or without endo H were further divided into two equal aliquots. Each aliquot was brought up to 500 μl by addition of 450 μl of extraction buffer. One sample was incubated with 5 μl of rabbit anti-gp120 serum, while other was incubated with 0.4 μg of soluble CD4 and 30 μl of OKT4 MAb at 4°C overnight. Complexes were harvested by addition of 5 mg of protein A-Sepharose CL-4B and analyzed by SDS-PAGE.

For the extracellular CD4-binding assay, infected cells were labelled with 250 μCi/ml of L-[^{35}S] methionine for 1 hr and then incubated for 2 hr in fresh TNM-FH medium. Culture medium was clarified and adjusted to 50 mM sodium phosphate, pH 5.0 and 1 mM PMSF with 1 M sodium phosphate, pH 5.0 and 100 mM PMSF. One half of each sample (1 ml) was incubated without enzyme, while the remaining half was incubated with either 10 mU of endo H or 2 U of endo FNG at 37°C for 16 hr. The supernatants were divided into two equal aliquots; one aliquot was incubated with 5 μl rabbit anti-gp120 serum, while the other was incubated with 0.4 μg of soluble CD4 and 30 μl of OKT4 MAb at 4°C for 14 hrs. Immunoprecipitation, electrophoresis and fluorography were performed as described above.

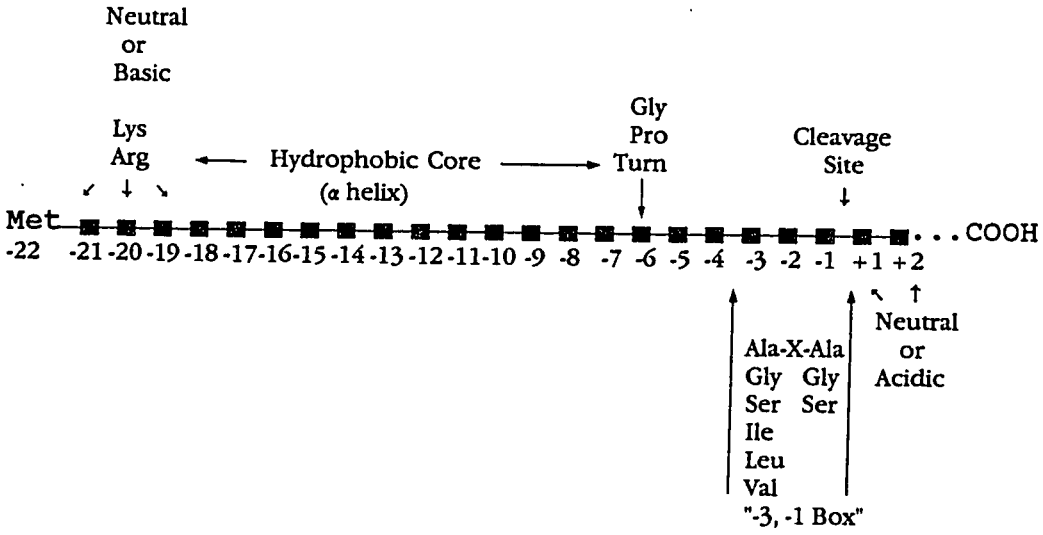
CHAPTER 3: EXPRESSION OF THE HIV-1 ENVELOPE GLYCOPROTEIN GP120 IN RECOMBINANT BACULOVIRUS-INFECTED INSECT CELLS

INTRODUCTION

Proteins destined to be secreted across membranes or integrated into membranes (including all viral glycoproteins) are synthesized on membrane-bound polyribosomes (Kreil, 1981). All these proteins are synthesized in a precursor form with an amino-terminal extension of 15-30 amino acids termed the " signal" or " leader" sequence (Milstein et al. 1972). These transient extra pieces of polypeptide have been demonstrated to initiate the first step of the secretory pathway: translocation across the membrane of the endoplasmic reticulum (ER) in eukaryotic cells or across the plasma membrane in prokaryotes.

A comparison of various signal sequences reveals no conserved features (Watson, 1984) such as those found in proteins which evolved from a common ancestor. However, certain structural features are shared among the signal peptides of bacterial and eukaryotic pre-proteins (Von Heijne, 1983; Perlman and Halvorson, 1983) (Fig. 8). These include: (a) a total length of 15-30 residues, (b) one or more basic amino acid residues at the extreme N-terminus, (c) a central hydrophobic core of eight or more apolar residues directly following the positively charged N-terminus, (d) a polar C-terminal ending with a cleavage site that can be

Figure 8. General features of a typical bacterial signal peptide. By convention, amino acid residues in the signal peptide and in the mature part of the polypeptide are numbered starting from the cleavage site. Adapted from Pugsley (1989).


Neutral
or
Basic
Lys
Arg
Hydrophobic Core
(α helix)
Gly
Pro
Turn
Cleavage
Site
Met
-22 -21 -20 -19 -18 -17 -16 -15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 +1 +2
. . . COOH
Neutral
or
Acidic
Ala-X-Ala
Gly Gly
Ser Ser
Ile
Leu
Val
"-3, -1 Box"

recognized by signal peptidase (Dierstein and Wickner, 1985) after translocation has occurred, and (e) in most signal peptides, a turn-inducing amino acid (e.g., proline or glycine) next to the central core region.

The envelope glycoprotein of HIV-1 is an integral membrane protein that has been shown to be involved in binding to the cellular receptor (CD4) on T helper lymphocytes and facilitating viral entry into the cell (Maddon et al. 1986; McDougal et al. 1986). Examination of the HIV-1 envelope glycoprotein sequence reveals that it contains a novel signal sequence; there is an unusually long hydrophobic domain at the N-terminus of the protein which is preceded by a highly charged region (Ratner et al. 1985). Although the exact nature of the translocation complex through which the extracellular domains of membrane proteins and secreted proteins are moved into the lumen of the rough endoplasmic reticulum (RER) is not known, recent experimental evidence has provided support for the earlier speculation (von Heijne and Blomberg, 1979; Inouye and Halegoua, 1980; Engelman and Steitz, 1981) that the nascent polypeptide chain of type I and type II proteins is inserted into the ER membrane by a common mechanism involving a hairpin loop structure (Shaw et al. 1988). HIV-1 env protein has been expressed in *E. coli* (Crowl et al. 1985; Putney, 1986), yeast (Barr et al. 1987; Haigwood et al. 1990), mammalian (Chakrabari et al.1986; Hu et al. 1986; Lasky et al. 1986; Dewar et al. 1987; Culp et al. 1991), and insect cells (Hu et al. 1987; Rusche et al. 1987;

Morikawa et al. 1990a; Wells and Compans, 1990) and its biosynthesis, processing and biological activities have been studied in these systems. However, these studies have shown that the level of expression of the protein appears to be very low and has led some researchers to speculate that the signal sequence of HIV-1 might account for the poor expression (Lasky et al. 1986). There is little direct experimental evidence regarding the role of the natural signal sequence in biosynthesis of the gp120 and how it might affect expression and secretion. Therefore, the first objective of this project was to study the role of the natural signal sequence of the HIV-1 envelope glycoprotein in expression and secretion by comparing the fate of proteins containing the HIV-1 glycoprotein signal sequence and heterologous signal sequences. If the HIV-1 signal sequence does limit expression and secretion of gp120, a second objective was to examine how and where expression and secretion of the protein is down-regulated.

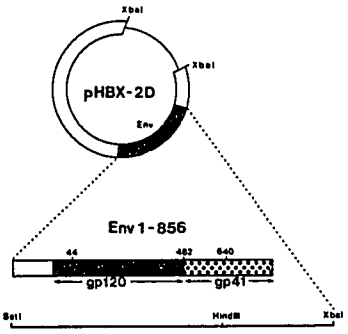
To address these questions, several independent HIV-1 gp120 gene constructs were designed: gp120 with its natural signal sequence (gp120-NSS); gp120 lacking the natural signal sequence(gp120-ΔS); and gp120 with signal sequences from honeybee melittin or murine interleukin 3 (gp120-MSS and gp120-IL3SS, respectively). The gp120 constructs were expressed in SF9 insect cells using recombinant baculoviruses and were used as models to study gp120 synthesis, processing, and transport through the secretory pathway of these cells.

RESULTS

1. Construction of a full length HIV-1 gp120 gene

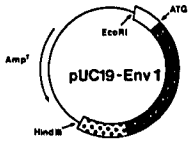
To construct a full length HIV-1 gp120 gene with the natural signal sequence, the plasmid pHXB-2D containing the entire HIV-1 genome was used as a source of the HIV-1 envelope glycoprotein coding sequence (Fisher et al. 1986b). As shown in Fig. 9, a 2.1 Kb SstI-HindIII gene fragment was isolated, treated with the Klenow fragment of DNA polymerase I and T4 DNA polymerase, and subcloned into the HincII site of pUC19, resulting in recombinant plasmid, pUC19-env1. The clone pUC19-env1 which contains approximately 220 bp upstream and 387 bp downstream of noncoding sequences would not be suitable for efficient expression of the 1533 nucleotide long env protein in the baculovirus system. Therefore, crossover linker mutagenesis, a rapid and elegant method for deletion of specific regions of DNA, was used to eliminate these non-translated regions (Sung et al. 1986; Luo et al. 1990). pUC19-env1 was digested with EcoRI and XbaI, and ligated with a double-stranded synthetic oligonucleotide crossover linker (YL1-YL2, see APPENDIX) containing an EcoRI sticky end, a BamHI site, a putative SF9 cell ribosome binding site (CCTATAAAT), and a translation initiation codon ATG, followed by 12 additional nucleotides of "homology searching sequences" that overlap with the N-terminus of the env gene. The ligation mixture was used to transform *E. coli* and a recombinant plasmid (pUC19-env2) was isolated (Fig. 9). pUC19-env2 was further modified to delete the remaining gp41 envelope coding

Figure 9. Strategy for construction of a full length HIV-1 gp120 gene by crossover linker mutagenesis. The black area corresponds to the HIV-1 gp120 coding sequences and the dotted area represents the sequence of gp41. The hatched and small dotted areas in the recombinant plasmid pAcgp120-NSS represent the polyhedrin promoter(pp) and polyhedrin gene sequence, respectively.

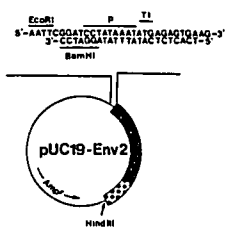


Cut with SstI & HindIII

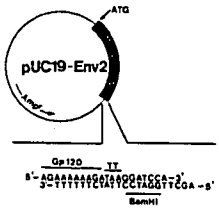
Filled with klenow & T4 DNA polymerase
Insert into HincII site of pUC19



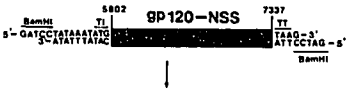
Synthetic linker ligation
& transformation



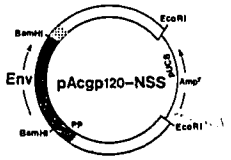
Cut with HindIII & dephosphorylation
Crossover linker ligation & transformation



Isolation of BamHI fragment



Insert into pAcYM1

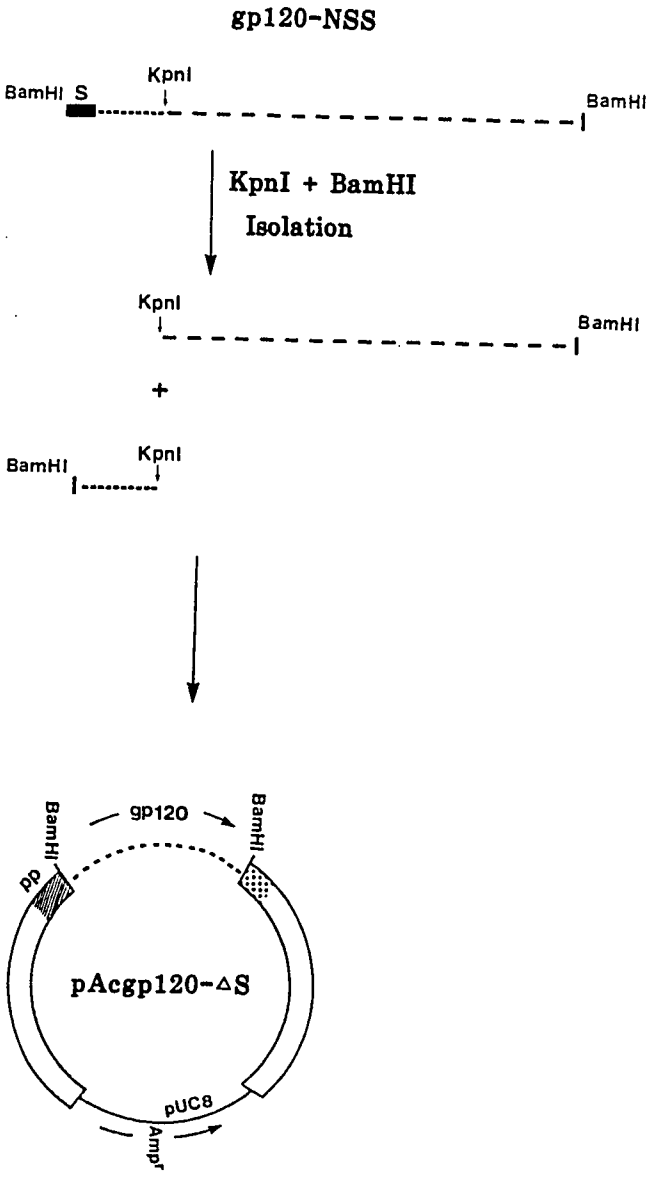


sequence by introducing an in-frame stop codon directly following the codon for arginine at position 518 of the HIV-1 env amino acid sequence [Ratner et al. (1985) numbering system]. Arginine 518 is the C-terminal residue in gp120 after cleavage of gp160 to gp120 and gp41 (McCune et al. 1988). pUC19-env2 was digested with PstI and HindIII and ligated with another double-stranded crossover linker (YL3-YL4; see APPENDIX) containing a HindIII sticky end, a BamHI site, a translation termination codon, and 12 nucleotides of "homology searching sequence" that recognize the C-terminal residues in gp120. After transformation and selection, the resulting recombinant plasmid (pUC19gp120-NSS) containing the putative ribosome binding site followed by the gp120 open reading frame; starting with the ATG (nucleotides 5802-5804) at the beginning of the natural signal sequence and ending with the translation termination codon TAA (nucleotide 7335-7337). The gp120-NSS cassette, which was flanked by BamHI sites was isolated and inserted into the BamHI site of the baculovirus transfer vector, pAcYM1, resulting in the recombinant plasmid pAcgp120-NSS (Fig. 9).

2. Construction of signal peptide deletion mutant, gp120-ΔS

To generate gp120 without the N-terminal 30 amino acids of the natural env signal sequence, a 1.4 kb KpnI-BamHI fragment from pUC19gp120-NSS was isolated (Fig. 10) and ligated with: (1) a synthetic double-stranded oligonucleotide linker (YL5-YL6, see APPENDIX) containing a BamHI sticky end, a putative SF9 ribosome binding site, and a translation initiation codon, followed by coding

Figure 10. Strategy for construction of the signal deletion mutant, gp120-ΔS. PP is the polyhedrin promoter and the dotted area is the polyhedrin gene sequence. The short dashed line represents the synthetic oligonucleotide linker containing the coding sequence of the mature env protein (amino acid residues Thr at position 31 to Val at position 42) and the long dashed line represents a KpnI-BamHI fragment isolated from the full length gp120 gene.


gp120-NSS
BamHI
S
KpnI
BamHI
KpnI + BamHI
Isolation
KpnI
BamHI
+
BamHI
KpnI
BamHI
gp120
BamHI
pp
pAcgp120-ΔS
pUC8
Ampr

sequences of mature env protein (starting with the threonine at position 31 and ending with a KpnI sequence encoding the valine at position 42) and (2) a linearlized pAcYM1 transfer vector after BamHI digestion, resulting in the recombinant plasmid pAcgp120-ΔS (Fig. 10).

3. Construction of an HIV-1 gp120 gene containing melittin or interleukin 3 signal sequences

To produce gp120 fused with the melittin signal sequence of *Apis melifica*, a 1.4 kb BamHI fragment from pAcgp120-ΔS was inserted into the BamHI site of the baculovirus secretion vector PVT-Bac (Tessier et al. 1990) (Fig. 11), which contains the melittin signal sequence under the control of the polyhedrin promoter, creating pAcgp120-MSS (Fig. 11). To generate a gp120 gene containing the murine interleukin 3 signal sequence, a modified pAcYM1 expression vector, pAcHK1 (Fig. 12), was digested with PstI and BamHI and ligated with a double-stranded synthetic oligonucleotide linker (JMC6-JMC7; see APPENDIX) containing a PstI sticky end, a putative SF9 cell ribosome binding site, and a translation initiation codon followed by IL3 signal coding sequence [from amino acid position -18 (Met) of the IL3 signal sequence to amino acid position 3 (Asp) of the mature IL-3 protein, and ending with a BamHI sticky end] (Fig. 12; Yokota et al. 1984). The resulting baculovirus secretion vector is designated pAcIL3 (Fig. 12). The 1.4 kb BamHI fragment of gp120-ΔS was then inserted into the BamHI site of pAcIL3, resulting in the recombinant plasmid pAcgp120-IL3SS (Fig. 12).

