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Structural, Immunological, and Functional Characterization of Simian Immunodeficiency Virus Proteins.

Mark Anton Miller

Louisiana State University and Agricultural & Mechanical College

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Structural, immunological, and functional characterization of simian immunodeficiency virus proteins

Miller, Mark Anton, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1992
STRUCTURAL, IMMUNOLOGICAL, AND FUNCTIONAL CHARACTERIZATION OF SIMIAN IMMUNODEFICIENCY VIRUS PROTEINS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Microbiology

by

Mark Anton Miller
B.S., West Texas State University, 1984
May 1992
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FOREWARD


Other parts of the data contained herein have led to the acceptance of a manuscript for publication in AIDS Research and Human Retroviruses (Miller, M. A., Murphey-Corb, M., Montelaro, R. C. 1992. Identification of broadly reactive continuous antigenic determinants of simian immunodeficiency virus glycoproteins. AIDS Res. Human Retroviruses; in press).

A manuscript is currently being prepared for submission which contains other parts of the data reported here (Miller, M. A., Mietzner, T. A., Montelaro, R. C. 1992. Potential role for human immunodeficiency virus (HIV) transmembrane glycoprotein in uncoupling lymphocyte signal transduction pathways. Manuscript in preparation).

Another manuscript is currently being prepared for submission which contains other parts of the data reported here (Miller, M. A., Liebman, J., Rinaldo, C. R., Cloyd, M. W., Montelaro, R. C. 1992.)
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ABSTRACT

Biochemical, immunological, and structural characterizations of the structural proteins of simian immunodeficiency virus (SIV) were performed. Various electrophoretic and chromatographic analyses of native and metabolically radiolabeled virus preparations coupled with immunological procedures such as Western blotting and radioimmunoprecipitation were utilized to identify SIV/DeltaB670-encoded polypeptides. Two-dimensional tryptic peptide mapping of each putative viral protein was employed to confirm that each of these components were unique and not cleavage products of precursor polyproteins.

Site-directed serological studies using synthetic peptides analogous to regions of the SU and TM protein predicted to have high antigenic potential were performed. These studies resulted in the identification of several group-specific and type-specific antigenic determinants of SIV envelope proteins and demonstrated serological similarities between SIV and several other lentiviruses.

The lentivirus lytic peptide (LLP), a segment within the carboxyl terminus of the TM protein which has high amphipathic potential and unusually high positive charge density, was identified during secondary structural modeling studies. Since the LLP appeared to share structural properties with natural cytolytic peptides such as magainins, cecropins, melittin, etc., synthetic peptides homologous to the LLP of HIV-1 and SIV were employed in standard assays designed to measure the ability of cytolytic peptides to inhibit prokaryotic or eukaryotic cells, and were found
to share functional characteristics with natural cytolytic peptides. Results from membrane permeability studies and $^{51}$Cr-release assays suggested that the LLPs inhibit cells via membrane perturbation.

The LLPs also share structural characteristics with calmodulin (CaM)-binding proteins. CaM is the primary sensor of Ca$^{2+}$ within eukaryotic cells. Since Ca$^{2+}$ is the primary signal for activation of many enzyme systems, CaM plays an essential role in the activation of many cellular enzymatic processes. Using gel mobility shift assays and standard phosphodiesterase competition assays, we have shown that LLPs bind CaM with high affinity and can inhibit CaM-mediated activation of CaM-dependent enzymes. LLP-mediated interference with enzyme activation and/or alteration of critical plasma membrane properties may explain many of the unusual manifestations of HIV-1-mediated disease and may represent a novel mechanism of pathogenesis.
CHAPTER 1

INTRODUCTION

Of the many known human pathogens, the retrovirus family of RNA viruses may be the greatest challenge to medical researchers to date. The members of this group of viruses contain an unusual enzyme, reverse transcriptase (RT), that allows them to reverse the usual flow of genetic information, hence the name "retrovirus". Using RT, retroviruses are able to convert their genomic RNA to a DNA intermediate which inserts into the genome of their host cell forming a stable provirus that may remain latent in the host for an extended period; the viral provirus is replicated along with the cellular genetic material and is passed to daughter cells of the host. Using virus-encoded genetic signals, all retroviruses utilize host-specific mechanisms and machinery to express their genes. Interestingly, retroviruses often mutate genes of the host and sometimes even capture cellular genes. These viruses have a very wide host range, as they have been isolated from a diverse group of vertebrate animals including birds, reptiles and mammals (Teich, 1982, 1985) resulting in oncogenic, non-oncogenic, and/or progressive disease syndromes.

The retroviruses comprise a family of structurally and conceptionally related viruses which have been divided into three subfamilies, onco, spuma and lentivirinae, based on morphological and biochemical properties. Until the mid 1980's, the oncovirinae subfamily of retroviruses had been the most thoroughly studied and well characterized
of these subfamilies. The oncoviruses typically establish persistent, slowly progressive infections that ultimately induce cell transformation in vitro, or malignancy in vivo (Gelderblom et al., 1990). The members of spumavirinae, or the foamy viruses have been isolated from many species, including cattle, cats, monkeys, and humans. Spumaviruses have been shown to produce characteristic cytopathic effects in vitro (Gelderblom and Frank, 1987; Hooks and Gibbs, 1975), but their pathogenic potential in vivo is still unknown (Weiss, 1988). The members of the lentivirinae subfamily of retroviruses, such as equine infectious anemia virus (EIAV), visna virus, and caprine arthritis and encephalitis virus (CAEV) have long been recognized as agents which induce slowly progressive but devastating diseases of ungulates. However, in 1983 Montagnier and associates (Barré-Sinoussi et al., 1983) isolated a retrovirus from an immunodeficient human patient that was later classified as a lentivirus and was determined to be the etiological agent responsible for acquired immunodeficiency syndrome (AIDS; Barré-Sinoussi et al., 1983; Gallo et al., 1984; Popovic et al., 1984). Since AIDS had become recognized as a worldwide epidemic (Gottlieb et al., 1981; Masur et al., 1981; Siegal et al., 1981; Lane et al., 1983; Ziegler et al., 1984), the human immunodeficiency virus (HIV) soon became one of the most intensely studied viruses known to man.

Since the initial isolation of the virus now known as HIV-1, new immunodeficiency viruses of man (HIV-2; Clavel et al., 1986), non-human primates (SIV; Daniel et al., 1985; Kanki et al., 1985a; Murphey-Corb et al., 1986; Fultz et al., 1986a), cats (FIV; Pederson et al., 1986), and cattle (BIV;
Gonda et al., 1988) have been discovered. HIV-1 and HIV-2 represent two distinct serotypes of lentiviruses that have been isolated from AIDS patients; those isolated in the United States, Europe, and central Africa are designated HIV-1 ( Barré-Sinoussi et al., 1983; Popovic et al., 1984) while those isolated in west Africa have been designated as HIV-2 (Clavel et al., 1986, 1987). These two distinct serotypes of HIV are closely related, as they share similar genetic organization and 50-60% primary DNA sequence identity (Guyader et al., 1987), and they cause a similar disease syndrome; the primary clinical difference between these virus types is that the course of HIV-2 infection can be attenuated (Kong et al., 1988). Simian immunodeficiency virus (SIV) was originally isolated in 1985 from immunodeficient Macaca mulatta (rhesus macaques; Daniel et al., 1985; Kanki et al., 1985a, Benveniste et al., 1986) and was later isolated from healthy Cercopithecus aethiops (African green monkeys; Ohta et al., 1988; Daniel et al., 1988a) and Cercocebus atys (African sooty mangabeys; Hirsch et al., 1989a; Fultz et al., 1986a; Lowenstine et al., 1986). Virologic and seroepidemiological surveys suggested that african monkeys were the natural hosts of SIV and act as asymptomatic carriers of the virus (Lowenstein et al., 1986; Kanki et al., 1985b; Schneider et al., 1987; Daniel et al., 1988b) while the Asian macaques, which are not natural hosts, experience a fatal AIDS-like syndrome upon infection with SIV (Letvin et al., 1985). Interestingly, SIV is approximately 80% homologous to HIV-2 at the level of DNA sequence and is very similar to HIV-2 serologically, but shares only 50% sequence identity and limited serological cross-reactivity with HIV-1 (Gardner and Luciw, 1988; Peterlin and Luciw, 1988). While
SIV is obviously most closely related to HIV-2, it is also very closely related to HIV-1 both structurally and pathogenically.

**Structural properties and classification of retroviruses**

Members of retrovirinae are enveloped, round or ovoid-shaped viruses that contain two copies of their 8-12 Kb RNA genome; a general model of retrovirus virions is presented in Figure 1. The viral genome contains three major genes (5'-gag-pol-env- 3') that encode the structural proteins of the virus. The *gag* gene encodes a precursor polyprotein that is processed into three or four internal proteins of the virus, including the matrix protein (MA), the major capsid shell protein (CA), and the RNA associated ribonucleoprotein (NC). The *pol* gene encodes a polyprotein that is processed to produce the protease (PR), the reverse transcriptase enzyme (RT), and the integration protein (IN). The *env* gene encodes a polyprotein which is processed to yield the two glycosylated envelope projections of the virus. The highly glycosylated surface glycoprotein (SU) is anchored to the virion by the slightly glycosylated transmembrane protein (TM) which is intimately associated with the viral lipid bilayer. Each virion contains several thousand copies of each of the *gag*-encoded proteins, five to ten copies of the RNA associated RT (Parekh *et al.*, 1980), and several hundred copies of each *env*-encoded protein (Parekh *et al.*, 1980; Gelderblom *et al.*, 1987; Özel *et al.*, 1988; Takahashi *et al.*, 1989). Retrovirus particles are composed of approximately 35% lipid, 60% protein, 3% carbohydrate, and 1% RNA, and it has been shown that viral lipids are derived from the host cell plasma membrane and that viral lipid composition resembles that of the host cell (Aloia *et al.*, 1988). Although all
Figure 1. **General structural model of a retrovirus virion.** This two-dimensional diagram of the HIV virion reflects the morphological and immunoelectron microscopic observations made during studies of retrovirus virion structure as well as specific studies of HIV and other lentivirus structure. General designations for proteins of all retroviruses are made according to the nomenclature devised by Leis et al. (1988). These acronyms are as follows: SU, surface unit; TM, transmembrane protein; MA, matrix protein; LI, link protein; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integration protein. Specific designations for HIV proteins reflecting the protein molecular weight are made according to the nomenclature of August et al. (1974); according to this nomenclature, "p" or "gp" stand for protein or glycoprotein, respectively, followed by the approximate molecular weight of the protein. Recently, a structure designated the core-envelope-link (CEL) has been described (Höglund et al., 1991); this structure is thought to be the remnants of of a morphopoietic protein that might play an essential role in the budding process. It has been speculated that the CEL is composed of LI (p6) in HIV. In support of this hypothesis, deletion mutations that truncate or delete LI negatively effect or eliminate the budding process, respectively (Göttlinger et al., 1990, 1991). LI proteins have been identified only in lentiviruses to date, and there appears to be no comparable protein in other retrovirus subfamilies. Structures known as lateral bodies have been observed in all lentiviruses, except maedi-visna virus, but are not observed in the other retrovirus subfamilies. It has been speculated that these structures are composed of excess gag protein or regulatory proteins, but there is little evidence to date. Several copies of the major histocompatibility complex (MHC) of the host cell become a component of the viral envelope during the budding process. (From Gelderblom, 1991)
retroviruses follow this basic structural model, there are several morphological characteristics that can be used to distinguish between different retrovirus types.