Figure 11. Strategy for construction of an HIV-1 gp120 gene containing the melittin signal sequence. The hatched and dotted areas correspond to the polyhedrin promoter and the polyhedrin gene sequences respectively. The black area represents the melittin signal sequence and the dashed line represents the gp120 gene without the signal sequence.

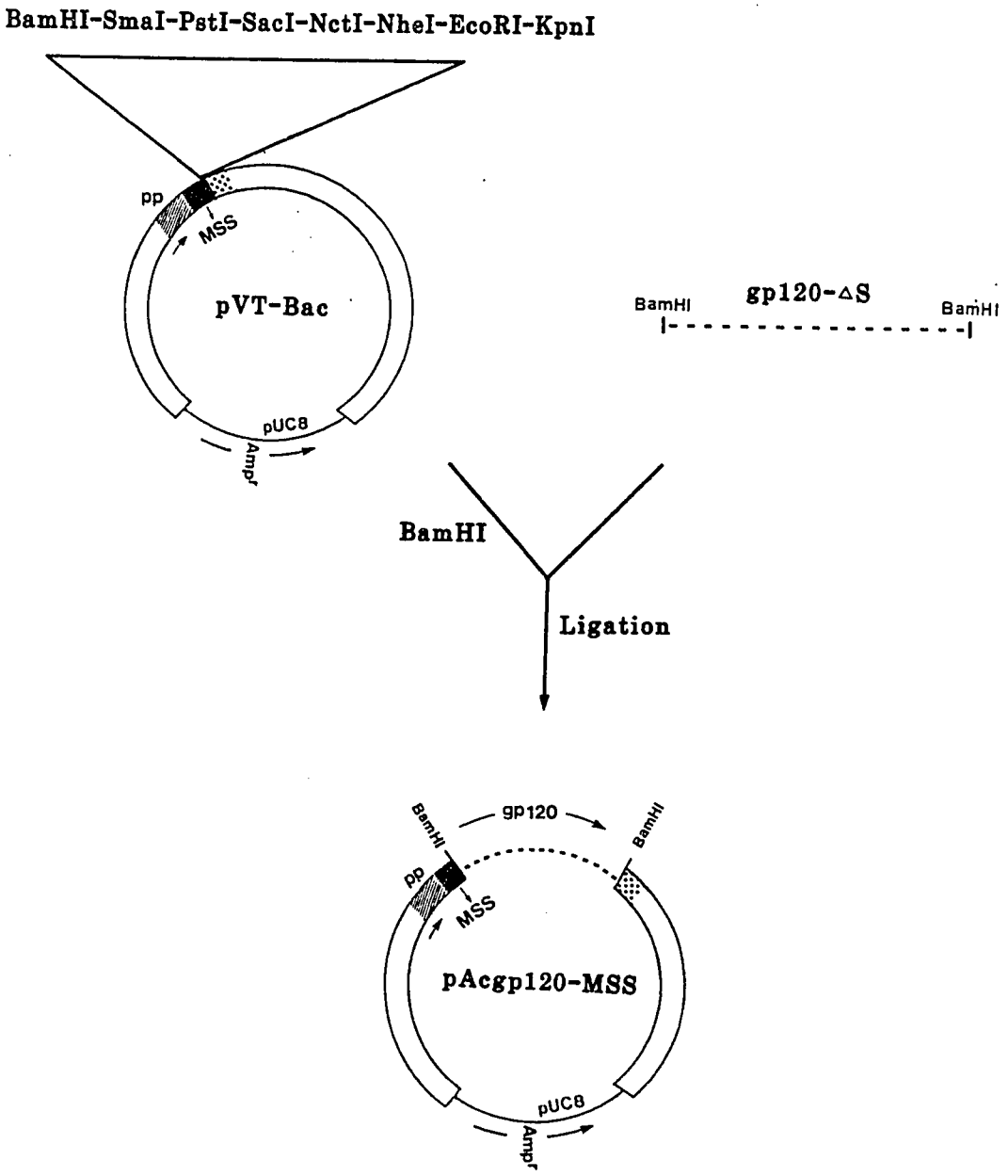
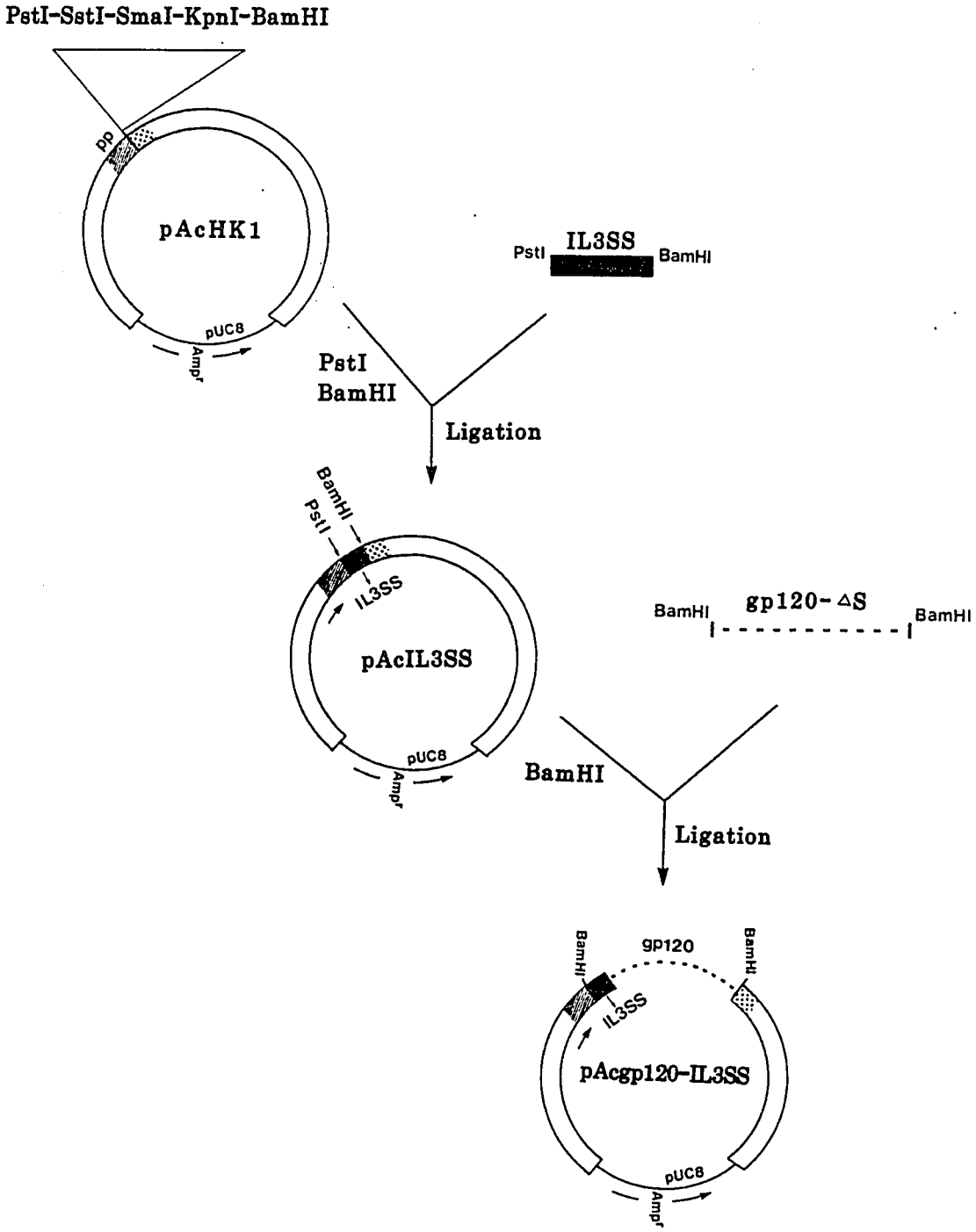

BamHI-SmaI-PstI-SacI-NctI-NheI-EcoRI-KpnI
pp
MSS
pVT-Bac
pUC8
Ampr
BamHI
gp120-ΔS
BamHI
BamHI
Ligation
BamHI
gp120
BamHI
pp
MSS
pAcgp120-MSS
pUC8
Ampr

Figure 12. Strategy for construction of an HIV-1 gp120 gene containing the interleukin 3 signal sequence. The hatched and dotted areas correspond to the polyhedrin promoter and the polyhedrin gene sequences respectively. The black bar represents the synthetic oligonucleotide linker coding the murine interleukin 3 signal sequence and the dashed line represents the gp120 gene without the signal sequence.


PstI-SstI-SmaI-KpnI-BamHI
pp
pAcHK1
pUC8
Ampr
PstI
IL3SS
BamHI
PstI
BamHI
Ligation
BamHI
PstI
IL3SS
pAcIL3SS
pUC8
Ampr
BamHI
gp120-ΔS
BamHI
BamHI
Ligation
BamHI
gp120
BamHI
IL3SS
pAcgp120-IL3SS
pUC8
Ampr

The nucleotide sequences at both ends of all of the gp120 gene constructs described above were confirmed by sequence analysis.

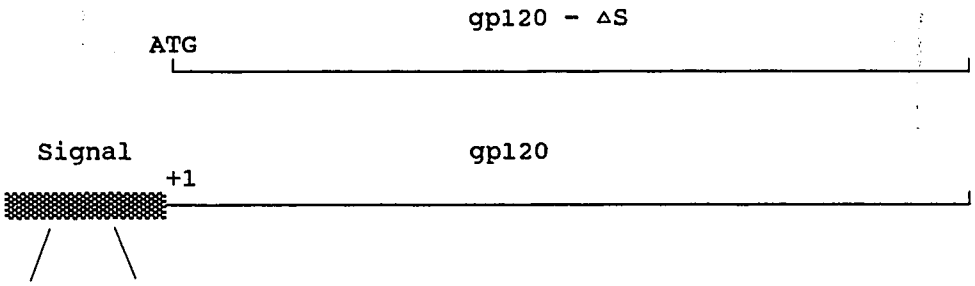
4. Expression of HIV-1 gp120 constructs by SF9 cells

To produce recombinant baculoviruses, SF9 cells were cotransfected with a mixture of wild type AcNPV viral DNA and each recombinant plasmid (Chapter 2). Three hours after the beginning of cotransfection, the inoculum was aspirated. Fresh TNM-FH medium (2 ml) was added to the transfected cells and incubation was continued at 27°C for 4 days. During the incubation period, the rare event of homologous recombination between the polyhedrin gene sequences on the plasmid and the baculovirus genome is expected to occur. As a result, the gp120 gene becomes part of a recombinant baculovirus genome which will be packaged into virions able to express the gp120 protein. The supernatant of cotransfected cells was collected and virus was plated on monolayers of SF9 cells to select each recombinant baculovirus. Recombinant plaques were picked, plaque purified 3 times and amplified for production of recombinant baculovirus stocks.

The resulting recombinant baculoviruses were designated vAcg120-NSS, vAcgp120-ΔS, vAcgp120-MSS, and vAcgp120-IL3SS as shown in Fig. 13. It should be noted that cleavage of the melittin or interleukin 3 signal sequence from gp120-ΔS leaves five and seven amino acids, respectively, preceding amino acid residue 1 (Thr) of mature gp120 (Fig. 13).

To examine the synthesis of gp120 proteins in insect cells by infection with

Figure 13. Schematic diagram of wild type HIV-1 gp120 and signal-deletion or signal-fusion mutant proteins. The natural signal sequence and melittin or IL-3 signal sequences are shown using the one-letter amino acid code. The site of cleavage between the signal sequence and the mature N-terminus of gp120 (+1) is indicated by an arrow. The underlined sequence is the result of linker insertion and indicates the five or seven amino acids between the signal sequence and the mature gp120 protein. Also depicted is the construct encoding truncated gp120 (gp120-ΔS) in which 30 amino acids of the natural signal sequence are deleted and a translation initiation codon (ATG) is added in frame.



1. HIV-1 Signal Peptide :

```
  +   + - +           +           +                                   +1
M R V K E K Y Q H L W R W G W R W G T M L L G M L M I C S A ▼T
```

2. Melittin Signal Peptide :

```
  +                                                 -           +1
M K F L V N V A L V F M V V Y I S Y I Y A ▼D P I N M T
```

3. Interleukin 3 Signal Peptide :

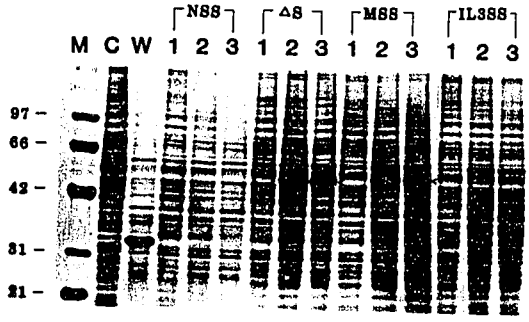
```
                                          + -         +1
M L L L L L M L F H L G L Q A S I S ▼G R D P I N M T
```

each of recombinant baculoviruses and to demonstrate immunoreactivity, time course experiments were performed from day 1 to day 3 after infection. SF9 cells were infected with wild type or recombinant virus and cells were harvested at different time intervals and washed twice with cold PBS. Whole lysates from uninfected, wild type-infected, and recombinant baculovirus-infected cells were analyzed by SDS-PAGE. A strongly stained protein band migrating at about 53 KDa was observed in the lysate of SF9 cells infected with vAcgp120-ΔS and vAcgp120-MSS at days 2 and 3 p.i. but there were no corresponding protein bands found in either vAcgp120-NSS or vAcgp120-IL3SS-infected cells (Fig. 14A, indicated by arrow). The 53 KDa proteins represent nonglycosylated forms of gp120 as predicted from the primary amino acid sequences of gp120-ΔS and gp120-MSS. Western blot analysis confirmed the identity of these proteins as HIV-1 envelope protein and also revealed a higher molecular weight form of the protein in vAcgp120-NSS, vAcgp120-MSS, and vAcgp120-IL3SS-infected cells at day 2 and 3 but not in vAcgp120-ΔS infected cells (Fig. 14B). The upper band, in each case, migrated close to the expected molecular weight for glycosylated gp120. The two major bands (nonglycosylated and glycosylated) were recognized not only by rabbit serum raised against the recombinant gp120 (see Chapter 2) but also by serum from HIV-1 positive patients (see Fig. 15).

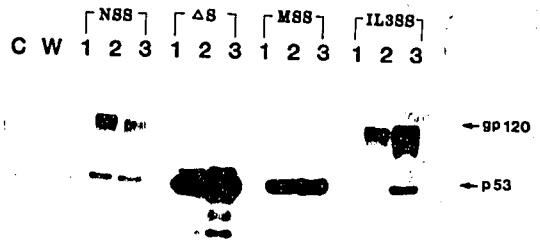
It was noted, however, that the amount of glycosylated gp120 was decreased in vAcgp120-NSS-infected cells by day 3 but increased in vAcgp120-IL3SS-infected cells. The amounts of gp120 in vAcgp120-MSS-infected cells was relatively constant.

Figure 14. Time course of HIV-1 gp120 expression in recombinant baculovirus-infected SF9 cells. Cell were either infected with wild type AcNPV (wt) or with recombinant viruses at a moi of 5 pfu/cell for 1 hr and incubated for the times (days) indicated above each lane. After incubation, whole cell lysates were analyzed by SDS-PAGE and Western blot as described in Chapter 2. (A) SDS-polyacrylamide gel stained with Coomassie blue showing whole cell lysates from uninfected cells (C), wild type AcNPV infected cells (W), vAcgp120-NSS infected cells (NSS), vAcgp120-ΔS infected cell (ΔS), vAcgp120-MSS infected cells (MSS), and vAcgp120-IL-3SS infected cells (IL-3SS). (B) Western blot analysis of the same lysates as shown in A. The blot was probed with rabbit anti-gp120 serum. Lane M shows a set of protein markers (Bio-Rad) the sizes of which are shown in kilodaltons (KDa). Location of the glycosylated gp120 and nonglycosylated (p53) forms of gp120 are indicated. All experimental results presented in this study were repeated at least twice.

A.


NSS
ΔS
MSS
IL3SS
M C W 1 2 3 1 2 3 1 2 3 1 2 3
97 –
66 –
42 –
31 –
21 –

B.


NSS
ΔS
MSS
IL3SS
C W 1 2 3 1 2 3 1 2 3 1 2 3
gp120
p53

The nonglycosylated form of gp120 was also detected in both vAcgp120-NSS and vAcgp120-IL3SS-infected cells and several smaller immunoreactive bands were found that probably are degraded products of the nonglycosylated form of gp120 (Fig. 14B). Several reports have suggested that the nonglycosylated form of a protein is more sensitive to protease than its glycosylated form (Olden et al. 1978; Schwartz et al. 1976). By comparison with the known amounts of standard protein markers, the total amount each of the nonglycosylated forms of gp120 produced by vAcgp120-ΔS and vAcgp120-MSS was estimated at 100-150 mg/1.0 x 10^8 cells/L.

These results clearly demonstrate that removal of the natural signal sequence or replacement of the natural signal sequence of gp120 with the melittin signal sequence or IL3 signal sequence resulted in dramatically increased production of either nonglycosylated or glycosylated forms of gp120.

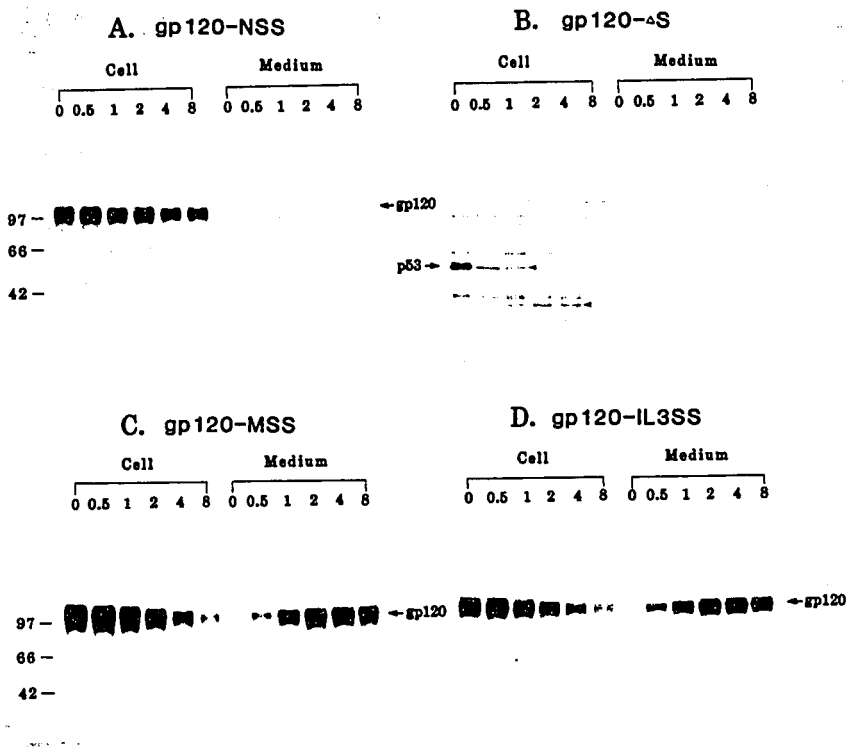
5. Kinetics of glycosylation and secretion of gp120 by SF9 cells

To examine if gp120 was secreted and to investigate the effects of substituting the natural gp120 signal sequence with either the melittin or interleukin 3 signal sequences on the expression and secretion of the protein, pulse-chase experiments were carried out to evaluate the kinetics with which the various gp120 molecules entered and exited the secretory pathway in SF9 cells. Recombinant baculovirus-infected cell cultures were pulse-labelled with [^{35}S]- methionine for 1 hr at 24 hr p.i. and chased for varying periods of time. Cell lysates and culture supernatants were separately immunoprecipitated with human HIV-1 anti-serum (Prince et al.

1988) and analysed by SDS-PAGE. Surprisingly, secretion of gp120 with its natural signal sequence (vAcgp120-NSS) was extremely slow. Secretion was first detected after 2 hr and increased slightly after 8 hr of chase. By 8 hr of chase, only about 10% of gp120 was secreted into the medium (Fig. 15A). Little or no additional secretion occurred after 24 hr of chase (data not shown). In contrast, gp120 with either the melittin or IL3 signal sequences (gp120-MSS and gp120-IL3SS) was rapidly glycosylated and secreted in significantly higher amounts than gp120-NSS (Fig. 15C and D). After 1.5 hr, the gp120 was distributed almost equally between the medium and cells. By 8 hr, more than 80% of either gp120-MSS or gp120-IL3SS had been secreted into culture medium. These results indicate that the kinetics of secretion of gp120 with the melittin or IL3 signal sequences from SF9 cells were nearly identical; both exhibited a half time of approximately 1.5 hr (Fig. 15C and D).

These results clearly demonstrate that recognition of the melittin or IL3 signal sequence occurs with high efficiency in SF9 cells, followed by rapid translocation across the membrane of the rough endoplasmic reticulum (RER) and N glycosylation, probably in a cotranslational fashion as previously described (Jarvis and Summers, 1989). With increasing time of chase, however, there was a clear decrease in the size of intracellular gp120 (Fig. 15A, C, and D). These results indicate that the newly synthesized cell-associated gp120 has undergone posttranslational cleavage of carbohydrates, most likely trimming of saccharide

Figure 15. Pulse-chase analysis of gp120 biosynthesis and secretion. SF9 cells were infected at a moi of 5 pfu/cell. At 24 hr p.i., cells were pulse-labelled for 1 hr with 250 μCi of [^{35}S]-methionine, and chased for the times (hr) indicated above each lane. gp120 was immunoprecipitated from cell lysates or culture supernatants with HIV-1 positive human serum and analyzed by SDS-PAGE. gp120-ΔS was immunoprecipitated with rabbit anti-gp120 serum. The numbers on the left represent molecular mass (in thousands) of marker proteins on each gel.


A. gp120-NSS
Cell
Medium
0 0.5 1 2 4 8
0 0.5 1 2 4 8
B. gp120-ΔS
Cell
Medium
0 0.5 1 2 4 8
0 0.5 1 2 4 8
97
66
42
gp120
p53
C. gp120-MSS
Cell
Medium
0 0.5 1 2 4 8
0 0.5 1 2 4 8
D. gp120-IL3SS
Cell
Medium
0 0.5 1 2 4 8
0 0.5 1 2 4 8
97
66
42
gp120
gp120

from high mannose N-linked oligosaccharide chains by exoglycosidase, which was also observed in both mammalian and insect system expressing HIV-1 env protein (Earl et al. 1991; Willey et al. 1991; Wells and Compans, 1990). An increase in electrophoretic mobility was not detected once the protein was released from cells, suggesting that further trimming of the oligosaccharide residues does not occur extracellularly.

In contrast, deletion of the signal sequence of gp120 (gp120-ΔS) resulted in production of a 53-KDa nonglycosylated form of gp120 (p53) which was exclusively immunoprecipitated from the intracellular fraction but not from the extracellular fraction (Fig. 15B). This clearly indicates that removal of the signal sequence from gp120 completely blocked gp120 access to the secretory pathway and thus the subsequent intracellular processing. Therefore, p53 was not glycosylated and not secreted. Some minor proteins, gradually increasing in size, were immunoprecipitated from the medium with rabbit anti-gp120 serum. The origin of these proteins is not yet known.

It was noted that substantial amounts of the nonglycosylated p53 protein of gp120 were initially immunoprecipitated from the NP40 soluble fraction but the amounts rapidly declined with time of chase (Fig. 15B). Therefore, the subcellular localization of the nonglycosylated form of gp120 was examined. The intracellular and insoluble fractions, as described by Jarvis and Summers (1989), were prepared from recombinant vAcgp120-NSS, vAcgp120-ΔS, and vAcgp120-MSS virus infected SF9 cells labelled with [^{35}S]-methionine and immunoprecipitated with rabbit anti-

gp120 serum. Immune complexes were analyzed by SDS-PAGE. As shown in Fig. 16, approximately equal amounts of newly synthesized gp120-ΔS were detected in intracellular and in insoluble fractions, whereas most of the nonglycosylated form of gp120-MSS was initially precipitated from the intracellular fraction. With increased time of chase, however, the amounts of the nonglycosylated forms of gp120 rapidly declined and these proteins were no longer found in the intracellular fraction. Instead, several small polypeptides largely in the insoluble fraction were specifically immunoprecipitated (Fig. 16). This indicates that the nonglycosylated gp120 is not secreted from SF9 cells; instead, it appears to be associated with an insoluble subcellular compartment and degraded rapidly. These findings, which are consistent with the observations of Jarvis and Summers (1989), suggest that N-glycosylation in SF9 cells is required directly or indirectly for the stabilization and secretion of gp120.

These data indicate that the natural signal sequence of HIV-1 gp120 is responsible for poor expression and secretion. This was further supported by examining the morphology of recombinant baculovirus-infected insect cells. As shown in Fig. 17, when SF9 cells were infected by recombinant baculovirus vAcgp120-NSS, the infected cells were completely lysed by day 3. In contrast, 70%-90% of SF9 cells infected with vAcgp120-MSS, vAc-gp120-IL3SS or vAcgp120-ΔS were still intact at day 3. This suggests that the natural signal sequence of HIV-1 gp120 may in some unknown way have a lethal effect on the cells.

In light of the apparent rapidity of translocation and glycosylation of

Figure 16. Specific immunodetection of gp120 in recombinant baculovirus-infected SF9 cells. Recombinant vAcgp120-NSS-, vAcgp120-ΔS-, and vAcgp120-MSS virus infected SF9 cells were pulse-labelled with [^{35}S]-methionine for 1 hr and chased for different times (Hours). Intracellular (IC) and insoluble (IN) fractions were prepared as described in Materials and Methods and proteins from each fraction were immunoprecipitated with rabbit anti-gp120 serum. Immune complexes were analyzed by SDS-PAGE.

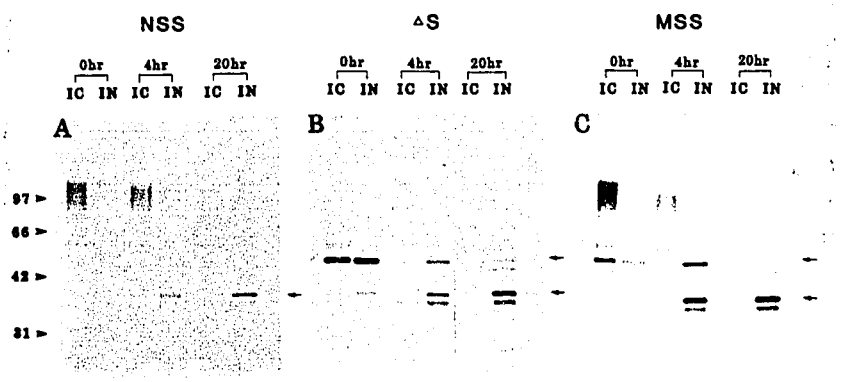
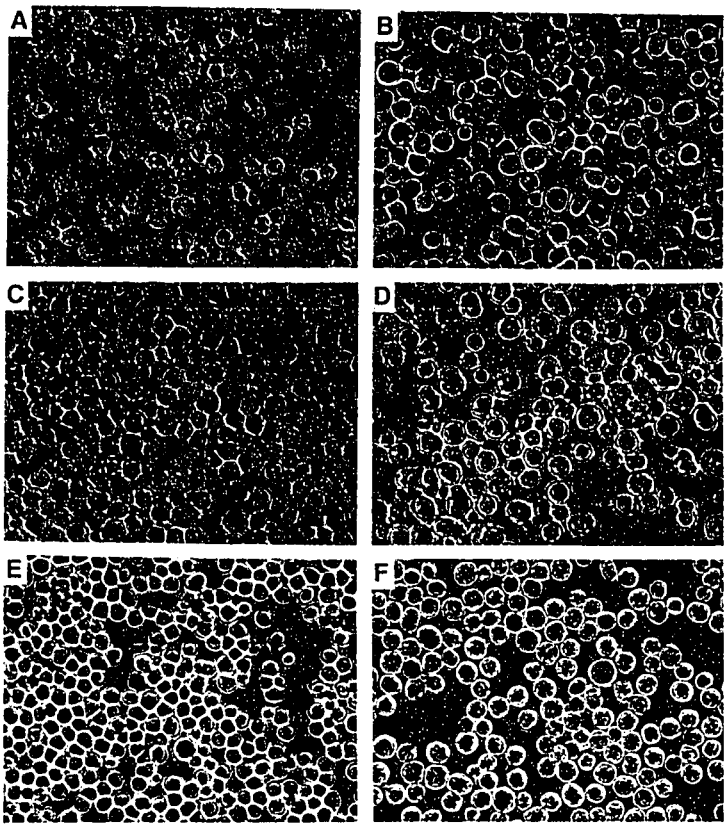

NSS
ΔS
MSS
0hr
4hr
20hr
IC IN IC IN IC IN
A
B
C
97 ►
66 ►
42 ►
31 ►

Figure 17. A phase-contrast view of recombinant baculovirus-infected cells. SF9 cells were infected with recombinant virus at a moi of 5 pfu/cell for 1 hr and incubated at 27°C for 72 hr. The infected cells were examined by phase-contrast microscopy. A. vAcgp120-NSS infected cells, B. vAcgp120-ΔS infected cells, C. vAcgp120-MSS infected cells, D. vAcgp120-IL-3SS infected cells, E. wild type AcNPV infected cells, F. uninfected control cells.


A
B
C
D
E
F

intracellular gp120-NSS (Fig. 15A), this result suggests that a rate-limiting step(s) in the secretion of gp120-NSS occurs later, during the subsequent processing of the polypeptide and its movement out of SF9 cells.

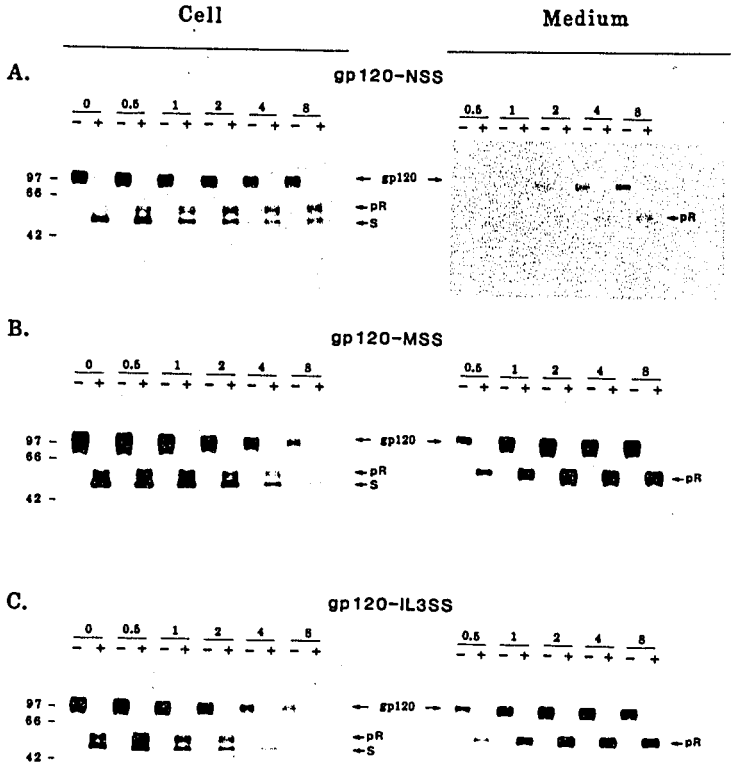
6. Acquisition of endo H resistance by gp120

HIV-1 gp120 is heavily glycosylated. Since carbohydrate modifications occur in specific subcellular compartments, intracellular transport of gp120 can be followed indirectly by monitoring changes in carbohydrate structure, using N-acetyl-β-endoglycosidase H (endo H). This enzyme cleaves high-mannose oligosaccharides added in the ER, but it does not cleave oligosaccharides which have been modified by the Golgi enzymes N-acetylglucosamine transferase and mannosidase II (Kornfeld and Kornfeld, 1985). The acquisition of endo H resistance by glycoproteins marks their arrival at the medial Golgi compartment. Therefore, the rate at which a synthesized molecule becomes resistant to the action of endo H is a measure of the rate at which it is transported from the ER to the Golgi. However, such an approach is valid only if certain precautions are taken as suggested by Yeo et al. (1985). For example, one must remember that significant amounts of protein may have already been secreted by the time that 50% of the total intracellular fraction becomes endo H resistant. Therefore, the endo H resistant extracellular fraction, which may be very large in some cases, must be included in the calculation of the time required for conversion of half of a secreted glycoprotein to an endo H resistant form.