Retroviruses have been classified into three subfamilies on the basis of morphological and morphogenic characteristics (Figure 2; reviewed in Gelderblom, 1991). One of these subfamilies, the oncoviruses, has been further broken into 4 subtypes (types A-D). The spumavirus subfamily is very similar to oncovirus type-B and D viruses, differing mainly in the length of their envelope projections. Members of the lentivirus subfamily are most similar to oncovirus type-C viruses, as the shape of their respective cores are the primary morphological difference.

Members of the lentivirus subfamily of retroviruses are virtually indistinguishable on the basis of morphology. Negative staining and thin section electron microscopy have revealed that the core (composed primarily of CA) of mature lentiviruses is a cone shaped structure that spans almost the entire diameter of the virion. The broad end of the core has an approximate diameter of 60-65 nm and it appears to be separated from the core-associated MA protein by a clear halo, while the narrow end of the core appears to be continuous with the MA protein (Chrystie and Almeida, 1988; Gelderblom et al., 1989). Computer emulation modeling has suggested that the MA protein of lentiviruses forms a 60-sided (32 vertices) icosahedral shell that directly underlies the lipid envelope (Marx et al., 1988); it is postulated that this shell plays a critical role in particle assembly and in arrangement of envelope knobs, however, direct interaction of MA with CA or TM has not been directly demonstrated.
Figure 2. Morphological classification of the retrovirus family. One of these subfamilies, the oncoviruses, has been further subdivided into four groups based on virion particle type. Type-A particles are morphologically completely assembled cores which then migrate to and bud from the plasma membrane to form type-B particles. Similarly, type-D particles arise from pre-formed cores that migrate to the plasma membrane and then bud from the host cell; type-D virions are distinguished from type-B particles by the length of their envelope projections as the envelope spikes of type-B viruses are approximately 4 nm longer than those of type-D oncoviruses. In contrast to type-B and type-D virions, the type-C particle core is formed concomitantly with the budding process, and following the budding process, the core condenses into an icosahedral structure. The morphological characteristics of the spumavirinae subfamily of retroviruses are similar to those of oncovirus types-B and D in that their cores are preformed and they migrate to and bud from the plasma membrane, however, the envelope projections of spumaviruses are 3-4 nm longer than those of type B and 7-8 nm longer than those of type D oncoviruses. Members of the lentivirus subfamily of retroviruses are morphologically most related to the type-C oncoviruses as lentivirus virion cores form at the plasma membrane during the budding process. Lentiviruses can be distinguished from type-C lentiviruses by the shape of the mature virion core as lentiviruses contain a cone-shaped core while type-C oncoviruses contain an icosahedral core. HTLV-I and II, BLV, and STLV-I appear to belong to an intermediate group of retroviruses, as they exhibit morphological features common to both lentiviruses and oncoviruses during assembly and following maturation. (From Gelderblom, 1991).
Using image analysis and rotational techniques, several groups have determined that HIV and SIV (and probably all lentiviruses) have 72 envelope projections per virion (Gelderblom et al., 1987; Özel et al., 1988; Takahashi et al., 1989). Examination of TEM micrographs of HIV thin sections and of negatively stained SIV have revealed triangular structures existing on the surface of virions suggesting that surface knobs reflect trimeric associations of env proteins (Gelderblom et al., 1988, 1989; Grief et al., 1989), while chemical crosslinking studies suggest a trimeric or tetrameric arrangement of SU and TM proteins on the virion surface (Pinter et al., 1989; Schawaller et al., 1989). The intense investigation of HIV and closely related viruses has rapidly led to an accumulation of fine structural details of lentivirus morphology and has revealed a fairly strict conservation of morphological characteristics across the lentivirus subfamily.

**Immunological properties of retroviral proteins**

In light of the structural and morphological similarities between the various members of the lentiviruses, it is not surprising that these viruses also share many immunological features. There is a high degree of immunological cross-reactivity between the gag and pol-encoded proteins of HIV-1, HIV-2, SIV, and EIAV (Clavel et al., 1986; Kanki et al., 1986; Barin et al., 1985; Montelaro et al., unpublished data). In fact, all of the major antigens of HIV-2 and SIV are serologically indistinguishable using typical serological procedures (Barin et al., 1988; Kanki et al., 1988). The kinetics of immune responses elicited by lentivirus infection appear to be similar among the different virus systems. Usually, the first detectable
lentivirus-specific antibody that can be identified reacts with the CA protein (Tindall and Cooper, 1991; Zhang et al., 1988). The next detectable response is usually TM specific, followed by MA and SU-specific responses. The SU protein elicits the highest titer antibody response, followed by the TM and CA proteins.

Western blot analysis of HIV-1 and HIV-2 reactive antisera have suggested that the major type-specific determinants of lentiviruses are located on the envelope glycoproteins (Barin et al., 1985). Since the surface glycoproteins of retroviruses are the primary target of host immune responses (Montelaro and Bolognesi, 1978) and since emergence of point mutations in the envelope proteins appears to be a mechanism of rapid antigenic variation in lentiviruses (Clements et al., 1980; Narayan et al., 1977; Salinovich et al., 1986) it is logical that lentiviral envelope glycoproteins are more diverse and should contain type-specific determinants. At least one type specific determinant that distinguishes HIV-1 from HIV-2 or SIV has been identified via site directed serology using synthetic peptides (Norrby et al., 1987 and 1989). The immunogenicity and variability of lentiviral envelope proteins suggest that they are the most important humoral immunological determinants of these viruses, and that further immunological characterization of these proteins is essential.

**Lentivirus genome organization**

While the genome organization of all retroviruses is very similar, the genomes of lentiviruses are more complex than the genomes of most other retroviruses. While all replication competent retroviruses encode
the \textit{gag}, \textit{pol}, and \textit{env} genes, members of the lentivirus subfamily typically encode several additional protein-coding genes. The primate lentiviruses each express at least six additional proteins, trans-activator (\textit{tat}), regulator of expression of virion proteins (\textit{rev}), virion infectivity factor (\textit{vif}), viral protein R (\textit{vpr}), and negative factor (\textit{nef}; Gallo \textit{et al.}, 1988) and either viral protein X (\textit{vpx}; SIV and HIV-2; Henderson \textit{et al.}, 1988b) or viral protein U (\textit{vpu}; HIV-1; Gallo \textit{et al.}, 1988) while an equine lentivirus, equine infectious anemia virus (EIAV), expresses three additional proteins (Rasty \textit{et al.}, 1990; Schiltz \textit{et al.} in press). Most of these additional proteins have been shown to have regulatory functions and are expressed from spliced, subgenomic-length mRNAs (Muesing \textit{et al.}, 1985; Guyader \textit{et al.}, 1987; Viglianti \textit{et al.}, 1990; Rasty \textit{et al.}, 1990; Schiltz \textit{et al.} in press). Obvious similarities in the organization of genetic material between several lentiviruses, most specifically the primate lentiviruses, can be readily seen upon comparison of genome maps (Figure 3). As mentioned earlier, SIV shares a higher degree of nucleotide sequence identity with HIV-2 than with HIV-1, however, all of these primate lentiviruses appear to be highly related both genetically and morphologically.

\textbf{Potential animal models for AIDS.}

As the AIDS epidemic continues to grow and to affect previously low-risk groups of people, development of chemotherapeutic and vaccine protocols becomes an even more essential goal. The most productive tool for testing the efficacy of drug and vaccine candidates would be an AIDS model in which HIV infects and induces an AIDS-like disease syndrome in a readily available laboratory animal. Although many attempts have
Figure 3. Genomic maps of several representative lentiviruses. In addition to the *gag*, *pol*, and *env* genes encoded by all retroviruses, lentiviruses possess several additional open reading frames (ORFs), most of which are known to code for regulatory proteins. The HIV-1 genome encodes six of these additional genes: virion infectivity factor (*vif*), virion protein R (*vpr*), virion protein U (*vpu*), trans-activator of transcription (*tat*), regulator of expression of virion proteins (*rev*), and negative factor F (*nef*). Similarly, the HIV-2 and SIV genomes encode *vif* (virion infectivity protein or *vip* for SIV), *vpr*, *tat*, *rev*, and *nef*, but do not code for *vpu*. However, HIV-2 and SIV do encode an ORF for viral protein X (*vpx*). EIAV, an equine lentivirus, encodes three additional ORFs designated *S1*, *S2*, and *S3*. 
been made to infect a variety of laboratory animals with HIV, the only non-human animals that have become reproducibly infected are chimpanzees (Alter et al., 1984; Fultz et al., 1986b; Gajdusek et al., 1984, 1985), gibbon apes (Lusso et al., 1988), and rabbits (Felice et al., 1988). Unfortunately, chimpanzees and gibbon apes are extremely scarce and they do not develop any clinical signs of HIV infection and, therefore, are not an ideal model for AIDS infections. Rabbits are the only small laboratory animal that can be infected with HIV, however, they do not develop a typical AIDS-like syndrome and, since their immune system is clearly less similar to that of humans than the closely-related non-human primates, the rabbit does not represent an ideal animal model.