To further characterize the effect of the different signal peptides on the intracellular transport of gp120, recombinant vAcgp120-NSS, vAcgp120-MSS, and vAcgp120-IL3SS virus infected SF9 cells were pulse-labelled with [^{35}S]-methionine for 1 hr at 24 hr p.i. The label was chased with nonradioactive methionine, and at intervals gp120 in cell lysates and medium was analyzed as described in Chapter 2. A time course of the acquisition of endo H resistance by gp120-NSS, gp120-MSS, and gp120-IL3SS is shown in Fig. 18. Newly synthesized gp120-NSS was found to be sensitive to endo H as indicated by a shift in the molecular mass from 120 KDa to 58 KDa. However, a small proportion of gp120 acquired partial endo H resistance within 0.5 hr as indicated by the presence of an intermediate band of approximately 61 KDa. After 2 hr of chase, about 50% of pulse-labelled gp120 (intracellular plus secreted) acquired partial endo H resistance ($t_{½}$ = 2 hr) and correspondingly, at this time point, only a very small amount of the partially endo H resistant form of gp120 was detected in the medium. By 8 hr of chase, approximately 70% of the molecules were partially endo H resistant. The persistence of fully endo H sensitive forms of gp120 during the entire chase time period suggests that a fraction of gp120 does not leave the ER (Wells and Compans, 1990; Willey et al. 1991).

In contrast, gp120-MSS and gp120-IL3SS exhibited markedly different kinetics of acquiring endo H resistance (Fig. 18B and C). Immediately after pulse-labelling, nearly 20% of cell-associated gp120-MSS and gp120-IL3SS was already partially endo H resistant. By 0.5 hr, 50% of gp120-MSS and gp120-IL3SS (intracellular plus

Figure 18. Time course of acquisition of endo H resistance by gp120. Recombinant virus-infected SF9 cells were pulse-labelled and processed as described in the legend to Fig. 3. One half of each immunoprecipitate was digested with endo H prior to SDS-PAGE. The plus signs indicate samples treated with endo H, and the minus signs indicate untreated control samples. pR. partially resistant to endo H, S, sensitive to endo H. The film for secreted gp120-NSS was exposed twice as long as the one for intracellular gp120-NSS (panel. A).


Cell
Medium
A.
gp120-NSS
0
0.5
1
2
4
8
97 -
66 -
42 -
gp120
pR
S
B.
gp120-MSS
C.
gp120-IL3SS

secreted) was partially endo H resistant ($t_{½}$ = 0.5 hr), and secreted gp120 was already being detected in the medium. After 8 hr of chase, almost all of the endo H sensitive forms of gp120 had been converted to endo H resistance and secreted into the medium (Fig. 18B and C). The observation that gp120-NSS is much more slowly converted from an endo H sensitive form to an endo H resistant form than are gp120-MSS and gp120-IL3SS, suggests that the gp120-NSS does not enter the medial saccules of the Golgi complex rapidly or efficiently. This would indicate that the intracellular transport of gp120-NSS is impaired at some point along the pathway from the ER to the medial Golgi complex.

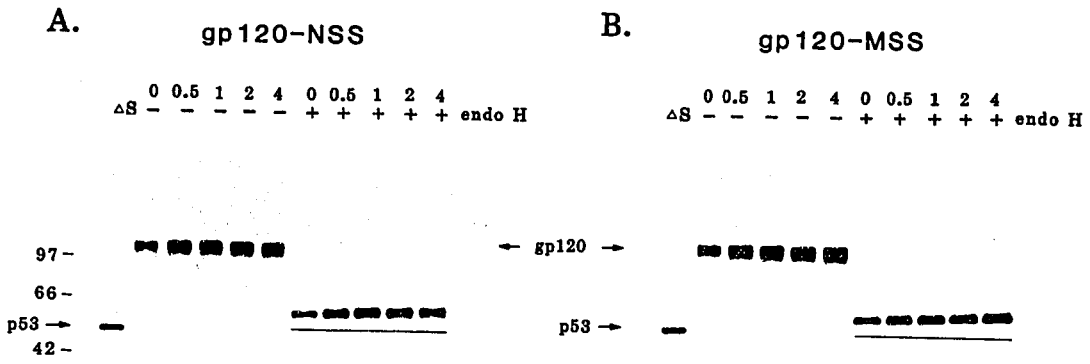
All gp120 molecules secreted into the culture medium appeared to be only partially resistant to endo H (Fig. 18A, B and C) (Earl et al. 1991; Wells and Compans, 1991). This suggests that these molecules have encountered enzymes involved in oligosaccharide processing during their transport through the exocytic pathway and may be a consequence of exoglycosidase trimming of high mannose oligosaccharides to endo H resistant trimannosyl cores or of the formation complex forms (Matthews et al. 1987; Geyer et al. 1988; Fenonillent et al. 1989; Wells and Compans, 1991). Thus, the acquisition of carbohydrate chains which are resistant to endo H digestion appears to be a prerequisite for secretion of gp120.

Lippincott-Schwartz et al.(1989, 1990) and Doms et al.(1989) have recently reported that mannosidase II was redistributed from the medial Golgi cisternae to the ER following treatment with brefeldin A (BFA), an inhibitor of transport between the ER and the Golgi apparatus which ultimately leads to dissolution of the

Golgi cisternae. One of the Golgi modifications that may be occurring is the conversion of the high-mannose N-linked oligosaccharide to complex forms. This can be tested by measuring the endo H sensitivity of the glycoprotein.

To investigate the possibility that the oligosaccharides on gp120 had been converted to complex forms, the oligosaccharide chains of gp120 expressed by baculovirus recombinants in BFA-treated SF9 cells were tested for endo H resistance. BFA (15 μg/ml) was added 2 hr before labelling and it was present throughout the labelling and chase periods. Following lysis, gp120 was immunoprecipitated, and one-half of each sample was digested with endo H. The primary product of gp120 synthesized in the presence of BFA was a 120 KDa, glycosylated protein with a high-mannose oligosaccharide. The electrophoretic mobility of cell-associated gp120 also increased with extended chase intervals (Fig. 19), indicating that high-mannose glycosylation was unaffected by BFA treatment. Consistent with the result described above (Fig. 15 and Fig. 18), the newly synthesized gp120 was sensitive to endo H digestion and gave rise to the expected 58 KDa deglycosylated form of gp120. However, as the chase continued in the presence of BFA, the endo H digestion product was larger than the 58 KDa deglycosylated form and appeared to be a doublet in gp120-NSS but not for gp120-MSS (Fig. 19A and B). These results showed that conversion of high-mannose to complex oligosaccharides occurred on gp120 and the oligosaccharides were no longer completely endo H sensitive.

Figure 19. Pulse-chase analysis of gp120 in BFA-treated SF9 cells. SF9 cells were infected with recombinant baculovirus at a moi of 5 pfu/cell. At 20 hr p.i., BFA was added at a concentration of 15 μg per ml. The cells were pulse-labelled at 23 hr p.i. for 1 hr and chased for the times (hr) indicated across the top of the lanes. Cells were lysed and gp120 was immunoprecipitated with serum from HIV-1 positive patients. Immunoprecipitates were divided in half and incubated in the presence (+) or absence (-) of endo H before analysis by SDS-PAGE. A. vAcgp120-NSS infected cells, B. vAcgp120-MSS infected cells.


A.
gp120-NSS
ΔS 0 0.5 1 2 4 0 0.5 1 2 4 endo H
97
66
p53
42
gp120
B.
gp120-MSS
ΔS 0 0.5 1 2 4 0 0.5 1 2 4 endo H
p53

DISCUSSION

The aim of the experiments described in this section was to study the role of the natural signal sequence of the HIV-1 envelope glycoprotein in expression and secretion and to ascertain if the signal sequence may account for poor expression and secretion of gp120. If so, it would be also be of interest to further examine where intracellular transport of gp120 is blocked.

1. Characterization of HIV-1 gp120 proteins produced in SF9 cells

Removal of the natural signal sequence of gp120 (gp120-ΔS) or replacement of the natural signal sequence with honeybee melittin or murine interleukin 3 signal sequences resulted in dramatically increased production of high molecular weight (about 120 KDa) and low molecular weight (about 53 KDa) protein products (Fig. 14). Western blot analysis further confirmed that both the higher and lower molecular weight versions of each recombinant protein corresponded to glycosylated and nonglycosylated forms of gp120 respectively. The differences in the level of synthesis of glycosylated and nonglycosylated forms of gp120 by the recombinant baculovirus could be explained by cell lysis (Fig. 17) and the different rates of secretion of gp120 (Fig. 15). SF9 cells infected with vAcgp120-NSS were completely lysed by 72 hours p.i., suggesting that the natural signal sequence of the gp120 may have a cytotoxic effect and lead to cell death (Fig. 17A). In contrast, with the melittin signal sequence, which is derived from another insect recognition

the signal sequence presumably occurs with high efficiency in SF9 cells (Fig. 15C) and the synthesis of recombinant protein gp120-MSS increases rapidly. large amounts of the nonglycosylated form of gp120-MSS is accumulated at later times of infection. The accumulation of gp120-MSS may simply saturate the SF9 cell secretory apparatus so that host cell metabolic processes may have shut down and the components needed for glycosylation have become limited.

2. Signal sequence is necessary for the translocation of gp120

The current form of the signal hypothesis (Briggs and Gierasch, 1985; Walter and Lingappa, 1986) states that the translocation of secretory proteins in higher eukaryotic cells should proceed in a cotranslational manner involving the sequential action of the signal recognition particle (Walter and Blobel, 1982) and docking protein (Meyer et al. 1982). According to this hypothesis, the signal peptide is absolutely necessary for the initiation of translocation. Thus, secretion should not occur in the absence of a functional signal sequence. However, only a few reports have analyzed the consequences of complete deletion of a signal peptide by examining either in vitro translocation or in vivo secretion of a protein (Kadonoga et al. 1984; Kaiser and Botstein, 1986; Townsend et al. 1986; Wiedman et al. 1986). The signal deletion mutant gp120-ΔS clearly showed that removal of the signal sequence of HIV-1 gp120 caused a complete block for entry of gp120 into the secretory pathway and subsequent intracellular processing, resulting in large quantities of a nonglycosylated and nonsecreted protein (p53) (Fig. 14 and

15B). This result further strengthens the signal hypothesis. The signal sequence is essential for initiation of the first step of the secretory pathway: translocation of protein into the endoplasmic reticulum where the protein receives N-linked core oligosaccharides (Blobel and Dobberstein, 1975; Briggs and Giersch, 1985; Walter and Lingappa, 1986). In addition, the fact that the cells infected with vAcgp120-ΔS are not lysed also supports the idea that the natural signal sequence is toxic for some reason.

The nonglycosylated protein was unstable and underwent rapid proteolytic degradation with time (Fig. 14B and 15B). Such degradation has been described for secreted proteins that are unable to move along or enter the exocytic pathway as a result of severe truncation (Doyle et al. 1986) or complete deletion of the signal peptide (Townsend et al. 1986). However, there were no degraded products found in vAcgp120-NSS-, vAcgp120-MSS-, and vAcgp120-IL3SS-infected cells (Fig. 15A, C, and D), suggesting that oligosaccharides protect the polypeptide from proteolytic degradation and, therefore, that the glycosylation is required for stabilization of the protein (Olden et al. 1978).

In addition, it was found that the nonglycosylated gp120 backbone was present in large quantities after 48-72 hours infection but with an altered intracellular distribution; it became largely insoluble in nonionic detergent (NP40) (Fig. 16). Pulse-chase experiments further showed that several small polypeptides were specifically immunoprecipitated from the NP40-insoluble fraction (Fig. 16). This is different from other reports that deletion of the signal sequence resulted in

production of cytoplasmic, nonglycosylated forms of proteins (Gething and Sambrook, 1982; Sekikawa and Lai, 1983). Thus, the abnormal intracellular distribution may reflect the structural characteristics of baculovirus or insect cells, since similar results have been observed when the HIV-1 pol gene was expressed in insect cells (Hu and Kang, 1991) and when human t-PA was expressed in tunicamycin treated SF9 cells (Jarvis and Summers, 1989).

3. The signal sequence of HIV-1 gp120 is responsible for poor expression and secretion of gp120

Although it is not clear if any features of signal peptides other than their hydrophobicity are recognized by the eukaryotic cell machinery, it has been observed in other system that different signal peptides give different levels of secretion (Kaiser and Botstein, 1990). Consistent with this observation and the results shown in Fig. 14, gp120 with its own natural signal sequence exhibited extremely low levels of secretion, whereas fusion of melittin or interleukin 3 signal sequences with gp120 (gp120-ΔS) resulted in high levels of expression and secretion of glycosylated gp120 (Fig. 15A, C and D). The same protein containing different signal sequences exhibited very different secretion rates ($t_{½}$ > 8 hr for gp120-NSS versus $t_{½}$ = 1.5 hr for gp120-MSS and gp120-IL3SS), which indicates that the formation of the native structure of the gp120 glycoprotein may not be responsible for the secretion-incompetence of the protein. Rather, the nature of the signal sequence appears to be a key factor determining whether gp120 can be

secreted efficiently.

The extremely slow rate of the secretion of gp120-NSS could be explained as being due to inefficient recognition of the natural signal sequence in insect cells. However, a nonglycosylated gp120 precursor was not detected in vAcgp120-NSS-infected cells, even when the cells were labelled for a very short time (see Fig. 23) and when rabbit antiserum, directed against denatured, nonglycosylated recombinant gp120 protein, was used for immunoprecipitation (see Fig. 23). This indicates that the natural signal sequence of HIV-1 gp120 was efficiently recognized in SF9 cells and that one result of this was the rapid translocation of the nascent polypeptide across the rough ER membrane where it became immediately available for N-linked glycosylation (Fig. 15A) (Jarvis and Summers, 1989). Thus, a rate-limiting step in the secretion of gp120 from SF9 cells probably occurs after translocation and N glycosylation. The site at which the secretion lag is most prominent seems to be transit from the RER to the Golgi.

4. <u>The limiting step in transport of gp120 is from RER to Golgi</u>

Previous studies have shown that many integral plasma membrane and viral surface glycoproteins mature at the cell surface at different rates; the distinctive and limiting step is exit from the RER (Fitting and Kabat, 1982; Schauer et al; William et al. 1985), whereas transport through the Golgi stack and to the cell surface occurs at essentially the same rate for all secretory and plasma membrane proteins in a given cell (Lodish et al. 1983). The kinetic analysis of intracellular transport

of gp120 with its natural signal sequence and gp120 containing the melittin or IL3 signal sequence, using primarily acquisition of resistant to endo H as a measure, further revealed that the half-time required for transport of gp120-NSS from RER to the Golgi was four-times longer than for gp120 with heterologous signal sequences ($t_{1/2}$ =120 min versus 30 min) (Fig. 18). These results support the idea that intracellular transport is a selective rather than a passive flow process and that structural characteristics (signals) of the protein determine their rates of transport (Fitting and Kabat, 1982). This suggests that differences in the rate of gp120 protein secretion from SF9 cells are due to variability in rates of transport from the RER to the Golgi; that is, retention in the RER is primarily responsible for overall rates of secretion (Lodish et al. 1983).

The same evidence could also indicate that although the different structural characteristics of the signal sequences indeed influence the rates of translocation gp120 and its rate of exit from the ER, they do not affect posttranslational modification (e.g., glycosylation pattern) once they enter the secretory pathway (Fig. 18).

It was noted that nearly 10-20% of pulse-labelled gp120-NSS had already acquired partial endo H resistance by 0.5 hr of chase, and this was maintained at a relatively constant level throughout the entire chase period. There was no corresponding accumulation of endo H resistant gp120, however, in the extracelluar fraction (Fig. 18A). One possible explanation is that the slow rate of secretion of gp120-NSS may not be due solely to differences in the rate of transport

from the RER to the Golgi but due to variability in the retention of gp120 within the Golgi, which has been observed with studies of secretory glycoproteins in human hepatoma cells (Yeo et al. 1985). Interestingly, the kinetics of intracellular transport, oligosaccharide maturation, and secretion of HIV-1 gp120 in insect cells were found to be remarkably similar to those events in lymphocytic A 3.01 cells infected with HIV-1 (Willey et al. 1988b) or in cells infected with a recombinant vaccinia virus expressing gp160 (Earl et al. 1991). The results of these studies have shown that although efficiency of cleavage of gp160 to gp120 was markedly cell type dependent, the fraction of gp120 shed from cells was relatively constant. gp120 was first detected in culture medium between 2 and 4 hr following expression. Afterwards, the remaining cell associated gp120 was not shed or was shed at a very slow rate (Earl et al. 1991). Approximately 30-50% of cell associated gp120 was slowly released into the medium over 24 hr. A similar observation was made by Wells and Compans (1990) in SF-8 insect cells using a recombinant baculovirus expressing gp160 and an anchor minus, truncated variant, gp160(t); the $t_{1/2}$ for transport of gp160 and gp160(t) from the RER to the Golgi was estimated at between 0.75 and 1 hr, whereas transport to the cell surface, as monitored by the release of gp120 and gp160(t) into the medium, took even longer (approximately 8.5 and 4 hr).

All of these studies and the results presented here show that endo H resistance is required for secretion of gp120 and gp160(t) (Lasky 1986, Wells and Compan 1990; Earl et al., 1991). The finding that SF9 cells are capable of

processing some N-linked oligosaccharide chains to a state of endo H resistance (Fig. 18) supports the previous findings that insect cells possess glucosidase and mannosidase activities (Jarvis and Summers 1989) whereas analysis of the oligosaccharide side chains of gp120 with endo H in BFA-treated SF9 cells further demonstrates that gp120 is processed by medial Golgi enzymes (Fig. 19). Although this modification observed in insect cells is not significant compared with those in mammalian cells (Lippincott-Schwartz et al. 1989, 1990; Doms et al. 1989), which probably is due to the low level of medial Golgi enzyme activities in SF9 cells, it is consistent with reports that Golgi enzymes are redistributed to the ER upon BFA treatment. Meanwhile, finding a doublet band for gp120-NSS but not for gp120-MSS in the presence of BFA following endo H treatment can be taken as presumptive evidence that the lower band represents some gp120 molecules that remain indefinitely in the ER (Fig. 19). Interestingly, a ER associated 120 kDa envelope protein has recently been reported in HeLa cells cotransfected with wild type and env mutant proviral clones (Willey et al. 1991).

CHAPTER 4: EXPRESSION AND CHARACTERIZATION OF CHARGE-ALTERED HIV-1 GP120

INTRODUCTION

Both statistical studies (Perlman and Halvorson, 1983; von Heijne, 1985) and mutational analysis (Bassford and Beckwith, 1979; Emr et al. 1980; Koshland et al. 1982) have demonstrated that the integrity of the signal peptide hydrophobic core is important for the initiation of secretion. However, the discovery of such hydrophobic sequences alone is not sufficient to unequivocally define their role. The role of the hydrophilic positively charged N-terminus of the signal peptide in the export of protein has also become clear (Boyd and Beckwith, 1990) (Fig. 8). Studies have shown that the net charge at the amino terminus of the signal sequence or of the mature sequence of bacterial secreted proteins are also important for the export process (Vlasuk et al. 1983; Puziss et al. 1989; Summers et al. 1989b; Li et al. 1988). Several possible roles for positively charged amino acids have been proposed to explain why improper charge distribution around the signal sequence blocks secretion (Boyd and Beckwith, 1990). However, some aspects of these proposals may not be relevant to eukaryotes because many eukaryotic signal peptides lack basic residues at their N-termini (von Heijne, 1984). Further examination of the HIV-1 gp120 natural signal sequence reveals that it contains five positively charged amino acid residues (three arginines and two

lysines) and one negatively charged residue (glutamic acid). In contrast, the melittin signal sequence contains only one positively charged amino acid residue (lysine) but there are no charged amino acids in the interleukin 3 signal sequence as shown in Fig. 13. This suggests that the positively charged amino acid residues in gp120 signal sequence may play a role in determining the low level of expression and secretion of the protein. To test this possibility, we have constructed four charge-altered forms of gp120 by using oligonucleotide-directed mutagenesis to alter the positively charged amino acids in the signal sequence of HIV-1 gp120. These charge-altered gp120s were used to examine the role of the positively charged amino acids in the expression, glycosylation, and secretion of the protein.

RESULTS

1. Construction of charge-altered gp120

To change the positively charged amino acids located in the natural signal sequence of HIV-1 gp120 into apolar amino acids as shown in Fig. 20A, oligonucleotide-directed mutagenesis was performed by polymerase chain reaction (PCR) in a Perkin Elmer Cetus thermal cycler. Four mutating oligonucleotide primers (YL-1, YL-2, YL-3,and YL-4; see APPENDIX) were designed to generate a series of mutations in a cDNA clone of HIV-1 gp120 (pUC19gp120-NSS; see Fig. 9). In addition, a universal primer (YL-5), which is complementary with the C-terminus of the gp120 envelope gene was used to obtain the full length of mutant gp120

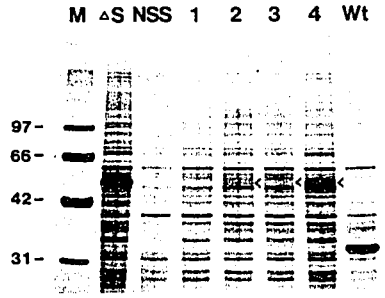
Figure 20. Construction and expression of charge-altered HIV-1 gp120. (A) Schematic diagram of the charge-altered HIV-1 gp120-1, 2, 3, and 4. The 30 amino acids residues of the signal sequence of HIV-1 gp120 are shown. Dashed horizontal lines denotes sequence identity of mutant 1-4 with wild type gp120, and substitutions are shown below their position in the gp120 signal sequence. (B) and (C) Expression of charge-altered gp120. SF9 cells infected with recombinant baculovirus containing gp120-NSS or charge-altered gp120 were harvested at day 3 p.i. Whole cell lysates were analyzed by SDS-PAGE and staining with Coomassie blue (B) or by Western blot probed with rabbit anti-gp120 serum (C) as described in detailed in Materials and Methods. The top arrow in panel C indicates gp120 aggregates that are too large to enter the gel. The location of glycosylated and nonglycosylated forms of gp120 are indicated. vAcgp120-ΔS-infected cell lysates were used as a marker. Lane M shows a set of marker proteins (Bio-Rad) the sizes of which are shown in kilodaltons (KDa).

A.

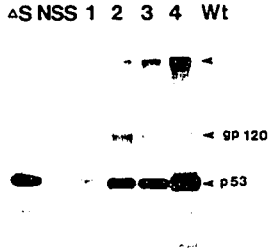
HIV-1 gp120 SIGNAL PEPTIDE

Mutant																							
			+		+	-	+				+			+									
-	M	R	V	K	E	K	Y	Q	H	L	W	R	W	G	W	R	W	G	T	M	L	L	G	M	L	M	I	C	S	A
1.	-	-	-	A	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.	-	I	-	-	-	-	-	-	-	-	-	I	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3.	-	-	-	V	-	I	-	-	-	-	-	I	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	-	I	-	A	-	I	-	-	-	-	-	I	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-

B.



C.



clones. The gp120 encoding sequence was amplified with the Geneamp kit by 30 cycles of PCR (the cycles were 94°C, 1 min; 45°C, 2 min; 72°C, 3 min) using 20 ng of HindIII-linearized pUC19gp120-NSS as template and 1.0 μM of each mutating primer and the universal primer. All primers had BamHI sites at the 5' and 3' termini so that the resulting gp120 DNA fragment could be inserted into the BamHI site of pAcYM1. All mutants had the expected mutations verified by dideoxy chain-termination sequencing (Sanger et al. 1977).

Using this approach, the five positively charged amino acid residues were systematically replaced with neutral amino acid residues, decreasing the positive charge in a stepwise fashion from +5 to +3, +2, +1, and 0 (Fig. 20A). The resulting recombinant baculoviruses containing each gp120 signal peptide mutations were designated vAcgp120-1, 2, 3, and 4 (which are simply indicated as numbers 1-4 in each figure).

2. Expression of charge-altered gp120

To examine the effect of charge alteration in the natural signal sequence on the synthesis of gp120, the level of the expression of the charge-altered gp120 protein was examined by comparison with the expression of unaltered gp120 (gp120-NSS) and gp120 without the signal sequence (gp120-ΔS). SF9 cells were infected with wild type virus or each recombinant baculovirus at a m.o.i. of 5 and infected cells were harvested at 3 days p.i.. Whole cell lysates from recombinant virus-infected cells were prepared and analyzed by SDS-PAGE followed by

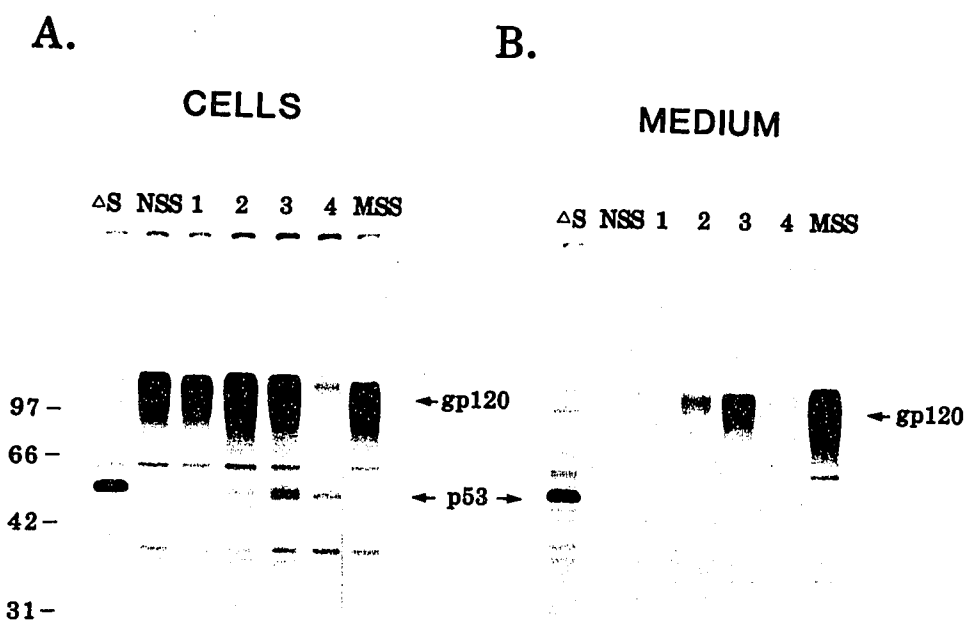
Coomassie blue staining. Figure 20B shows that substitution of all three arginines (gp120-2), or two lysines and the last two arginines (gp120-3) or all five positively charged amino acid residues (gp120-4) with neutral amino acid residues resulted in protein profiles that were significantly different from those of gp120-NSS or gp120-1 (with only two altered lysines). As indicated in Fig. 20B, a protein band migrating at about 53 KDa, like gp120-ΔS, was observed in the lysates from gp120-2, 3, and 4 but not in lysates of gp120-NSS and gp120-1. Western blot analysis of the same samples further showed that gp120-1 (retaining three arginines) exhibited nearly the same level of expression of both glycosylated and nonglycosylated forms of gp120 as gp120-NSS (Fig. 20C). However, gp120-2, 3, and 4 which, respectively, retain two lysines, one arginine, or no positive charged amino acid, exhibited remarkable increases in the expression of both glycosylated and nonglycosylated forms of gp120 (Fig. 20C). Interestingly, the level of expression of gp120 by gp120-4 was dramatically increased in its nonglycosylated form but decreased in its glycosylated form (Fig. 20C and see Fig. 21). In addition, it was observed that gp120-2, 3 and 4 produced large aggregates of gp120 products which could not enter the gel.

3. Effects of charge alteration on the secretion of gp120

To examine effects of changing of the positively charged amino acid residues in the signal sequence of gp120 on secretion of gp120, SF9 cells infected with recombinant viruses vAcgp120-NSS, vAcgp120-MSS, and viruses containing each of

four altered gp120 genes were pulse-labelled with [^{35}S]-methionine for 1 hr and chased for 2 hr with cold methionine. Cells lysates and medium were separately immunoprecipitated with rabbit anti-gp120 serum and analyzed by SDS-PAGE. As shown in Fig. 21A, alteration of positively charged amino acid residues did not interfere with the glycosylation of gp120 (gp120-1) but rather improved its efficiency of glycosylation (gp120-2 and 3), which are consistent with the results shown in Fig. 20C. However, changing of all five positively charged amino acid residues led to a severe effect on translocation of gp120 and subsequently the extent of glycosylation of the protein (gp120-4)(Fig. 21A); as result, large amounts of the nonglycosylated form accumulated in cells (Fig. 20B and C). This result indicates that a minimum number of positively charged residues in the signal sequence may be required for efficient translocation of the protein across the ER membrane. Therefore, complete removal of positively charged residues, and a net negative charge in the signal sequence may block translocation of gp120 into the ER and thus block its subsequent intracellular processing. The results of the pulse-chase experiments further showed that the products of all charge-altered forms of gp120 (1-4) were all secreted. However, the level of secretion was increased as the number of positive charges was reduced (Fig. 21B). Particularly, gp120-3, which retained only the first arginine, showed much more efficient secretion, which was still about 50% of the level secretion as compared for gp120 containing the melittin signal sequence (Fig. 21B). Interestingly, gp120-4, which did not contain any positive charge but retained a net negative charge, was still capable of being secreted.

Figure 21. Pulse-chase analysis of biosynthesis and secretion of charge-altered gp120. Infected cells were labelled with [^{35}S]-methionine for 1 hr and chased for 2 hr. Proteins were immunoprecipitated from infected cell lysates and extracelluar medium with rabbit anti-gp120 serum. The immunoprecipitates were analyzed by SDS-PAGE. Radiolabelled immunoprecipitates from vAcgp120-ΔS-infected (ΔS) cell lysates and samples from vAcgp120-MSS-infected (MSS) cell lysates and medium were used as markers.


A.
CELLS
ΔS NSS 1 2 3 4 MSS
97 –
66 –
42 –
31 –
gp120
p53
B.
MEDIUM
ΔS NSS 1 2 3 4 MSS
gp120

Furthermore, it was noted that reducing the number of arginines resulted in more efficient secretion than reducing the number of lysine residues (Fig. 21B, gp120-1 and 2). This suggests that different positively charged amino acids have different effects on secretion.

These data demonstrate that the positively charged amino acid residues in the natural signal sequence of HIV-1 gp120 are a major factor resulting in its poor expression and secretion. This observation supports the conclusion that the positively charged amino acids are not essential for signal function but, rather, could be responsible for its efficiency (Vlasulk et al. 1983; Puziss et al. 1989). Furthermore, it was noted that the charge-altered gp120 (2, 3 and 4), which retained two lysines, one arginine or no positively charged amino acid residues, exhibited faster electrophoretic mobility in their nonglycosylated forms (which had electrophoretic mobility similar to gp120-ΔS) as compared with gp120-NSS or gp120-1 retaining all three arginines (Fig. 20C and Fig. 21A). These results suggest that changing positively charged amino acid residues in the natural signal sequence may results in cleavage of signal sequence of gp120.

4. Characterization of gp120 upon tunicamycin or endoglycosidase treatment

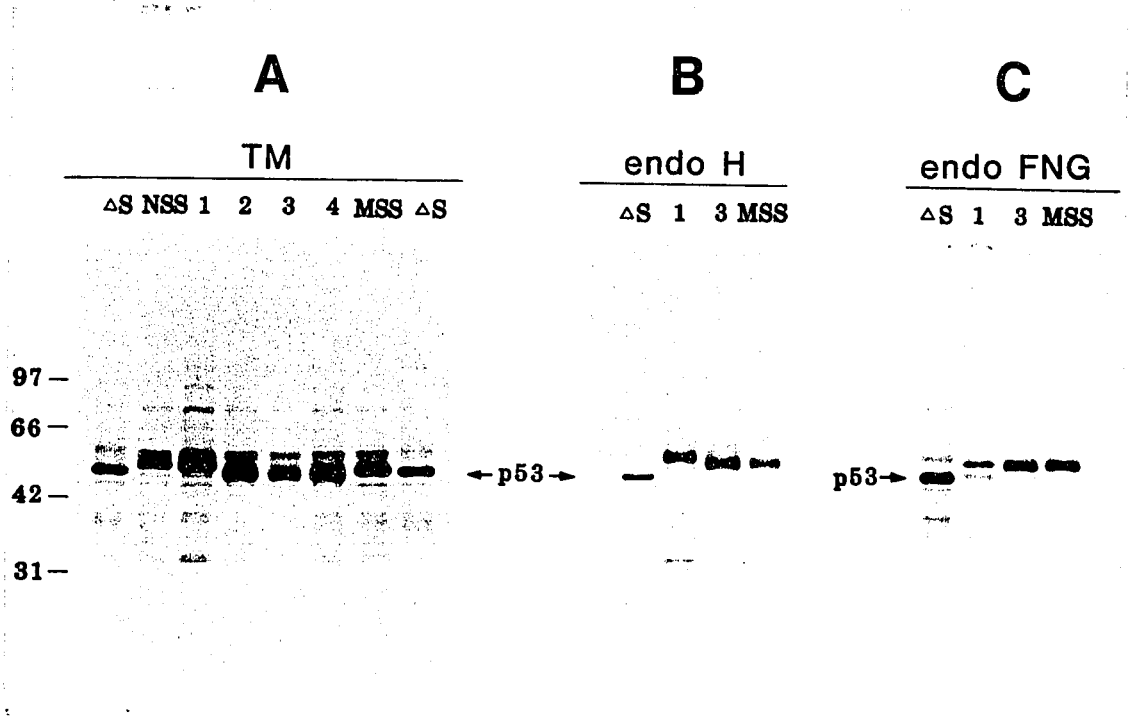
To provide indirect evidence for proteolytic cleavage of charge-altered gp120, the electrophoretic mobility of gp120-NSS was compared with the mobilities of charge-altered gp120 in the presence of the glycosylation inhibitor tunicamycin or (TM) or after treatment with endoglycosidase H (endo H) or a mixture of

endoglycosidase F/N glycanase (endo FNG). As shown in Fig. 22A and B, both nonglycosylated gp120-NSS and gp120-1 produced in the presence of TM or after endo H treatment had slower electrophoretic mobilities than gp120-2, 3 or 4 and gp120 with melittin signal sequence. The same results were obtained when all the intracellular gp120 proteins were treated with endo FNG, which cleaves all glycan moieties from the glycoprotein (data not shown; Tarentino et al. 1989). However, once secreted, the extracellular proteins exhibited the same electrophoretic mobilities after treatment with endo FNG (Fig. 22C), which further supports the speculation that proteolytic cleavage of the signal sequence occurred for gp120-MSS and gp120-2, 3 or 4 but not for gp120-NSS and gp120-1. In addition to the major deglycosylated form of gp120 detected for gp120-1, another faint protein band, with a mobility between the deglycosylated and nonglycosylated gp120-ΔS, was also immunoprecipitated for reasons not yet understood.