Since there is not an ideal animal model for HIV infection, it is important to investigate alternative lentivirus systems in which an AIDS-like disease results from infection with the particular virus. As was previously discussed, SIV is a naturally-occurring lentivirus isolated from many species of non-human primates that is morphologically and genetically similar to HIV-1 and HIV-2. This virus induces a fatal AIDS-like syndrome in macaques, and among animal lentiviruses, the disease syndrome induced by SIV most closely approximates infection of humans with HIV (Desrosiers and Letvin, 1987; Schneider and Hunsmann, 1988; Gardner and Luciw, 1989). Similarities in the disease include CD4+ lymphoid and macrophage cell tropism, CD4+ cell depletion, similar serological and immunological responses, and similar pathology (including neuropathology and opportunistic infections). Since SIV does not induce disease in its natural hosts (the african monkey species) but
does induce lethal AIDS-like disease in Asian monkey species, study of this virus system may lead to discovery of the differences in immune responses produced by African species that lead to protection against SIV-induced pathology.

**RESEARCH OBJECTIVES**

The main objectives of the body of research presented here were to characterize the protein composition of SIV/DeltaB670 and to identify determinants of the virus that play an important immunological or pathogenic role(s) in SIV infection and disease progression. More specific questions regarding these objectives are listed below.

1. What is the protein composition of SIV/DeltaB670? Can we identify structural determinants of these proteins that may play an important role in SIV-induced pathogenicity. Answers to these questions were necessary to determine whether SIV/DeltaB670 would be a desirable model for HIV; in addition, identification of important structural determinants of SIV would lead to further study of these determinants and possibly to testing of chemotherapeutic and vaccine strategies based on these determinants.

2. Using synthetic peptide methodologies coupled with site-directed serology, can we identify immunological determinants that may play an important role in immunological management of SIV infection? Due to obvious risks associated with attenuated or killed whole virus vaccines, subunit vaccines that induce protection against HIV/SIV must be the ultimate goal of AIDS vaccine research. The proposed studies may lead to the identification of subunit vaccine candidates, but at the very least, they
will enable us to evaluate the immunological relatedness between SIV and HIV.

(3) Does the region near the carboxyl terminus of the TM of SIV and other lentivirus, that was identified while answering question #1 above, play a role in viral cytopathicity? Does this segment of TM share functional, as well as structural similarities to natural cytolytic peptides? Answers to this question could shed light on one of the primary mysteries confronting AIDS researchers: "How do the immunodeficiency viruses kill their host cell?" Several lines of evidence have implicated the carboxyl end of TM as the mediator of cytopathicity. Identification of the exact region of TM that contributes to cytopathicity, and its mechanism of action, may lead to efficacious therapeutic intervention strategies.

(4) Does the potentially cytopathic segment of TM also play a role in perturbation of enzyme functions in lentivirus-infected cells by competing with calmodulin-dependent enzymes for calmodulin binding? Many enzymatic pathways in all eukaryotic cells require calmodulin as a Ca\(^{2+}\)-second messenger. As intracellular Ca\(^{2+}\) concentration is a major signalling mechanism for many cellular enzyme systems, and more specifically, is the primary signal for lymphocyte activation, any interruption in the delivery of this signal could result in cellular dysfunction. Evidence that HIV induces enzymatic dysfunction in cells is mounting, and by answering this question, we may identify a mechanism by which lentiviruses induce this dysfunction.
CHAPTER 2

MATERIALS AND METHODS

Propagation of virus

SIV/DeltaB670 was originally isolated from a rhesus monkey with AIDS-like disease syndrome (Baskin et al., 1986). SIV/Delta B670 virions were purified from infected H9 or CEM cell cultures three to four days after subculture. Tissue culture supernatants were clarified to remove cellular debris and then virions were concentrated using a tangential-flow filtration device (Millipore, Bedford MA). Virions were then pelleted by centrifugation at 53,000 X \( g \) for 90 min over a 20% glycerol cushion in PBS. Pelleted virus was resuspended gently in PBS and banded by centrifugation at 250,000 X \( g \) in a 30-45% sucrose step gradient prepared in 0.01 \( M \) phosphate buffer (pH 7.4). Virus at the gradient interface was collected, diluted three-fold in 0.01 \( M \) phosphate buffer, and pelleted by centrifugation at 250,000 X \( g \) for 60 min to remove residual sucrose. The resulting virion pellet was resuspended in 0.01 \( M \) phosphate buffer and stored at -70°C until use.

Affinity purification of viral glycoproteins

Lentil lectin affinity chromatography was performed as previously described (Montelaro et al., 1983). Briefly, lentil lectin-sepharose 4B, packed in a glass column (0.8 cm diameter) to a bed volume of approximately 5 ml, was equilibrated in start buffer (0.02 \( M \) tris, 0.1 \( M \) sodium chloride, 0.1% deoxycholate, pH 8.3). Virions were disrupted and lipids were removed by precipitation in 10 volumes cold acetone prior to
chromatography. Viral proteins were diluted to 0.5 mg/ml in disruption buffer (start buffer supplemented with 0.5% deoxycholate), loaded onto the column at approximately 10 ml/hr, and lectin non-binding proteins were eluted with start buffer. Glycoproteins were eluted from the column using elution buffer (0.2 M methylglucoside in start buffer). Lentil lectin binding and non-binding protein fractions were then dialyzed extensively, lyophilized to dryness, and resuspended in 0.01 M phosphate buffer.

**SDS-PAGE and Western analysis.**

The procedures for SDS-PAGE electrophoresis and Western blotting has been previously described (Montelaro et al., 1984; Lo et al., 1990). Briefly, SDS-PAGE was performed on 10:0.8% acrylamide:BIS slab gels containing 0.1 M phosphate buffer using a 0.01 M phosphate running buffer at pH 7.2. Electrophoresis was performed on a Protean II electrophoresis unit (BioRad Laboratories, Richmond, CA). Protein bands were visualized by staining with Coomassie brilliant blue for 30 min, followed by immediate destaining with multiple changes of methanol-acetic acid-water (10:3:27). To identify immunoreactive components, SDS-PAGE resolved proteins were transferred to nitrocellulose in 150 mM Glycine (BioRad Laboratories), 20 mM Trizma Base (Sigma Chemical Co.) using a Hoefer Transphor Unit (Hoefer Scientific, San Francisco, CA) as previously described (Burnette, 1981), immunoblotted with either reference serum from an experimentally-infected macaque (#B845) or ammonium sulfate concentrated hybridoma supernatants containing monoclonal antibodies. Immune complexes were detected using I\(^{125}\) labeled protein A, or a variety of secondary antibody-enzyme conjugates. Prior to immunoblotting, glycosylated proteins were detected using a previously
described procedure (Clegg, 1982) while total protein profile was visualized via reversible staining with Ponceau S staining as previously described (Salinovich and Montelaro, 1986).

Metabolically radiolabeled virus preparations were analyzed on 200-mm cylindrical gels; the gels were then fractionated into 1-mm fractions with an automatic fractionator (Gibson Medical Electronics, Middleton, Wisc.), and the fractions were assayed for radioactivity (Montelaro et al., 1978; Montelaro and Bolognesi, 1978; Parekh et al., 1980).

**HPLC-purification of SIV/DeltaB670 proteins**

Viral proteins were separated via reverse-phase high pressure liquid chromatography (RP-HPLC) on a Waters 600E HPLC system employing a 8 X 100-mm µBondapak phenyl Radial-pak cartridge with the Radial Compression Module-100 (Waters). Gradient-purified virions in 0.01 M sodium phosphate, pH 7.4 were disrupted in two volumes of 6 M guanidine hydrochloride, pH 3.1 immediately prior to injection. Separations were performed using a multistage gradient and a mobile phase consisting of water with 0.1% TFA (solvent A) and 2-propanol with 0.1% TFA (solvent B) at a flow rate of 0.7 ml/min. The gradient was modified from that reported by Ball and associates (1988) for purification of EIAV proteins.

**Two-dimensional tryptic peptide mapping**

SIV/DeltaB670 proteins were purified from gradient-purified virion preparations via reverse phase high pressure liquid chromatography (RP-HPLC) followed by SDS-PAGE. Protein bands were excised directly from Coomassie blue-stained SDS-PAGE gels, and tryptic peptide mapping was performed as previously described (Elder et al., 1977a; Elder et al., 1977b;
Montelaro et al., 1984; Salinovich et al., 1986). Briefly, following extensive washing in 25% isopropanol and 10% methanol, protein bands were labeled by in situ radioiodination using the chloramine T method (Greenwood and Hunter, 1963) and then washed thoroughly in 10% methanol. The labeled proteins were then subjected to exhaustive digestion with trypsin (DPCC-treated; Sigma) and then protein fragments were eluted from the gel slice. The peptides were applied to a 20 X 20 cm cellulose-coated thin-layer chromatography plate (EM Industries, Darmstadt, Germany) and separated in the horizontal direction by high voltage electrophoresis in solvent consisting of 15% glacial acetic acid, 5% formic acid, and 80% H$_2$O. The peptides were then separated in the vertical direction via liquid chromatography in 40% n-butanol, 30% pyridine, 6% glacial acetic acid, and 24% H$_2$O. The separated peptides were detected by autoradiography with Kodak X-Omat film (XRP-5).

**Gas phase N-terminal amino acid sequencing**

Protein sequencing was performed on an Applied Biosystems 470-A gas phase protein sequencer furnished with a fraction collector capable of accommodating the Waters WISP 710B limited volume inserts. Phenylthiohydrantoin (PTH)-derivitized amino acids generated by the sequencer were analyzed using a Waters HPLC system equipped with two high performance pumps (Waters Model 510), a temperature control module, a variable wavelength ultraviolet detector (Waters, Model 481), an automatic sample injection module (WISP 710B), and a Waters 840 computer interface for data acquisition and processing.

Soluble protein samples, purified via RP-HPLC as previously described (Ball et al., 1988), were applied directly to TFA-treated glassfiber
discs and inserted directly into the sequenator reaction cartridge. Sequencing was performed according to manufacturer's instructions. PTH derivatives of the amino acids cleaved from the amino terminus during each sequencing cycle were identified by RP-HPLC using a 3.8 X 15 cm Novapak C18 column (Waters) via linear gradient separation with a mobile phase consisting of 0.014 M sodium acetate containing 5% aqueous tetrahydrofuran and acetonitrile.

**Secondary structure predictions**

The predicted amino acid sequence of SIVmac251 (Hirsch *et al.*, 1987) was analyzed manually, using Chou-Fasman rules for prediction of protein conformation (Chou and Fasman, 1978), to predict regions which have high potential to form α-helical, β-sheet, and turn secondary structure motifs. These sequences were also analyzed using the UWGCG PeptideStructure/PlotStructure protein structure prediction algorithms (Starcich *et al.*, 1986) on a VAX 750 host system; these programs predict secondary structure by the Chou-Fasman (1978) and the Garnier-Osquethorpe-Robson methods (Garnier *et al.*, 1978) along with the Hopp-Woods (1981) and Kyte-Doolittle (1982) algorithms for calculating hydropathy. In addition, the computer algorithm AMPHI (Margalit *et al.*, 1987) was used to predict regions of the predicted SIVmac251 *env* and *gag* sequences that have high potential to form amphipathic helical segments. The output from each of these computer algorithms was considered as composite secondary structure predictions were made.

**Prediction of epitopes**

The predicted amino acid sequences of SIVmac (Chakrabarti *et al.*, 1987; Hirsch *et al.*, 1987) and SIVsm (Hirsch *et al.*, 1989a) *env* polyproteins
were analyzed using the SurfacePlot computer algorithm which was designed to predict continuous epitopes on the basis of hydrophilicity (Parker et al., 1986), flexibility (Karplus and Schulz, 1985), and accessibility (Janin, 1979). Sequence segments assigned antigenic index values over 50 on the 60% composite SurfacePlot profile were considered strongly predicted as potential epitopes. These sequences were also analyzed using the UWGCG PeptideStructure/PlotStructure protein structure prediction algorithms (Starcich et al., 1986) on a VAX 750 host system. The UWGCG algorithms use the Jameson and Wolf method (1988) of epitope prediction which consolidates calculated values for hydrophilicity (Hopp and Woods, 1981), surface probability (Emini et al., 1985; Janin et al., 1978), and flexibility (Karplus and Schulz, 1985). The results obtained for each sequence from both of these computer algorithms were used to predict a composite antigenicity profile of the SIVmac251 env sequence.

**Synthesis, purification, and characterization of peptides**

All synthetic peptide sequences used in these studies were synthesized as amides either manually on a Rapid Multiple Peptide Synthesizer (DuPont, Boston, MA), utilizing F-moc chemical strategies as described previously (Atherton et al. 1988; Carpino and Han, 1972; Fontenot et al., 1991), or using a SAM II automatic peptide synthesizer (Biosearch, San Rafael, CA) employing t-BOC chemical strategies (Barany and Merrifield, 1979; Fontenot et al., 1991; Merrifield, 1963). Following cleavage from supporting resins, all peptides were initially subjected to gel filtration using Sepadex G25 (Sigma Chemical Co., St. Louis, MO) in a glass column (3 cm X 53 cm) with a mobile phase consisting of 10-50%
acetic acid depending on peptide solubility. Eluting peptides were detected using a UA-5 absorbance detector (ISCO, Lincoln, NE).

Gel filtered peptide synthesis products were analyzed via reverse-phase high pressure liquid chromatography (RP-HPLC) using a Delta Prep 3000 preparative chromatography system (Waters, Milford, MA) employing a 0.39 X 15 cm µbondapak C18 column (Waters). Separation was accomplished using linear gradient elution of the mobile phase beginning with initial conditions of 95% solvent A (0.1% TFA in water) and 5% solvent B (0.1% TFA in acetonitrile) with a flow rate of 1.5 ml/min. Typically, a 2%/min linear gradient was employed until all peptide products eluted from the column. Major components were collected manually upon elution and lyophilized to dryness. To identify the peptide component corresponding to the theoretical molecular weight, mass spectrometry was performed on HPLC-fractionated peptides using a 252Cf Plasma Desorption Mass Spectrometer BIO ION 20 employing an acceleration voltage of 15 Kv with 8 k channels for a duration of one million counts. The BIO ION 20 was interfaced with a PDP 11/73 based data acquisition and processing system. Samples in a solution of ethanol: 0.1% TFA in water (50:50 ratio) were applied to a nitrocellulose coated foil, allowed to absorb for 15 minutes, spun dry, and placed in the instrument for analysis. Once the desired peptide products were identified by mass spectroscopy, they were purified via preparative RP-HPLC on a 1.9 X 15 cm µbondapak C18 column (Waters) using linear 1%/min gradient elution at a flow rate of 10 ml/min. Eluting peptide was collected manually, frozen, and lyophilized to dryness. Purified peptide was then subjected to analytical RP-HPLC to access purity.
Magainin-2 peptide was purchased commercially (Calbiochem, La Jolla, CA). RP92 and RP93 peptides were a gift from Drs. Scott Putney and Kashi Javharian of Repligen Corporation, Boston, Mass.

**Peptide enzyme-linked immunosorbent assay (P-ELISA)**

Poly-L lysine was diluted in 0.05 M bicarbonate buffer (pH 9.6) to a final concentration of 10 ng/50 μl/well. This solution was allowed to adsorb onto the wells of a 96-well Immulon I microtiter plate for 1 hr at room temperature. The wells containing adsorbed poly-L lysine were washed once with phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4), treated with 50 μl 1% glutaraldehyde in phosphate buffered saline (PBS) for 15 min at room temperature, then washed once with PBS. Peptides corresponding to predicted epitopes of SIV and diluted to a final concentration of 10 ng/50 μl/well in PBS were dispensed into the poly-L lysine coated wells. The plate was sealed and the peptide allowed to adsorb overnight at room temperature. The plate was washed 2 times with PBS using a Corning 26305 ELISA plate washer (Corning, Corning, NY). In order to minimize non-specific adherence of antibody to the wells, 1 M glycine (100 μl/well; Bio-Rad, Richmond, CA) in PBS was added and incubated for 1 hr, followed by treatment with 100 μl 2.5% non-fat dry milk and 0.5% gelatin (Difco, Detroit, MI) in PBS. A 50 μl sample of test serum diluted in 5% non-fat dry milk in PBS (blotto) was then added to each well and incubated for 1 hr at room temperature. After washing 5 times with approximately 200 μl ELISA wash solution (E-wash; 1 M NaCl, 0.05% polyoxyethylene-sorbitan monolaurate (Tween 20; Sigma Chemical Co.) using an Ultra Wash 2 microplate washer (Dynatech Laboratories, Torrance, CA) to remove
unbound antibody, 50 μl of urease-conjugated goat anti-human IgG (Sigma Chemical Co.) diluted 1/1000 in blotto (to detect monkey primary Ab) or 50 μl of peroxidase-conjugated goat anti-rabbit (Sigma Chemical Co.) diluted 1:1000 in blotto (to detect rabbit primary Ab) were added and incubated for 1 hr at room temperature. Unbound urease-antibody conjugate was removed by washing 3 times with approximately 200 μl E-wash followed by 3 washes with water. The antibody reactivity was then detected by addition of 100 μl substrate solution (40 mg bromocresol purple (Sigma Chemical Co.), 500 mg urea (Bio-Rad), 35 mg EDTA, 500 ml H2O; pH to 5.0 with 0.1 N NaOH). The calorimetric reaction in each well was recorded after 1 hr and then again after 4 hrs to assure detection of weak reactivities. Absorbance measurements were made at 570 nm with an automatic ELISA plate reader (model MR700, Dynatech Laboratories).

Titration of rabbit anti-peptide responses were performed as described above except that the secondary Ab used was peroxidase-conjugated goat anti-rabbit and the substrate consisted of 40 mg 4-chloro-1-napthol, 5 ml cold HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ), 25 ml PBS, and 15 μl H2O2 (Sigma Chemical Co.). Absorbance measurements were made at 490nm.

**SIV-infected monkey serum panels.**

Four distinct panels of SIV-infected monkey sera were employed in these analyses: SIVmac-infected and SIV/DeltaB670-infected rhesus macaques suffering from AIDS-like syndrome, and asymptptomatically infected sooty mangabeys and African green monkeys. The macaque sera were obtained from animals housed at the Tulane Regional Primate Center. The mangabey monkey sera used for this study were all obtained
from Yerkes Regional Primate Research Center. The African green monkeys used for this study were obtained from many sources, both African and Asian, and the source of virus infection is unknown in all cases.

**Anti-peptide antisera production**

Peptide specific antisera (SAM35, SAM26, and SAM56) was produced in New Zealand white rabbits (Hazelton Research Products, Denver, PA) by multiple intramuscular injections (hip) of peptide in MDP adjuvant (Syntex, Cambridge, MA). The initial dosage of peptide was 5 mg. Three weeks after the initial dosage, a 5 mg boost was administered. A second boost consisting of 2 mg of peptide was given following another three week period. 5 weeks after the second boost, weekly 1 mg dosages were administered for 4 weeks and the animals were sacrificed. All other peptide-specific antisera were produced in New Zealand white rabbits (East Acres Biologicals, Southbridge, MA) by multiple IM injections of peptide glutaraldehyde-crosslinked to keyhole limpet hemocyanin (Sigma Chemical Co.). Inoculations with approximately 1.5 mg of peptide-carrier complex were performed at three week intervals and serum samples were collected weekly. Serum samples from all animals, taken prior to each injection of peptide antigen, were screened for anti-peptide antibody via P-ELISA.