5. Kinetics of the intracellular folding of gp120

The intracellular folding of glycoprotein destined for the cellular secretory pathway has been primarily studied by using either conformation-dependent monoclonal antibodies or by assessing protein oligomer formation. Such studies with influenza virus HA and VSV G protein have demonstrated that correct folding and oligomerization are prerequisites for transport from the ER to the Golgi complex (Gething et al. 1986; Kreis and Lodish. 1986). Failure to attain the proper three-dimensional structure would thus result in retention by default.

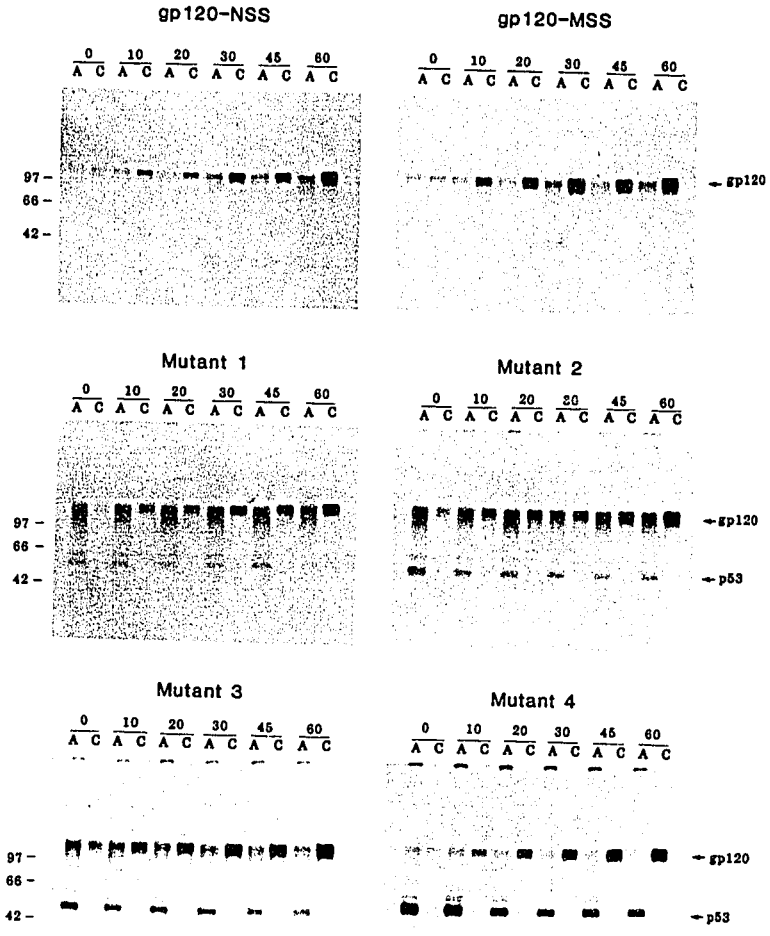
Figure 22. Comparison of the electrophoretic mobility of gp120 upon tunicamycin or endoglycosidase treatment. Immunoprecipitates from radiolabelled TM-treated cells (A) or after digestion with endo H (B) or endo FNG (C) were analyzed by 10% SDS-PAGE. Recombinant baculoviruses used in this experiment were indicated on the top of the gel: ΔS, vAcgp120-ΔS; NSS, vAcgp120-NSS; MSS, vAcgp120-MSS; and the number 1-4 represents, respectively, the charge-altered gp120-1, 2, 3 and 4.


A
B
C
TM
endo H
endo FNG
ΔS NSS 1 2 3 4 MSS ΔS
ΔS 1 3 MSS
ΔS 1 3 MSS
97
66
42
31
p53
p53
p53

Since the comparative analysis of secretion and intracellular transport of gp120-NSS, gp120-MSS, and gp120-IL3SS with endo H indicated that intracellular transport was interrupted at a point between the ER and the medial Golgi, the slow and inefficient transport of gp120-NSS from cells could reflect a defect in the folding of the molecule, as it contains 18 cysteine residues. To address this question, the kinetics of intracellular folding of gp120-NSS and gp120-MSS were examined by measuring their abilities to bind to CD4 as a functional assay for correct conformation (Fennie and Lasky, 1989). Recombinant baculovirus-infected SF9 cells were pulse-labelled for 10 min and lysed at different times of chase, after which one half of each lysate was immunoprecipitated with rabbit anti-gp120 serum , while the remaining half was incubated with excess soluble CD4, followed by coprecipitated with a monoclonal antibody OKT4, specific for CD4.

As described previously, the antiserum we used was raised directly against the denatured and nonglycosylated form of recombinant gp120 and thus it only recognized the linear epitopes on gp120. The amount of gp120 immunoprecipitated with this antiserum remained relatively constant throughout the entire chase period. On the contrary, the amount of newly synthesized gp120 able to bind CD4 was very low, but the ability of its binding to CD4 gradually increased with time (Fig. 23). This suggests that gp120 is originally synthesized as a highly glycosylated but unfolded linear molecule which has not attained conformation which is necessary to bind CD4. With time, however, the protein has probably folded into the proper conformation and efficiently binds CD4 (Fig.

Figure 23. Pulse-chase analysis of intracellular folding of gp120. Infected SF9 cells were pulse-labelled for 10 min with [^{35}S]-methionine at 24 hr p.i. and chased for the indicated time (minutes). Equal portions of cell lysates were either immunoprecipitated with rabbit anti-gp120 serum (A) or with OKT4 after complexing with soluble CD4 (C). The precipitated material was then analyzed by 10% SDS-PAGE and visualized by fluorography.


gp120-NSS
gp120-MSS
0 10 20 30 45 60
A C
97 –
66 –
42 –
gp120
Mutant 1
Mutant 2
p53
Mutant 3
Mutant 4

23). By 30 min of chase, gp120-MSS rapidly reached its maximal CD4 binding capacity and then binding slightly declined due to secretion of gp120 into the culture medium. In contrast, the folding of gp120-NSS was considerably slower and its maximal binding to CD4 was not observed until 60 min after the beginning of the chase period. As measured by this assay, the half-time of folding for gp120-MSS appeared to be approximately 15 min, which was much faster than that of gp120-NSS ($t_{½}$ > 30 min) (Fig. 23). This finding, is again consistent with the results obtained from kinetic analysis of intracellular transport and secretion of gp120-NSS (Fig. 15 and Fig. 18), indicates that the different signal peptides rather than disulfide bond formation may play a role in affecting gp120 protein folding and thereby its transport. To further test this possibility, the intracellular folding of the four charge-altered forms of gp120 was examined. Consistent with the results of the secretion (Fig. 21), gp120-1 exhibited almost the same folding rate as gp120-NSS. gp120-2 and 3, retaining two lysines or one arginine in their signal sequences were more efficiently secretion and folded more rapidly than gp120-NSS or gp120-1. Interestingly, although only low amounts of glycosylated gp120-4 were produced, it still exhibited nearly the same folding rate as did gp120-2 and 3 (Fig. 23).

Previous results (Fig. 22) suggested that the intracellular form of gp120-NSS and gp120-1 still retain the signal sequence, while gp120-2, 3 and 4 do not. These results are consistent with other reports that failure to process signal peptides sometimes affects secretion kinetics (Haguenauer-Tsapis and Hinnen, 1984; Schauer et al. 1985; Haguenauer-Tsapis et al. 1986).

DISCUSSION

1. Positively charged residues in the natural sequence are a key factor determining the poor secretion of gp120

The lack of a clear explanation for what characteristics of the signal sequence are responsible for the very slow kinetics of gp120-NSS in its secretion and intracellular transport prompted us to look for distribution of charged amino acids in the signal sequence, which may affect for the processes. Computer analysis reveals that, unlike most eukaryotic signal sequences (von Heijne, 1984) such as the melittin or interleukin 3 signal sequence presented in this study, the HIV-1 gp120 signal sequence contains five positively charged amino acids and one negatively charged amino acid at its N-terminus--an unusually high numbers (Fig. 13). This unusual feature indicates that positive charges may play an important role in secretion of the protein. To this end, four charge-altered forms of gp120, gp120-1, 2, 3 and 4 were constructed by oligonucleotide-directed mutagenesis to determine if the positive charges account for the poor expression and secretion of the protein.

Western blot analysis showed that substitution of the positively charged amino acids with neutral amino acids resulted in dramatic differences in both glycosylated and nonglycosylated gp120 protein production (Fig. 20C). Secretion of gp120 was progressively increased by decreasing the positive charges in a stepwise fashion from +5 to +3, +2, and +1 (Fig. 21B, secretion of gp120-3 > gp120-2 > gp120-1 > gp120-NSS). This provides, for the first time, direct evidence why this HIV-1

envelope glycoprotein has been refractory to efficient expression in recombinant system (Lasky et al. 1986). These results, consistent with the statistical studies (von Heijne, 1984) and mutational analysis (Vlasulk et al. 1983; Puziss et al. 1989) indicate that the positively charged amino acids are not essential for signal sequence function but, rather improve its efficiency (Boyd and Beckwith, 1990). The finding that signal mutant 4, even without basic amino acids but with a net negative charge, still can be secreted provides further evidence that a mutated signal sequence containing a net negative charge is still capable of prompting secretion, but with considerably slower kinetics (Vlasuk et al. 1983; Puziss et al. 1989; Boyd and Beckwith, 1990).

We found that the basic side chains of arginine and lysine are not equivalent in terms of their effect on secretion; reducing the number of arginine residues results in more efficient secretion than only reducing the number of lysine residues (Fig. 21B, secretion of gp120-2 > gp120-1) (Li et al. 1988; Summers et al. 1989; Summers and Knowles, 1989). This suggests that the pKa of the amino acids (arginine has a higher pKa than lysine) may also be a determining factor. Recent studies with bacterial secretory proteins have shown that the net positive charge within the first five amino acids of mature proteins also have a blocking effect on the export process (Li et al. 1988; Summers et al. 1989; Summers and Knowles). However, computer analysis of the amino acid sequence of HIV-1 envelope protein shows that this constraint is not found at the amino terminus of the mature part of gp120, which further supports our conclusion that the key factor affecting the poor expression and secretion of gp120-NSS is contributed only to the highly charged

N-terminal region of signal sequence.

Strikingly, computer analysis of the sequences of HIV-1 signal peptides from different strains have revealed that highly positive charges with an average of +3 to +5 are a unique feature of HIV-1 signal peptides (Myers et al. 1988). In contrast, analysis of the HIV-2 signal sequence from different isolates shows that they have fewer positively charged amino acids than does the signal sequence of HIV-1. In good agreement with this analysis and the results presented here, when HIV-2 gp120 gene, which has only three positively charged amino acids (lysine) in its signal sequence, was expressed under the same conditions described in this study, it exhibited a higher level of expression and secretion than HIV-1 gp120 (see Fig. 25A). This result is consistent with the observation made by Earl et al. (1991) that the shedding efficiency of gp120 is more likely to be dependent on the strain of HIV used; >90% of HIV-2 gp120 is shed as compared with 59% of HIV-1 gp120. Although, at present, we do not understand the significance of the highly charged amino acids in the signal sequence with respect to HIV replication, based on the above observation it may play an important role in viral pathogenicity.

2. Signal sequence affects the folding of gp120 and thereby its exiting from RER

It has been suggested that a delay in secretion might reflect a need for secretory proteins to interact with a specific receptor in the RER for transport to the Golgi (Fitting and Kabat, 1983; Lodish et al. 1983). Alternatively, protein may be retained in the ER until they have folded correctly; different folding kinetics may result in different retention times in the ER (Kreis and Lodish, 1986; Gething et al.

1986; Williams et al. 1988). Several studies with naturally existing integral membrane proteins have indicated that native folding of the polypeptide chain and oligomerization of the monomers are sequential and prerequisite events for intracellular transport out of the ER (Gething et al. 1986; Kreis and Lodish, 1986; Copeland et al. 1986; Doms et al. 1988). Most misfolded and nonoligomerized integral membrane proteins including HIV-1 glycoprotein (gp160), fail to be transported and exhibit prolonged associations with the ER resident protein GRP78-BiP (Copeland et al. 1986; Gething et al. 1986; Machamer et al. 1990; Ng et al. 1990; Earl et al. 1991) Indeed, comparison of folding kinetics of gp120-NSS with that of gp120-MSS showed that the long folding time for gp120-NSS correlated with its long transit time from the RER to the Golgi (Fig. 18 and Fig. 23). These findings support the conclusion that gp120 can fold correctly in the absence of the C-terminal gp41 region and that gp120 is folded within the RER and before transport to the Golgi apparatus, as reported recently by Fennie and Lasky (1989). However, since gp120 binds to CD4 as a monomer, the observation that misfolded influenza HA proteins (Gething et al. 1986) are incapable of exiting from the RER would appear not to apply to the HIV gp120 molecule (Fennie and Lasky, 1989). In contrast, the fact that replacement of the natural signal sequence of gp120-NSS with melittin (Fig. 23) or IL3 signal sequences (data not shown) and reducing the number of positively charged residues in the signal sequences (Fig. 23) results in faster folding than that of gp120-NSS strongly suggests that signal peptide rather than unique structural aspects of gp120 (i.e., formation of nine disulfides) appear to be an important factor affecting the gp120 folding.

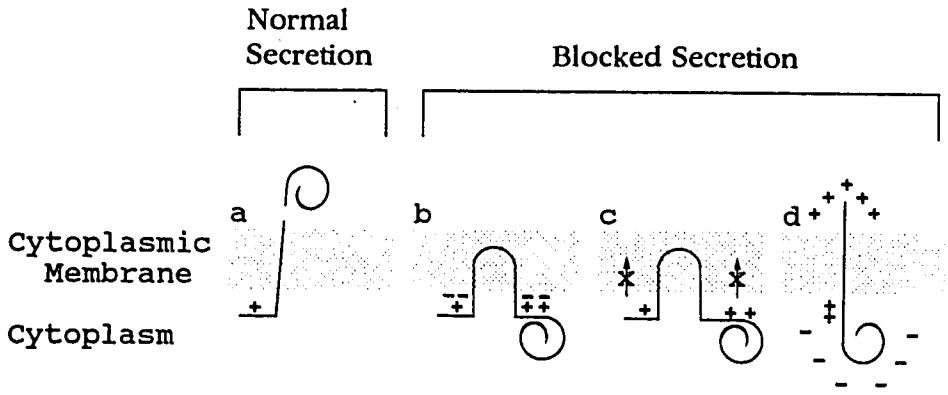
Studies with VSV G and influenza HA proteins have led to the similar conclusion that loop structure of the signal sequence, depending on the timing of signal sequence cleavage, may have an important function in directing protein folding (Wilson et al. 1981; Shaw et al. 1988). Furthermore, studies from another group, by comparing the folding kinetics of purified mature and precursor maltose-binding proteins or ribose-binding proteins in vitro, have provided direct evidence that signal peptides indeed retard the folding of the mature part of the polypeptides (Park et al. 1988). Thus, the finding that the extracellular and intracellular forms of gp120-NSS and gp120 signal mutant 1 had different apparent molecular masses (61 KDa versus 58 KDa) whereas extracellular and intracellular forms of gp120-MSS and gp120 signal mutants 2-4 had the same molecular masses (58 KDa) after TM or endo H and endo FNG treatment (Fig. 22) indicated that the difference in their masses for gp120-NSS and gp120-1 was not related to a difference in N-linked glycosylation. Rather, this supports the speculation that the intracellular forms of gp120-NSS and signal mutant 1 retain, while their extracellular forms lack, the amino-terminal signal sequence of 30 amino acids. The fact that none of the 58 KDa forms of gp120-NSS (data not shown) and gp120-1 was not detected intracellularly and instead detected extracellularly (Fig. 22B and C) suggests that the structure modification accounting for their lower masses occurs simultaneously with or shortly after their exit from the cell. Consistent with the results presented here, it has been reported that human t-PA protein secreted from insect cells has a lower molecular mass than the cell-associated form, which has been attributed to late cleavage of the signal peptide (Jarvis and Summers, 1989) and others have

shown that failure to process signal peptides affect secretion kinetics by delaying release of the proteins from ER (Haguenauer-Tsapis and Hinnen, 1984; Schaner et al. 1985; Haguenauer-Tsapis et al. 1986). Taken together, the data presented here clearly indicate that the uncleaved signal peptide may act as retention signal that retards the folding and secretion of gp120-NSS and signal mutant 1. However, direct peptide sequencing of the N-terminal portion of the recombinant gp120 proteins will be necessary to elucidate their precise structures.

On the other hand, while cleavage of the signal sequence plays an important part in the kinetics of gp120 release from ER membrane, this cleavage appears not to be an obligatory step in the routing of the protein to its final destination (Fig. 15 and Fig. 18; Haguenauer-Tsapis et al. 1984, 1986).

At present, it is not known how the positively charged amino acid residues in the natural signal sequence of HIV-1 gp120 participates in the initial stages of secretion. However, several possible roles for positively charged amino acids, mostly based on the results from studies with bacterial membrane and secreted proteins, have been proposed to explain why improper charge distribution around signal sequence blocks secretion (Boyd and Beckwith, 1990, and Fig. 24). According to the loop model (Fig. 24b; Inouye and Halegoua, 1980), the basic amino acids preceding the signal sequence interact with the acidic head groups of the phospholipids in the membrane, thus anchoring the amino terminus of the protein in the cytoplasm. On the other hand, basic amino acids at either end of the signal sequence are unable to penetrate the lipid bilayer because of their high pKa, particularly arginine, which would make their translocation energetically more

Figure 24. Models to explain why improper charge distribution around the signal sequence blocks secretion. Normally, the body of the protein (spiral) would be translocated across the membrane and cleaved from the signal sequence (a). In the later three cases (b, c, and d), the body of protein remains in the cytoplasm rather than being secreted due to improper charge distribution. Modified from Boyd and Beckwith (1990).


Normal
Secretion
Blocked Secretion
a
b
c
d
Cytoplasmic
Membrane
Cytoplasm

costly if they had to be neutralized in order to penetrate an apolar environment (Fig. 24c; Summers et al. 1989). Finally, an altered charge distribution around the signal sequence causes the hydrophobic sequence to insert in the wrong orientation via its interaction with the charge difference across the membrane (Fig. 24d; Li et al. 1988).

Although some aspects of these proposals may not be relevant to eukaryotes and it is not known whether the retention of the N-terminal positively charged domain of HIV-1 gp120 is due to binding by cytoplasmic factors or if a local electrical potential across the membrane makes translocation of this region thermodynamically unfavourable, the finding that reducing of positive charges in the natural signal sequence results in dramatically releasing of gp120 into medium, in good agreement with the models, provides further evidence that HIV-1 gp120 and bacterial membrane and secreted proteins may share a common mechanism for determining the efficiency of protein secretion by which the N-terminal positively charged residues provide a barrier to translocation and thus block secretion. In the case of prokaryotes, recent studies have suggested that the *E. coli* secA protein directs protein translocation by recognizing N-terminal positive charges within a signal sequence (Akita et al. 1990), and it seems possible that an analogous protein may operate similarly in eukaryotes. Thus, the models (Boyd and Beckwith, 1990) could be adopted as a good interpretation of results presented here.

It is clear that the N-terminal region of HIV-1 gp120 glycoprotein plays an

important role in initiating the secretion of the protein. Further studies, which are currently underway on examination of the effect of charge-altered signal mutants generated from HIV-1 proviral DNA clone on the viral infection and of whether the presence of an insect cell version of GRP-BiP or secA-like protein directing protein translocation by recognizing N-terminal positive charges within signal sequence, will help elucidate the mechanism involved in this process.

CHAPTER 5: A ROLE OF N-LINKED GLYCOSYLATION OF GP120 IN BINDING TO CD4

INTRODUCTION

The interaction of envelope glycoprotein gp120 of the human immunodeficiency virus (HIV) with it receptor, CD4, represents the initial step in virus infection and accounts for HIV's tropism and cytopathic effect (Dalgleish et al. 1984; Klatzmann et al. 1984; Sodroski et al. 1986). This interaction is a high affinity event that appears to be completely dependent upon the overall conformation of the envelope glycoprotein (McDongal et al. 1986; Lasky et al. 1987). gp120 is a highly glycosylated molecule and has more than 20 potential N-linked glycosylation sites. The N-linked glycans represent almost 50% of the apparent molecular weight of the protein and were shown to be of both high mannose and the complex branched types (Fenonillent et al. 1989; Matthews et al. 1987, Geyer et al. 1988). Therefore, carbohydrates are likely to be prominent structures on the surface of HIV, but their precise biological functions remain elusive.

There has been a great deal of interest in the requirements for glycosylation of both CD4 and gp120 in their successful interactions. For CD4, the sites of interaction with gp120 have been clearly defined (Peterson and Seed, 1988; Arthos

et al. 1989; Camerin and Seed, 1990) and there is no requirement for either of the two N-linked carbohydrate side chains (Arthos et al. 1989). For gp120, however, the nature of the site of interaction with CD4 is more complex (Lasky et al. 1987; Cordonnier et al. 1989). Although it has been shown that glycosylation is not required for induction of neutralizing antibodies (Putney et al. 1986), the exact role of carbohydrate on gp120 in complex formation remains unclear. Conflicting results have been reported regarding this issue; some reports claim that nonglycosylated gp120 produced in bacteria (Putney et al. 1986; Morikawa et al. 1990b) and deglycosylated gp120 with endoglycosidase are unable to bind to CD4 (Matthews et al. 1987; Fennine and Lasky, 1989). In contrast, other reports claim that enzymatic removal of carbohydrate from gp120 does not significantly reduce its ability to bind to CD4 (Fenouillet et al. 1989, 1990). In view of these apparent discrepancies, as well as the importance of virus-host cell receptor interactions associated with virus infection, it was necessary to reexamine the nature of the interaction between gp120 and CD4. To this end, we have employed recombinant baculoviruses, as shown in Fig. 13, to prepare nonglycosylated and fully glycosylated forms of gp120, and also deglycosylated gp120 using endoglycosidase, we then tested the ability of glycosylated, nonglycosylated, and deglycosylated gp120 to bind to CD4 molecules. In addition, HIV-2 gp120 was also expressed in SF9 cells and used in the CD4 binding assay.

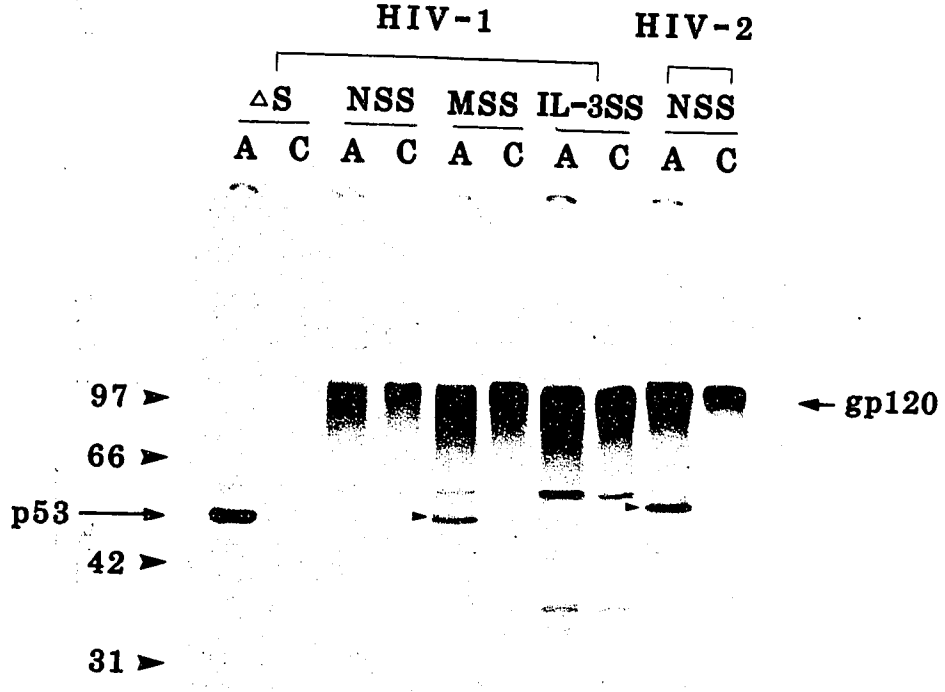
RESULTS

1. Examination of CD4 binding properties of recombinant gp120

To study receptor binding of nonglycosylated and glycosylated gp120, we used an assay system based on the ability of monoclonal antibody against CD4 (OKT4) to immunoprecipitate the CD4-gp120 complex (McDougal et al. 1986; Lasky et al. 1987). SF9 cells infected with the different recombinant baculoviruses were labelled with [^{35}S]-methionine for 1 hr and membrane and cytosol of ^{35}S-labelled cells were extracted with 1% Nonidet P-40. One half of the cytosolic fraction was analyzed for total gp120 by immunoprecipitation with rabbit antiserum directed against recombinant gp120. The remaining half of the sample was incubated with soluble CD4, and the formation of gp120-CD4 complexes was assessed by immunoprecipitation with excess MAb OKT4. The intensity of the band co-migrating with gp120 is an indication of the quantity of gp120 that bound to CD4.

As shown in Fig. 25, only glycosylated forms of gp120 bound to CD4 while both nonglycosylated and glycosylated forms of gp120 were immunoprecipitated with rabbit anti-gp120 serum. In contrast, nonglycosylated gp120 generated by the deletion of the signal sequence (gp120-ΔS), nonglycosylated forms of gp120 from recombinant baculovirus carrying HIV-1 gp120 gene with the signal sequence from melittin, and nonglycosylated HIV-2 gp120 did not bind to CD4. A higher molecular weight protein detected in the vAcgp120-IL3 lysate with OKT4 was probably a cellular protein that was nonspecifically immunoprecipitated because

Figure 25. CD4 binding of recombinant gp120 expressed in SF9 cells. Recombinant baculovirus-infected SF9 cells were labelled for 1 hr with [^{35}S]-methionine and lysed with 1% NP40. One half of each cell lysate was immunoprecipitated with rabbit anti-gp120 serum (A). The remaining material was incubated with soluble CD4, and the complex was coprecipitated with MAb OKT4 (C) and protein A-Sepharose CL-4B. Samples were analyzed by SDS-PAGE, and visualized by fluorography. ΔS, gp120 without a signal sequence; MSS, gp120 containing the melittin signal sequence; IL-3SS, gp120 containing the interleukin 3 signal sequence; NSS, gp120 with either HIV-1 or HIV-2 signal sequence. The numbers on the left represent molecular mass (in thousand) of protein standards.

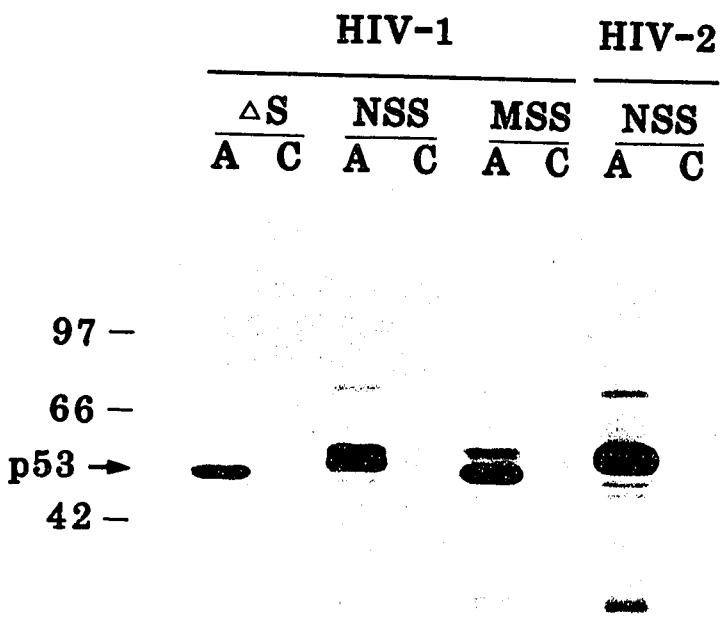

HIV-1
HIV-2
ΔS
NSS
MSS
IL-3SS
NSS
A C A C A C A C A C
97
66
p53
42
31
gp120

this protein was also present in all extracts from recombinant virus-infected cells (Fig. 25). Furthermore, the precipitation of glycosylated gp120 by OKT4 was dependent on the addition of exogenous CD4 (data not shown). These results are consistent with the recent reports (Morikawa et al. 1990a; Dirckx et al. 1990; Wells and Compans, 1990) that although gp120 produced in insect cells has the altered carbohydrate structure such as being high in mannose, lacking sialic acid and less processed than those produced in mammalian cells (Luckow and Summers, 1988), it exhibits excellent CD4 binding characteristics as reported for gp120 expressed in mammalian cells (Fennie, and Lasky, 1989). Conversely, the nonglycosylated gp120 backbone is unable to bind to CD4 (Putney, et al. 1986; Morikawa et al. 1990b).

2. Analysis of the effect of tunicamycin treatment on gp120 binding to CD4

To further study the necessity of glycosylation of gp120 for binding to CD4, we expressed gp120 in the presence of tunicamycin. As can be seen in Fig. 26, gp120 produced in the presence of tunicamycin was nonglycosylated and similar in size to the nonglycosylated protein lacking the signal sequence. This protein failed to bind to CD4. These results suggest that glycosylation of gp120 is essential to create a conformational epitope where CD4 can bind. To examine the folding of gp120 without glycosylation, we prepared total cell extracts from vAcgp120-ΔS-infected cells in the presence or absence of reducing agent (5% β-mercaptoethanol) and examined migration of the gp120-ΔS by SDS-PAGE and Western blot analysis.

Figure 26. Effect of tunicamycin treatment on gp120 binding to CD4. SF9 cells were infected with recombinant baculovirus at a m.o.i. of 5. At 20 hr p.i. tunicamycin was added and maintained at a concentration of 15 μg per ml. The cells were labelled with [^{35}S]- methionine (250 μCi/ml) for 2 hr at 24 hr p.i. An equal portion of intracellular material was either incubated with rabbit anti-gp120 serum (A) or with CD4 molecules followed by OKT4 (C).

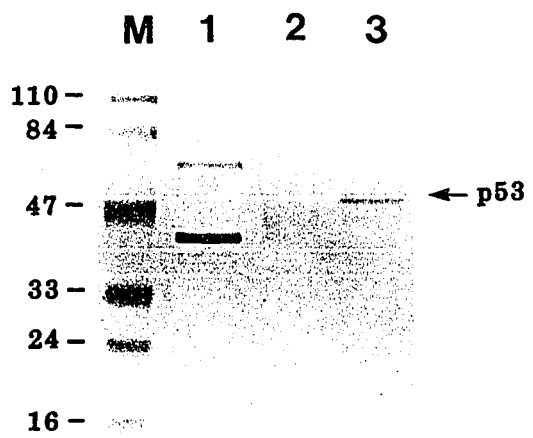

HIV-1
HIV-2
ΔS
NSS
MSS
NSS
A C A C A C A C
97 –
66 –
p53 →
42 –

The banding pattern for gp120-ΔS changed substantially when the extract was not reduced (Fig. 27), suggesting that gp120-ΔS made in insect cells forms disulfide bonds. Thus, the fact that the nonglycosylated gp120 was unable to bind to CD4 is considered to support the hypothesis that the absence of N-linked oligosaccharide may result in aberrant formation of disulfide bonds that results in incorrect folding of the gp120 which may destroy functional conformation of the protein (Vidal et al. 1989; Grigera et al. 1991).