**Neutralization assays**

The procedures for SIVmac251 neutralization assays have been previously described (Langlois et al., 1991). Briefly, peptide-specific rabbit antibody preparations were diluted two-fold in microtiter plates. A 30 µl aliquot of virus, containing 100 syncytia-forming units, was added to the
wells and the plates were incubated for 30 minutes at 37°C. HUT-78 target
cells (3 X 10³ cells in 30 μl) were added to each well. Syncytia were
enumerated 3-4 days post-challenge. The neutralization titer was
expressed as the reciprocal serum dilution inhibiting syncytium
formation by 90%.

Neutralization blocking experiments were performed by pre-
incubation of a neutralizing macaque reference serum with each of the
synthetic peptides described in this study prior to addition of the sera to the
assay. All remaining steps were performed as described above.

**Identification of potential amphipathic α-helices**

The transmembrane protein sequences of HIV-1 (Ratner et al.,
1985), HIV-2 (Guyader et al., 1987), SIV (Hirsch et al., 1987), and EIAV
(Rushlow et al., 1986) were analyzed using the UWGCG Peptide
Structure/Plot Structure secondary structure prediction algorithms on a
VAX 750 host system. Regions within the carboxy terminal end of the
transmembrane protein predicted to form α-helical structures were
further analyzed using the Amphi algorithm (Margalit et al., 1987) to
identify amphipathic α-helical regions. Each helical region was modeled
using the SYBYL molecular graphics software on an Evans and
Sutherland PS 390 interactive graphics system with a VAX 750 host
system; α-helical secondary structure was assumed for construction of
these models and residue polarity assignments were made according to
the polarity scale of transmembrane helices (Engleman et al., 1986).

**Prokaryotic killing assays**

The specific cytopathic activity of synthetic peptides for prokaryotic
cells was assayed using standard procedures (Lehrer et al., 1983). Briefly,
one ml suspensions of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in 10 mM K$_2$HPO$_4$ buffer were incubated for one hour at 37°C with SIV-L, HIV-L, Magainin-2, RSV-Pep, HTLV-1-Pep, or Control-Pep at 1 μM, 10 μM, and 100 μM concentration; three different concentrations of bacteria were treated. Each peptide was dissolved in 0.01% acetic acid solution to assure complete solubility prior to its addition to bacterial suspensions. Following incubation, the bacterial suspensions were serially diluted (10-fold) and 100 μl of each dilution was spread evenly over the surface of a TSA plate and then incubated overnight at 37°C. Colonies were then counted to determine the number of survivors of each treatment.

**Eukaryotic killing assays**

The specific cytopathic activity of synthetic peptides with eukaryotic cells was assayed using standard procedures previously for studies of retrovirus-induced cell killing (Rasheed *et al.*, 1986; Temin and Kassner, 1974). One ml cultures (5 X 10$^5$ cells/ml) of the RH9 subclone of HuT78 cells were incubated with HIV-L, SIV-L, RSV-pep, HTLV-1-pep, or control-pep at the indicated concentrations. Each peptide was dissolved in 0.01% acetic acid solution to assure complete solubility prior to its addition to bacterial suspensions. Following incubation for 24 hours at 37°C in RPMI 1640 medium with 10% serum supplement, the cells were stained with the vital exclusion dye trypan blue and the numbers of viable cells were determined by counting in a hemocytometer.

**$^{51}$Cr-release assays**

Fresh human peripheral blood leukocytes, obtained from various donors, were grown in the presence of $^{51}$Cr for 24 hours. The cells were
washed and then cultured until confluent growth in 96 well plates was attained. Various peptides, including LLPs were added exogenously to the cell cultures to a final concentration of 10 and 100 \(\mu M\) followed by a 24 hour incubation period. Following incubation, the supernatants were clarified and assayed for radioactivity to determine the amount of \(^{51}\text{Cr}\) that was released by the cells. Viability of the cells was then measured by counting the cells in a hemacytometer following staining with the vital exclusion dye trypan blue. Cell pellets were lysed and assayed for radioactivity to determine the amount of label not released from the cells.

**Membrane permeability assays**

Membrane permeability was assessed by quantitating the influx of radiolabeled molecules into cells by methods previously described (Cloyd and Lynn, 1991; Bashford *et al.*, 1983; Smolen *et al.*, 1986). Briefly, 30 \(\mu l\) test peptide suspension was added to suspensions of 1.2 \(X\) \(10^6\) CEM cells in 120 \(\mu l\) of PBS lacking \(\text{Ca}^{2+}\) at 0\(^\circ\)C. Between 10 and 20 microcuries of \(^{45}\text{Ca}^{2+}\), \([\text{C}^{14}]\text{sucrose}, \text{or [C}^{14}]\text{inulin} were then added to these suspensions. To assay for permeability of membranes, an aliquot containing 2 \(X\) \(10^5\) cells (20 \(\mu l\)) were removed from the suspension every 30 seconds following addition of radioactive compounds. These cells were pelleted through silicone oil to separate the cells from their aqueous environment. The tip of the microfuge tube containing the cell pellet was then excised and placed in a scintillation tube. The cell pellet was allowed to soak overnight in scintillation solvent and was then assayed for radioactivity. The cpm of \(^{45}\text{Ca}^{2+}\), \([\text{C}^{14}]\text{sucrose}, \text{or [C}^{14}]\text{inulin in pellets from peptide treated cell suspensions were compared with those from untreated cells and any increase in cpm indicated increased membrane permeability. As a
control for maximum permeability, 1 µl of 200 µM digitonin was added to
the last aliquot of cell suspension and allowed to incubate for 1 minute,
and the influx of radioactive compounds was assayed as before. In
addition, one aliquot of cell suspension was placed directly into
scintillation vial/solvent and assayed to determine background cpm in the
cell suspension prior to cell separation.

**Gel mobility shift assays**

Gel mobility shift assays were performed as previously described
(Head and Perry, 1974; Erickson-Viitanen and DeGrado, 1987). Briefly,
when investigating calmodulin (CaM) binding in the presence of calcium,
calmodulin was incubated for 1 hour at room temperature in the presence
of test peptide in binding buffer consisting of 100 mM Tris base (Sigma), 4
M urea (Bio-Rad), and 0.1 mM CaCl₂ at pH 7.2. Once the binding reaction
was complete, the calmodulin/peptide mixture was analyzed via non-
denaturing PAGE on 12.5% gels containing 0.375 M Tris pH 8.8 (Sigma), 4
M urea (Bio-Rad), and 0.1 mM CaCl₂ using electrophoresis buffer
containing 25 mM Tris (Sigma), 192 mM glycine (Bio-Rad), and 0.1 mM
CaCl₂ at pH 8.3. When investigating CaM-binding in the absence of
calcium, the calcium-specific chelator ethylene glycol-bis(b-aminoethyl
ether) n,n,n',n'-tetraacetic acid (EGTA, Sigma) was substituted for
calcium in the binding buffer, resolving gel, and electrophoresis buffer at 2
mM concentration.

**Identification of calmodulin-binding proteins on blots**

Extracts from HIVIIIB, HIVRF, and SIV/DeltaB670-infected CEM
cells, as well as gradient purified HIV and SIV virions were resolved on
12% SDS-PAGE as previously described (Laemmli, 1970) and transferred to
nitrocellulose as previously described (Burnette, 1981). As previously described (Billingsley et al., 1990), the membrane was pre-incubated for 1 hr in blocking solution (50 mM Tris-HCl [pH 7.4] containing 150 mM NaCl, 2 mM CaCl₂, and 1% BSA). Following three 10 min washes in washing buffer (50 mM Tris-HCl [pH 7.4] containing 150 mM NaCl), the membrane was incubated with biotinylated calmodulin (0.5 µg/ml in blocking buffer) for 1 hr. Following three 10 min washes with washing buffer, the membrane was incubated with Vectastain avidin-biotin-alkaline phosphatase conjugate (Vector Laboratories, Inc., Burlingame, CA) for 30 min. Following three washes in washing buffer, the membrane was incubated in alkaline phosphatase substrate kit II (Vector) prepared in 100 mM Tris-HCl with 20 mM CaCl₂ until banding was complete.

Precipitation of calmodulin-binding proteins of HIV-1 and SIV/DeltaB670 virus preparations.

Extracts from HIVIIIb, HIVRF, and SIV/DeltaB670-infected CEM cells, as well as gradient purified HIV and SIV virions were disrupted in 0.1% deoxycholic acid. Approximately 80 µg of each protein sample was pipetted into an eppendorf tube and the total volume of each sample was adjusted to 100 µl with 0.1% DOC/2 mM CaCl₂. 100 µl calmodulin-sepharose 4-B beads (Pharmacia), washed several times with 0.1% DOC/2 mM CaCl₂ and saturated in same buffer was added to each sample. The samples were vortexed gently overnight at 4°C. The beads were washed 3X in 0.1%DOC/2 mM CaCl₂. During the final wash, the beads were transferred to a fresh eppendorf tube to assure that only CaM-sepharose bound proteins would be analyzed further. The beads were resuspended in electrophoresis sample buffer (10% SDS; 10% glycerol; 100 mM Tris, pH 6.8;
0.001% bromphenyl blue; 0.5% 2-mercaptoethanol), boiled for three minutes to remove proteins bound to sepharose beads and to reduce disulfide crosslinking, and the proteins were analyzed via SDS-PAGE as previously described (Laemmli, 1970). Resolved proteins were then electrophoretically transferred to nitrocellulose or immobilon membrane and immunoblotted using sera from HIV-1 or SIV immune reference sera as described by Burnette, 1981). Immune complexes were detected using either biotin-conjugated anti-human (Sigma) or peroxidase-conjugated anti-human (Sigma) secondary antibody preparations along with matching substrate.

**Phosphodiesterase assays**

To demonstrate the ability of LLPs to bind to and inhibit normal activities of CaM, and to determine the affinity of LLPs for CaM relative to a known CaM-binding peptide (DG-A, described in Chapter 6), LLPs were employed in standard phosphodiesterase (PDE) assays as previously described (Schiefer, 1986). To establish a calibration curve of PDE activity in the presence of CaM, these solutions were added sequentially to a cuvette:

1. 100 μl 0.1 M glycylglycine (Boehringer Mannheim, Indianapolis, IN) pH 7.5;
2. 700 μl 0.1 M glycylglycine/3 mM CaCl₂;
3. 30 μl 40 mM MgSO₄ (Sigma);
4. 10 μl 400 kU/l adenosine deaminase (ADA, Boehringer Mannheim);
5. 20 μl 1500 kU/l alkaline phosphatase (AP, Boehringer Mannheim);
6. 100 μl CaM (Boehringer Mannheim) standard solution (diluted 10-fold from a 1 mg/ml stock);
7. 10 μl 0.01% acetic acid;
8. 10 μl 0.3 U/ml PDE in 0.1 M glycylglycine, pH 7.5. The reaction was started by addition of (7) 30 μl 2.9 mM cyclic adenosine 3':5'-monophosphate (cAMP;
Boehringer Mannheim) followed by thorough mixing. The resulting decrease in absorbance at 265 nm was monitored over a 12 minute period using a Shimadzu UV160U visible recording spectrophotometer (Shimadzu, Kyoto, Japan) with a light path length of 10 mm. Inhibition assays were performed by adding 10 μl test peptide solution (diluted series performed in 0.01% acetic acid) instead of adding solution 5 (10 μl 0.01% acetic acid) and adding 100 μl of the standard CaM dilution required for 50% maximal PDE activity (as determined from PDE-CaM calibration curve). Absorbance change was monitored as previously described.
CHAPTER 3

CHARACTERIZATION OF SIV/DELT A PROTEINS

Introduction

In 1986, Murphey-Corb and associates at the Tulane Primate Center reported the isolation of an HIV-related retrovirus, SIV/Delta, from asymptomatic sooty mangabeys (Cerocebus atys) and rhesus macaques (Macaca mulatta) suffering from a transmissible AIDS-like syndrome (Murphey-Corb et al., 1986). The immunosuppressive syndrome experienced by macaques consisted of opportunistic infections, lymphoid atrophy or hyperplasia, wasting, and syncytium formation, along with greatly reduced OKT4/OKT8 ratios. Preliminary immunological studies using sera from these infected monkeys established the relationship between this SIV isolate and HIV and revealed the presence of antibody to SIV proteins with estimated molecular masses of 110,000 Daltons (110-kDa), 60-kDa, 45-kDa, 35-kDa, 26-kDa, and 16-kDa (Murphy-Corb et al., 1986).

As previously discussed, the social and economic implications of the rapidly growing AIDS epidemic have made the development of a representative animal model for vaccine and chemotherapeutic investigations essential. The rhesus macaque is the most thoroughly characterized non-human primate and it displays an immune system remarkably similar to that of humans (Letvin et al., 1985; Martin et al., 1983). The established morphological and immunological relationship
between HIV and SIV (Benveniste et al., 1986; Murphey-Corb et al., 1986), along with the clinical and pathological similarities between their respective disease syndromes, affirm the need for an extensive characterization of the SIV/Delta system. When we embarked on this project in 1986, isolation and nucleotide sequencing of only a few strains of SIV had been reported. Since that time, many more strains of SIV have been isolated, but very few protein characterization studies have been reported. The establishment of a virion protein model for other lentiviruses, such as equine infectious anemia virus (EIAV; Parekh, et al., 1980; Montelaro et al., 1982), have been instrumental in the investigation of these viral systems. The data presented here describe a virion protein model for SIV/Delta that reveals obvious similarities to that of HIV and other lentiviruses, and should represent a useful tool for study of the SIV system.
Results

Analysis of SIV/Delta proteins by SDS-PAGE.

When subjected to analysis by SDS-PAGE, SIV/Delta yields a complex profile that resembles those of HIV and EIAV (Figure 4). Several major protein bands that appeared to correlate with the gag-encoded proteins of the other lentiviruses were evident. Due to the complexity of this profile, SIV/Delta was metabolically labeled with [S\(^{35}\)]-methionine and with [\(^{3}H\)]-leucine to specifically label viral proteins. These labeled virus preparations were analyzed by SDS-PAGE in cylindrical gels (20 cm in length) which were sliced into 1 mm fractions which were assayed for radioactivity (Figure 5A & B). To identify the glycosylated viral proteins, virus preparations were metabolically labeled with [\(^{3}H\)]-glucosamine and analyzed in the same manner (Figure 5C). The resulting profiles contained radioactive species corresponding to proteins of approximately 43-kDa, 26-kDa, 17-kDa, 14-kDa, and 9-kDa and glycoproteins of 110-kDa and 35-kDa; the heterogeneity of the 110-kDa component is probably due to variations in glycosylation pattern. Interestingly, the radioactive species corresponding to a 43-kDa component was highly variable from one virus preparation to the next, depending on the stage of virus production. When label was added to cells prior to optimal virus production, the 43-kDa protein typically comprised a high percentage of the label recovered, whereas upon addition of label during optimal virus replication, the 43-kDa protein was usually only a minor component of the preparation (data not shown). To determine whether the 43-kDa protein was of viral or
Figure 4. SDS-PAGE analysis of gradient purified SIV/Delta virions. 10% SDS-PAGE analysis of gradient purified (E) EIAV, (H) HIV-1, and (S) SIV/Delta virions. The approximate molecular weights of markers are labeled on the left.
Figure 5. SDS-PAGE analysis of metabolically radiolabeled SIV/Delta. Labeled virus preparations were analyzed via SDS-PAGE on tube gels 20 cm in length. Following electrophoresis, the cylindrical gels were sliced into 1 mm fractions and the fractions were assayed for radioactivity. SIV/Delta-infected H9 cells were metabolically labeled with S^{35}-methionine (Panel A), H^{3}-Leucine (Panel B), and H^{3}-glucosamine (Panel C). Mock-infected H9 cells were metabolically labeled with S^{35}-methionine, a mock-purification was performed, and the mock-infected H9 cell preparation was analyzed using similar procedures (Panel D).
cellular origin, [S\textsuperscript{35}]-methionine was added to mock-infected H9 cells. SDS-PAGE analysis of this preparation revealed that a high percentage of label was incorporated into the 43-kDa component (Figure 5D) indicating that this protein was probably cellular. However, the possibility that a virus-encoded protein was co-migrating with this 43-kDa cellular contaminant could not be ruled out on the basis of these results.

**Analysis of SIV/Delta proteins by lentil lectin affinity chromatography.**

To further evaluate the glycoprotein composition of SIV/Delta, virus preparations were subjected to lentil lectin affinity chromatography and analyzed by SDS-PAGE procedures. When visualized by coomassie blue staining, the lectin binding profile contained two distinct bands corresponding to proteins of approximately 110-kDa and 35-kDa molecular mass, and the lectin non-binding profile revealed bands of 26-kDa, 17-kDa, 14-kDa, and 9-kDa (Figure 6). When visualized by a glycoprotein-specific staining procedure (Clegg, 1982), the non-binding profile contained no prominent bands while the binding fraction contained bands representing proteins of 110-kDa and 35-kDa, and a faint band of approximately 9-kDa molecular weight (data not shown). [S\textsuperscript{35}]-methionine labeled viral preparations were also subjected to lentil lectin fractionation and analyzed on cylindrical gels. The resulting profiles were similar to previous results indicating that there are at least three virus-encoded lentil lectin non-binding proteins, 26-kDa, 17-kDa, and 14-kDa, and at least two lentil lectin-binding viral glycoproteins of 110-kDa and 35-kDa molecular mass (Figure 7). The 35-kDa component appears as a doublet on Coomassie stained gels, indicating that both putative glycoproteins are heterogeneous. In addition
Figure 6. **Analysis of protein fractions obtained from lentil-lectin affinity chromatography of SIV/DeltaB670.** Panel A: SDS-PAGE analysis of protein standards (lane 1) with molecular weights as indicated, SIV/DeltaB670 gradient-purified whole virus (lane 2), lentil-lectin non-binding (lane 3) and binding (lane 4) pools. The total protein profile was visualized by staining with Coomassie blue. Panel B: Immunoblot analysis of whole virus (lane 1), lentil-lectin non-binding (lane 2) and binding (lane 3) of SIV/DeltaB670 using a reference serum from an SIV-infected macaque and 125I-labeled protein A. Thirty-six micrograms of purified whole virus, 35 μg of the lentil-lectin non-binding fraction, and 24 μg of the lentil-lectin binding fraction was loaded in each respective well. The characteristic migration positions of each of the SIV polypeptides are designated on panels A and B. Similar immunoblots using pre-infection monkey sera show no reactivity with any components of these SIV protein pools (data not shown).
to the 110-kDa and 35-kDa components, a small amount of radioactive species corresponding to a protein of approximately 43-kDa was evident in the lentil lectin binding fraction of this virus preparation (Figure 7C). This glycosylated 43-kDa component, which appears to co-migrate with the 43-kDa cellular contaminant of these SIV virion preparations, may represent a third glycoprotein of SIV. However, the relationship of these glycoproteins is not clear from these analyses.

**Serological analysis of SIV/Delta proteins.**

Whole SIV/Delta virion preparations and lentil lectin pools were analyzed by western blotting techniques (Burnette, 1981) using reference sera obtained from SIV-infected macaque #B845 (Baskin et al., 1986). Immune sera reacted specifically with polypeptides of approximately 110-kDa, 43-kDa, 35-kDa 26-kDa, 17-kDa, 14-kDa, and 9-kDa molecular masses (Figure 6). The relative intensities of the bands indicated that the 110-kDa and 35-kDa components are highly immunogenic and the broadness of these bands depict their heterogeneity. This conclusion was drawn due to the apparent low copy number of the envelope proteins per virion, as suggested by whole virus profiles on coomassie stained gels and as definitively shown by electron micrographic analyses (discussed in Chapter 1). The 26-kDa component, which is present in high copy number, is also highly immunoreactive while the 17-kDa, 14-kDa, and 9-kDa polypeptides appear to be less immunogenic. The specific reactivity of immune sera with a 43-kDa protein contained in both whole virus and the lentil lectin binding fraction of SIV/Delta suggested that the three glycoproteins identified in preparations of SIV are produced during viral
Figure 7. Analysis of protein fractions obtained from lentil-lectin affinity chromatography of $^{35}$S-methionine labeled SIV/DeltaB670. Metabolically radiolabeled SIV/DeltaB670 preparations were subjected to lentil-lectin affinity chromatography. Whole SIV starting material (Panel A), lentil-lectin non-binding (Panel B), and binding (Panel C) pools were analyzed via SDS-PAGE on 20 cm cylindrical gels. Gels were sliced into 1 mm fractions and the fractions were then assayed for radioactivity.
replication and are probably encoded by the SIV genome. Sera from healthy macaques failed to recognize any of these proteins confirming that antibody specific for these proteins are elicited by SIV/Delta infection (data not shown).