3. Characterization of the effect of deglycosylation of gp120 on binding of CD4

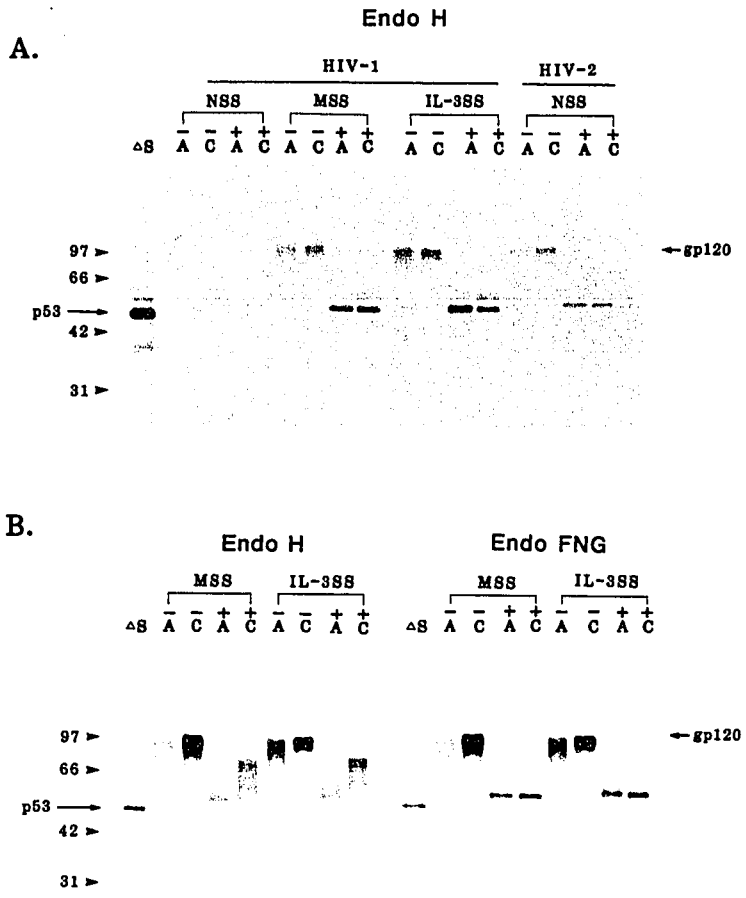
Previous studies (Fig. 15 and Fig. 18) have shown that a subset of N-linked carbohydrate residues on gp120 produced in insect cells was found to be modified post-translationally, most likely by exoglycosidase trimming of the high mannose N-linked oligosaccharide to endo-H resistant trimannosyl core (Jarvis and Summers, 1989; Wells and Compans, 1990). Therefore, the next series of experiments was designed to determine the effect of various types of oligosaccharide side chains on the ability of gp120 to bind to CD4. By using a similar but modified method described by Fennie and Lasky (1989), endo H digestion of intracellular gp120 was first conducted on the gp120-CD4 complex in the presence of SDS (0.5%). After digestion, the gp120-CD4 complex was dissociated by boiling at 100°C for 3 min. Equal portions of samples were diluted to a final SDS concentration 0.02% and then incubated with either rabbit anti-gp120 serum or with soluble CD4 followed by OKT4. To compare and characterize the efficiency of the deglycosylation, SDS-

Figure 27. Comparison of the electrophoretic mobility of gp120-ΔS in the presence or absence of β-mercaptoethanol (βMe). Lane 1, HIV-2 gag protein; lane 2, gp120-ΔS without βMe; lane 3, gp120-ΔS with βMe. Lane M shows a set of prestained protein makers (Bio-Rad) the sizes of which are shown in kilodaltons.


M
1
2
3
110
84
47
33
24
16
p53

PAGE analysis of these molecules was performed before and after endoglycosidase treatment. As shown in Fig. 28A, untreated total gp120 and its coimmunoprecipitated counterpart with OKT4 had similar characteristics. However, after treatment of the gp120-CD4 complex with endo H under denaturing conditions in the presence of SDS, two forms of faster migrating polypeptides appeared when coprecipitated with OKT4; one is a predominantly endo H-sensitive form with a molecular mass of 58 kDa that is consistent with that estimated from the amino acid sequence of the protein backbone (Matthews et al. 1987; Fennie and Lasky, 1989); the second is a minor partially endo H-resistant form with a molecular mass of 61 kDa (Fig. 28A). Interestingly, not only the partially deglycosylated form (partial endo H resistant) but also the completely deglycosylated form of gp120 (endo H sensitive) exhibited the high CD4-binding activity as compared with that of the intensity of nonglycosylated gp120 reacting only with rabbit antiserum (Fig. 28A). However, since it has been shown that secreted gp120 molecule has encountered enzyme involved in oligosaccharide processing during its transport through the exocytic pathway and appeared to be partially endo H resistant (Fig. 18). It was thus important to demonstrate whether or not deglycosylated, secreted gp120 is still capable of binding to CD4. Therefore, the secreted gp120 was treated with endo H or endo F/N glycanase in the absence of SDS. The enzyme treated and untreated gp120 were then analyzed for CD4 binding property. As shown in Fig. 28B, upon endo H treatment, rabbit antiserum recognized only the completely deglycosylated form of gp120 but not the partially

Figure 28. Effect of deglycosylation of gp120 on binding of CD4. Panel A, the intracellular CD4-binding assay. Radiolabelled immunoprecipitates (Materials and methods) were divided into two equal aliquots. One aliquot was digested with endo H(+), while the other served as control(-), after which samples were further divided into two equal aliquots. One aliquot was incubated with rabbit anti-gp120 serum (A), while other was incubated with soluble CD4 followed by OKT4 (C). Panel B, the extracellular CD4-binding assay. Recombinant baculovirus-infected SF9 cells were labelled with [^{35}S]-methionine and chased for 2 hr, after which half the medium was incubated without enzyme (-), while the remaining half was incubated with either endo H or endo FNG (+). After digestion, each sample was further divided into two equal aliquots. One aliquot was incubated with rabbit anti-gp120 serum (A), and other half was incubated with soluble CD4 followed with OKT4 (C). Immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography. For sake of comparison, radiolabelled-gp120-ΔS immunoprecipitated with rabbit anti-gp120 serum was used as a marker.


A.
Endo H
HIV-1
HIV-2
NSS
MSS
IL-3SS
NSS
ΔS A C A C A C A C A C A C A C A C
97
66
p53
42
31
gp120
B.
Endo H
Endo FNG
MSS
IL-3SS
MSS
IL-3SS
ΔS A C A C A C A C ΔS A C A C A C A C
97
66
p53
42
31
gp120

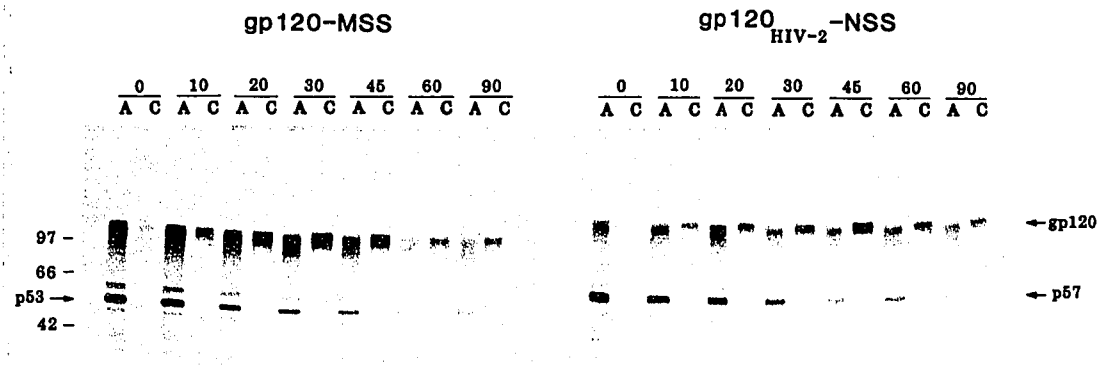
endo H resistant forms. In contrast, CD4 recognized heterogeneous sizes of glycoproteins which migrated from approximately 58 to 76 kDa range (Fig. 28B). Interestingly, the major binding component within this smear appeared to be of a higher molecular weight (about 61 and 76 kDa) and probably represents the partially deglycosylated gp120 molecules. In contrast, when secreted gp120 was treated with endo F/N glycanase, which cleaves all glycan moieties from the molecule (Tarentino, et al. 1989), gp120 migrated to the same position as the nonglycosylated gp120 and still displayed a specific activity in CD4-binding (Fig. 28B). These results clearly demonstrate that, in contrast to previous reports (Matthews et al. 1987; Fennie and Lasky, 1989), deglycosylation of intracellular and extracellular recombinant gp120 with endoglycosidase H or endoglycosidase F/N glycanase under denaturing condition in the presence of SDS or nondenaturing conditions does not abrogate their ability to bind to CD4. Conversely, these results suggest that the carbohydrate moieties on gp120 appear not to be required to maintain the conformation of a CD4-binding domain once it has formed (Fenouillet, et al. 1989; Fenouillet, et al. 1990). It seems likely that N-linked oligosaccharides on the gp120 molecule may play a role in correct folding of the protein to provide the proper conformation so that the protein can be recognized by CD4 receptor.

4. Kinetics of intracellular folding of recombinant gp120

To substantiate further that the folding of gp120 plays an important role in

binding to CD4, the intracellular folding of gp120 expressed by recombinant baculoviruses, vAcgp120-MSS and HIV-2 vAcgp120-NSS was analyzed by their ability to bind to CD4 as a functional assay for overall conformation (Fennie and Lasky, 1989). Recombinant baculovirus-infected SF9 cells were pulse-labelled with [^{35}S]-methionine for 10 min and lysed at different times of chase, after which one half of the lysate was immunoprecipitated with rabbit anti-gp120 serum, while the remaining half of the material was incubated with excess soluble CD4, followed by immunoprecipitation with OKT4. As described previously, the antiserum used was raised directly against denatured nonglycosylated recombinant gp120-ΔS and recognized linear epitopes on gp120 (Materials and Methods); it reacted efficiently with both nonglycosylated and high-mannosylated precursor glycopeptide of gp120, but reacted poorly with its native glycosylated counterparts (Schwartz et al. 1979; Vidal et al. 1989). Significant amounts of nonglycosylated and glycosylated gp120 polypeptides were immunoprecipitated with the antiserum at early pulse-chase period, and thereafter gradually decreased with time (Fig. 29). In contrast, newly synthesised gp120 was unable to bind to CD4, although it was highly glycosylated. The ability of its binding to CD4 was gradually increased with time. This suggests that gp120 is first synthesized as a highly glycosylated but unfolded linear molecules that have not yet attained a native conformation that can be recognized by CD4. With increasing time, however, the protein folded properly to give rise to a correctly folded CD4-binding domain (Fig. 29). This suggests that maturation of the carbohydrate chain confers conformational features on the mature glycoprotein

Figure 29. Time course of intracellular folding of gp120. Infected SF9 cells were pulse-labelled for 10 min with [^{35}S]-methionine at 36 hr p.i. and chased for the indicated time (minutes). Equal portion of cell lysates was either immunoprecipitated with rabbit anti-gp120 serum (A) or OKT4 after complexing with soluble CD4 (C). The precipitated material was then analyzed by SDS-PAGE and visualized by fluorography.


gp120-MSS
gp120HIV-2-NSS
0 10 20 30 45 60 90
A C A C A C A C A C A C A C
97 –
66 –
p53 →
42 –
←gp120
←p57

not present in the nonglycosylated species. Whereas the different folding rate between gp120 with melittin signal sequence and HIV-2 gp120 with its own natural signal sequence has been found to be the consequence of different signal sequences (Fig. 22 and Fig. 23).

DISCUSSION

The role of the carbohydrate on gp120 of HIV and other virus surface proteins is not firmly established. The lack of crystallographic data for HIV gp120 makes it difficult to predict the effects of N-linked oligosaccharides on the interaction of gp120 with CD4. Recent information clearly indicates that N-linked oligosaccharides of different viral glycoproteins play a significant role in determining properties of viral glycoproteins which are dependent on folding to form the proper conformation (Machamer and Rose, 1988; Rose and Doms, 1988; Sodora et al. 1989; Vidal et al. 1989; Wright et al. 1989). Our results agree with those of previously published data (Fenouillet et al. 1989, 1990) that enzymatic removal of glycans in the presence or absence of ionic detergent (SDS) does not significantly affect the native conformation of recombinant gp120; deglycosylated gp120 still retains the structural and biological properties of the native glycoprotein, which can bind to CD4. This indicates that carbohydrate side chains per se are unlikely to be involved in direct contact with CD4 (such as sugar-lectin interaction), as the composition of the oligosaccharide chains can be varied without

loss of CD4 binding ability reported in this study and others (Cordonnier, et al. 1989, Fennie and Lasky, 1989, Dirckx, et al. 1990, Morikawa, et al. 1990a). Rather, N-linked carbohydrate, as others have suggested (Fennie and Lasky, 1989; Morikawa, 1991), may act as molecular "padding", required for gp120 to take up the correct folding to form the proper conformation for CD4 binding. Thus, one explanation for these results would be that N-linked glycosylation is essential for promoting correct folding of gp120. Once the protein folds properly, removal of preformed carbohydrate chain with endo H or endo F/N glycanase has little effect on the interaction between gp120 and CD4. Recent studies on the effect of glycosylation on the reactivity of vesicular stomatitis virus (VSV) G protein with MAbs directed to conformational epitopes have provided evidence that the carbohydrate chains per se do not influence the antigenic specificity of VSV G protein, suggesting that N-linked glycosylation plays a key role in promoting the formation and the stability of disulfide bonds that determine epitope-specific conformational integrity of the VSV glycoprotein (Grigera, et al. 1991).

Based on these facts (Matthews, et al. 1987; Fennie and Lasky, 1989; Fenouillet, et al. 1989, 1990; Vidal et al. 1989; Morikawa et al. 1991), our data are compatible with the hypothesis that N-linked glycosylation of HIV gp120 plays a crucial role in the folding of the protein and its stabilization (Fenouillet, et al. 1989). Further characterization of the requirements for attainment of appropriate gp120 to attain the correct tertiary conformation will not only clarify intracellular pathways of glycoprotein folding, but may also lead to a clear picture as to how the critical primary event of virus-cell interaction in HIV infection occurs.

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APPENDIX: OLIGONUCLEOTIDE SEQUENCES

Crossover linkers

5'-end YL1 5'-AAT TCG GAT CCT ATA AAT ATG AGA GTG AAG-3'

YL2 5'-CAC TCT CAT ATT TAT AGG ATC CG-3'

3'-end YL3 5'-CAG AGA GAA AAA AGA TAA GGA TCC A-3'

YL4 5'-AGC TTG GAT CCT TAT CTT TTT TCT CT-3'

Linkers for direct insertion

gp120-ΔS linkers:

YL5 5'-GAT CCT ATA AAT ATG ACC GAG AAG CTC TGG GTG ACC GTG TAC TAC GGG GTA C-3'

YL6 5'-CCC GTA GTA CAC GGT CAC CCA GAG CTT CTC GGT CAT ATT TAT AG-3'

Interleukin 3 signal sequence linkers:

JMC6 5'-GTA TAA ATA TGC TGC TCC TGC TCC TGA TGC TCT TCC ACC TGG GAC TCC AAG CTT CAA TCA GTG GCC GG-3'

JMC7 5'-GAT CCC GGC CAC TGA TTG AAG CTT GGA GTC CCA GGT GGA AGA TCA GGA GCA GGA GCA GCA TAT TTA TAC TGC A-3'

Primers used for site-directed mutagenesis

YLS-1 5'-AAT TCG GAT CCT ATA AAT ATG AGA GTG GCG GAG ATA TAT CAG CAC-3'

YLS-2 5'-AAT TCG GAT CCT ATA AAT ATG ATA GTG AAG GAG AAA TAT CAG CAC TTG TGG ATA TGG GGG TGG ATA TGG GGC ACC-3'

YLS-3 5'-AAT TCG GAT CCT ATA AAT ATG AGA GTG GCG GAG ATA TAT CAG CAC TTG TGG ATA TGG GGG TGG ATA TGG GGC ACC-3'

YLS-4 5'-AAT TCG GAT CCT ATA AAT ATG ATA GTG GCG GAG ATA TAT CAG CAC TTG TGG ATA TGG GGG TGG ATA TGG GGC ACC-3'

YLS-5 5'-AGC TTG GAT CCT TAT CTT TTT TCT CTC TGC ACC ACT CT-3'

Primers used for sequencing gp120 gene and flanking regions

YM1-UP-3 5'-GTT GCT GAT ATC ATG G-3'

YM1-DOMN-2 5'-TAC GTA CAA CAA TTG TCT GT-3'