**Two-dimensional peptide mapping of tryptic digests.**

Each of the putative SIV/Delta proteins were analyzed by peptide mapping procedures in order to examine whether these protein components were unique or were precursors or cleavage products of other viral proteins. Following SDS-PAGE analysis with coomassie staining, SIV-specific protein bands were excised directly from the gel, radiiodinated and trypsinized in the gel slice, and then the peptides were eluted from the slice. These tryptic peptide fragments were subjected to electrophoresis in one direction and then liquid chromatography in a perpendicular direction. Preliminary results revealed that excision of homogeneous bands from these complex mixtures was difficult, if not impossible. Therefore, we subsequently used a combination of reverse phase high pressure liquid chromatography (RP-HPLC) purification procedures, as described by Ball *et al.* (1988) for EIAV proteins, and SDS-PAGE to generate homogeneous protein bands. Resulting two-dimensional peptide profiles (Figure 8) demonstrated the uniqueness of each of these proteins with the exception of the 35-kDa and 34-kDa components. As expected, these two profiles were very similar and seemed to differ only in their relative degrees of glycosylation. Since glycopeptides do not migrate in the chromatography solvent and can be seen along the horizontal origin (Salinovich *et al.*, 1986; Payne *et al.*, 1987),
Figure 8. Tryptic peptide maps of $^{125}$I-labeled SIV/DeltaB670 proteins. The two-dimensional analysis consisted of electrophoresis in the horizontal direction and liquid chromatography in the vertical direction. Samples were applied in the lower left corner. The identity of the proteins analyzed are as follows: 110-kDa (Panel A), 35-kDa (Panel B), 34-kDa (Panel C), 26-kDa (Panel D), 17-kDa (Panel E), 14-kDa (Panel F), and 9-kDa (Panel G).
these peptide mapping procedures can be useful in evaluating the relative extent of glycosylation of glycoproteins. The 110-kDa profile also revealed the presence of glycopeptides along the horizontal origin; the intensity of this "layer" of glycopeptide suggests that the 110-kDa component is heavily glycosylated. As expected the 26-kDa, 17-kDa and 14-kDa profiles are less complex than those of the glycoproteins; however, the 9-kDa profile is highly complex suggesting a heterogeneous protein band. In fact, the heavy concentration of peptides at the origin of the 9-kDa peptide map (Figure 8G) are indicative of glycopeptides. Therefore, the heterogeneity of the 9-kDa band could be due to co-migration of glycoprotein breakdown products, as glycoproteins typically are extremely labile.

**Direct amino acid sequencing of putative SIV virion proteins.**

To confirm that each of the putative SIV proteins were in fact virus-encoded, we attempted to perform amino-terminal amino acid sequencing of the 110-kDa, 35-kDa, 26-kDa, 17-kDa, and 9-kDa proteins discussed above. Since protein samples for sequencing must be extremely homogeneous, protein samples were purified via RP-HPLC, subjected to SDS-PAGE, and electrophoretically transferred to an activated glassfiber sheet for sequencing. Sequencing of the 26-kDa protein yielded interpretable data from 15 of the first 16 cycles attempted (Figure 9). Thirteen of these 15 residues matched the predicted CA protein sequence of SIVmac251 (Hirsch *et al.*, 1987); unfortunately, no sequence data from SIV/Delta isolates were available for comparison. Attempts to obtain sequence data from the 17-kDa component were unsuccessful indicating that this protein was probably amino-terminally blocked. Since the MA proteins of other
| 26kDa NH$_2$- Pro-Val-Asn-Asn-Val-Gly-Gly-Asn-Tyr-Thr-His-Leu-Pro-Leu-? -Pro |
| 17kDa NH$_2$- blocked, no sequence obtained |
| 14kDa NH$_2$- Val-Leu-Ser-? -?-Asp |
| 9kDa NH$_2$- Val-Thr-Asn-Gly-Asn-Arg-Glu-Thr-Ile-lys-?-?-?-? -Gly-Leu |

Figure 9. **Amino-terminal sequencing of the putative SIV/DeltaB670 proteins.** SIV/DeltaB670 proteins were purified via RP-HPLC and soluble samples were directly applied to TFA treated glassfiber discs and then subjected to gas-phase sequencing. The amino acid sequence obtained from each of the indicated proteins is listed. All residues in bold print differ from the reported amino acid sequence of SIVmac251.
lentiviruses are myristylated at their amino terminus, and since those MA proteins are similar in molecular weight to this 17-kDa component, this protein is probably the MA protein of SIV/Delta. Analysis of the 14-kDa component yielded only four interpretable cycles out of six cycles that were attempted (Figure 9). Surprisingly, this 4/6 residue sequence could not be located in the gag-encoded polyprotein sequence suggesting that the 14-kDa component was not a gag-encoded protein. Sequencing of the 9-kDa component yielded interpretable data from 12 of the 16 cycles performed (Figure 9). However, this sequence did not correspond to any region of the predicted amino acid sequences of SIVgag or env polyproteins. Attempts were made to obtain sequence data from the 110-kDa and 35-kDa components. However, due to the requirements for relatively large amounts of extremely pure protein, these attempts were soon aborted and no sequence data was obtained.

Secondary structural modeling of putative SIV proteins.

Gallaher and associates have previously performed extensive secondary structural modeling of several lentivirus TM (Gallaher et al., 1989) and SU (Gallaher et al., 1991) proteins. These studies have revealed striking similarities between lentivirus envelope proteins. Here, we have performed similar modeling studies of the SIVmac251 envelope proteins for comparative purposes. Composite secondary structure models for both SU and TM (Figure 10A & B) were prepared by analyzing predicted amino acid sequences of SIVmac251 both manually, via application of Chou-Fasman methodology (Chou and Fasman, 1974), and using several computer algorithms designed to predict secondary structure (detailed
discussion in Chapter 4). Disulfide linkages were predicted on the basis of similarities with HIV-1 SU linkages which have been experimentally derived (Leonard et al., 1990) and from the predicted disulfide linkages of HIV-2 and SIVmac SU (Hoxie, 1991).

Although results obtained from these predictive algorithms are extremely speculative, the resulting structural models were strikingly similar to those of HIV-1, HIV-2, and EIAV, and they may be useful tools for experimental design. On the basis of mutational (Kowalski et al., 1987; Laskey et al., 1987; Willey et al., 1988) and immunological (O'Brien et al., 1990) analyses, as well as sequence and predicted structure comparisons, lentiviral SU proteins have been conceptionally subdivided into three regions (Gallaher et al., 1991). The amino-terminal one-third of SU appears to consist of a relatively constant core structure that contains the TM contact sites. The central one-third of the SU protein generally contains sequences important to both humoral and cellular immune responses, such as the principle neutralizing domain and several potentially amphipathic sequences that may act as T-cell epitopes. The carboxy-terminal one-third of SU appears to contain the binding site for the CD4-receptor; the structural characteristics of this binding site include a core of β-sheet which forms a hydrophobic pocket that is stabilized by disulfide crosslinking. Lentiviral SU proteins have also been divided into five disulfide-bonded domains that are well conserved. Gallaher and associates (1989) have also identified some highly conserved structural features within the amino terminal one-half of lentiviral TM proteins. These structures include the fusion domain (Gallaher, 1987), an extended
α-helical region, a putative disulfide "loop" which stabilizes an immunodominant humoral antigenic determinant (Norrby et al. 1989, 1991), and finally, a hydrophobic α-helical membrane spanning domain. The secondary structural investigations of Gallaher and associates identified no common structural motifs within the carboxy-terminal one-half of the TM protein.

The secondary structure predictions reported here are consistent with those reported by Gallaher and associates, further demonstrating the similarities between SIV and the other lentiviruses (Figure 10A & B). Similar to predictions for other lentivirus SU proteins, the SIVmac SU can be subdivided into five domains on the basis of predicted disulfide linkages. A putative CD4-binding domain can easily be identified from these modeling studies as an extended hydrophobic β-sheet, lying between disulfide bonded domains IV and V, appears to form a binding pocket or cleft that is stabilized by disulfide bonding; this structure is very similar to that predicted for HIV-1. The disulfide domain IV of SIV is also highly analogous to that predicted for HIV-1 as it contains a structure similar to the principle neutralizing determinant of HIV-1. The structural features of the SIVmac TM protein are predicted to be similar to those predicted for other lentiviruses as well. The five residue core sequence of the fusion domain (FLGFL) in SIVmac is identical to that of HIV-1 and, also similar to HIV-1, it begins at position 7 of the TM sequence. However, the fusion domain of SIV is predicted to form a β-sheet structure while other lentiviruses are predicted to assume a random coil conformation in this region. The SIVmac model also contains structures closely resembling
the disulfide loop-stabilized immunodominant antigenic determinant and the hydrophobic helical transmembrane membrane-spanning domain that is characteristic of other lentiviruses (Gallaher et al., 1989). These studies did identify an amphipathic α-helical region near the carboxy-terminus of SIVmac and several other lentiviruses that may play an important role in the cytopathicity of lentivirus infections (Miller et al., 1991; Miller and Montelaro, 1991). This structural motif of SIV and other lentiviruses was examined in detail, and these studies are described in Chapters 5 and 6.
Figure 10 Secondary structural models of SIVmac251 SU and TM proteins. SIVmac251 SU and TM protein sequences (Hirsch et al., 1987) were analyzed via Chou-Fasman rules manually and with the PeptideStructure / PlotStructure computer algorithms designed to predict secondary structural elements from primary amino acid sequences (Starcich et al., 1986). In addition, the sequences were analyzed using the AMPHI algorithm which is designed to locate amphipathic protein segments (Margalit et al., 1987). The composite secondary structure models of the SU (A) and TM (B) envelope proteins are presented here. Amino acid side chain characteristics were assigned according to the polarity scale of transmembrane helices (Engleman et al., 1986). Intramolecular disulfide linkages, which are indicted by a double line, were predicted on the basis of similarities with HIV-1 linkages which have been experimentally derived (Leonard et al., 1990) and from the predicted disulfide linkages of HIV-2 SU (Hoxie, 1991).
Discussion

As a result of the protein characterization and immunological studies reported here, we propose a partial model for the structural components of the HIV-related SIV/Delta virion. The high immunogenicity and extensive glycosylation of the 110-kDa component isolated from SIV/Delta, implies its role as the external envelope glycoprotein (SU). The slightly glycosylated and less immunogenic 35-kDa protein probably represents the transmembrane protein or TM. Similar to other lentiviruses, the most abundant protein present in SIV/Delta preparations has an apparent molecular mass of 26-kDa. Serological analyses revealed that infected monkeys produce high levels of antibodies specific for this protein, while amino acid sequencing revealed that this protein is encoded by the gag gene. Together, these data suggested that the 26-kDa protein is the major capsid component (CA) of SIV/Delta. The 17-kDa component was predicted to be fatty acylated due to its failure to yield a sequence upon automated sequencing and is therefore thought to be the matrix protein (MA). Due to their SDS-PAGE migratory properties, the 110-kDa, 35-kDa, 26-kDa and 17-kDa protein were predicted to be the SU, TM, CA, and MA proteins, respectively, prior to these studies; the results of these studies confirmed those predictions.

Similarly, the 14-kDa and 9-kDa components of SIV/Delta virus preparations were predicted to be the nucleocapsid (NC) protein and a minor core component, respectively. However, results of the experiments described here implied that these predictions were incorrect. These
studies suggested that the 14-kDa and 9-kDa proteins, which are relatively abundant components of SIV/Delta virus preparations, are not encoded by the *gag* gene. Neither of these proteins appeared to be breakdown products of the SU or TM either, as the partial amino-terminal sequences obtained from these components did not correlate with any region of the predicted amino acid sequences of the envelope polyprotein.

The results reported here have been corroborated, and in some cases clarified by reports from other laboratories. Henderson *et al.* (1988a) performed extensive amino acid sequencing studies using HPLC-purified proteins, as well as protein fragments generated by enzymatic cleavage, and found that the 26-kDa and 17-kDa components of SIVmne were the CA and MA protein, respectively. Neither the studies reported here, or those of Henderson and associates (1988a) suggested that the 14-kDa SIV protein was encoded by the *gag* gene. However, a later report identified the 14-kDa protein as the product of a unique ORF found in SIV and HIV-2, but not HIV-1, this ORF was designated *vpx* (Henderson *et al.*, 1988b). This protein appears to be required for efficient viral replication in fresh monocytes and macrophages and may function by activating the expression of cellular factors that facilitate viral replication (Yu *et al.*, 1991). It has been speculated that this *vpx* protein, and other regulatory proteins, may compose the lateral bodies of lentivirus virions (Gelderblom, 1991). We are currently employing 14-kDa-specific monoclonal antibodies in immune-electronmicroscopic analyses in an attempt to localize the *vpx* protein within virions. Since the 9-kDa protein does not correspond to any of the known SIV-encoded proteins, we speculate that it may represent a
virion-associated cellular protein that either is specifically or non-specifically incorporated into virions at relatively high levels. Using monoclonal antibodies generated to putative SIV glycoproteins (110-kDa and 35-kDa), Veronese et al. (1989) performed immunoprecipitations coupled with SDS-PAGE and then excised precipitated proteins from the gel, eluted the protein from the gel slice, and then analyzed the protein via amino-terminal sequencing. Sequencing results indicated that the 110-kDa and 35-kDa SIV proteins are indeed the SU and TM products of the env gene, respectively, thus corroborating the observations reported here.

Nucleotide sequence analysis of SIVmac has revealed that the env gene contains an in-frame stop codon that truncates the cytoplasmic domain of the TM protein (Hirsch et al., 1987) resulting in the expression of a protein of approximately 35-kDa. This is consistent with data presented here. However, Mullins and associates reported that truncated TM protein is the primary form of TM observed in vitro, but in vivo a full length TM of approximately 41-kDa predominates (Hirsch et al., 1989b). The 43-kDa component that we observed in lentil lectin binding fractions of SIV/Delta may represent a residual level of the full length TM protein. It has been demonstrated that growing SIV in cultured human cells selects for TM mutants that produce a truncated TM protein. SIV produced in macaque PBLs in vivo retains a full length TM protein (Hirsch et al., 1989b).

Secondary structural modeling of SIV proteins has further shown the close resemblance of SIV proteins to those of the other lentiviruses, specifically HIV-1, HIV-2, and EIAV. These structural models also
represent a useful tool for experimental design as they reveal structural features of SIV proteins that may be essential for infectivity and cytopathicity of the virus. Further characterization of these structural features may lead to a better understanding of lentivirus pathogenic mechanisms and development of novel therapeutic measures designed to alter lentivirus-induced disease progression.
CHAPTER 4

IDENTIFICATION OF CONTINUOUS EPITOPE S OF SIV ENVELOPE PROTEINS

Introduction

The simian immunodeficiency virus (SIV) system constitutes an important animal model for examining mechanisms of HIV pathogenesis and for developing strategies for AIDS vaccine development (Gardner and Luciw, 1988, 1989; Desrosiers and Letvin, 1987; Daniel and Desrosiers, 1989; Desrosiers and Ringler, 1989). A critical component to both of these aspects of AIDS research is a characterization of SIV immunogenicity in monkeys and how specific immune responses may correlate with the progression of persistent viral infection and the clinical course of disease. Infections of various monkey species with a particular isolate of SIV can result in a diverse disease spectrum, ranging from asymptomatic infections of sooty mangabeys (Murphey-Corb et al., 1986; Fultz et al., 1986a; Lowenstine et al., 1986) and African green monkeys (Ohta et al., 1988; Daniel et al., 1988a) to the development of fatal immunodeficiency disease in rhesus macaques (Letvin et al., 1985). In addition, characterization of SIV strains isolated from various species of monkeys has revealed a remarkable degree of variation in viral genetic and biological properties (Regier and Desrosiers, 1990). Because of the variations intrinsic to SIV infections, it has been difficult to identify common themes in viral immunogenicity and to characterize immune responses that can influence the course of SIV infection and disease. Thus, the identification
of group and type site-specific antigenic determinants of SIV is an important foundation for more detailed studies of how host immune responses may affect virus replication and disease.

The kinetics of SIV-induced immune responses has typically been studied using immunological techniques such as Western blotting and radioimmunoprecipitation (Zhang et al., 1988). Results from these types of studies have shown that core-specific responses, directed primarily toward the 26-kDa major core protein (CA), are detectable prior to glycoprotein-specific responses which are directed toward the 110-kDa external envelope glycoprotein (SU), the 32-kDa truncated transmembrane protein (TM32), and the full-length 41-kDa transmembrane protein (TM41; Hirsch et al., 1986; Hirsch et al., 1987). In animals infected with non-pathogenic strains of SIV, these responses persist throughout the course of infection. Analysis of serial serum samples from animals infected with pathogenic strains of SIV revealed that responses specific for some of these proteins are transient, and the fluctuations observed may be predictive of disease progression (Zhang et al., 1988).

While the aforementioned immunological assays are effective for monitoring humoral responses specific for individual proteins, they yield no information concerning epitope-specific responses. In the current study we have utilized synthetic peptide methodologies to characterize linear B-cell determinants in the envelope glycoproteins of SIV. Toward this goal, we have performed a detailed computer-aided analysis of potential antigenic sites on the SIV envelope glycoproteins and have synthesized selected peptides for further analyses. The SIV envelope
peptide antigens were then assayed for their serological reactivity with defined panels of immune serum from various species of SIV-infected monkeys to identify group and type specific B-cell determinants. In addition, the candidate peptide antigens were examined for their ability to induce neutralizing antibodies in rabbits and to block serum neutralization of virus in vitro.
Results

Predicting B-cell and T-cell epitopes of SIV.

Analysis of deduced amino acid sequences of the env polyproteins of two SIVmac isolates (Hirsch et al., 1987; Chakrabarti et al., 1987), SIV/DeltaB670 (Murphey-Corb et al., 1986) and a SIVsm isolate (Hirsch et al., 1989a) using the SurfacePlot (Parker et al., 1986; Karplus and Schultz, 1985; Janin, 1979) and UWGCG PeptideStructure / PlotStructure computer algorithms (Starcich et al., 1986) identified several protein segments with high potential to form epitopes on the surface proteins of SIV. The SurfacePlot profiles (Figure 11) reveal several protein segments corresponding to composite surface values over 50, thus predicting those amino acid sequences to be antigenic regions of the external glycoproteins. Analysis of these sequences with the PeptideStructure/PlotStructure algorithms yielded similar results (data not shown). Results obtained from computer-aided analysis of each of these SIV sequences were compiled to form a consensus antigenic profile of SIV, and this profile was used to select potential epitope regions from SIVmac251.

At least nine regions of the SIV env polyprotein were predicted to have high potential to form antigenic sites. Seven of these were within the SU protein and two were identified in the TM portion of the polyprotein sequence. Peptides corresponding to all nine of the high-index regions of the env polyprotein were produced synthetically according to the SIVmac251 sequence reported by Hirsch et al. (1987): residues 46-66 (SAM42), 112-143 (SAM35), 178-207 (SAM26), 269-289 (SAM45), 365-389
Figure 11. **Prediction of SIV B-cell Epitopes.** SurfacePlot 60% composite profiles from analysis of the deduced amino acid sequences of the envelope polyprotein of (Panel A) SIVmac251 (Hirsch et al., 1987), (Panel B) SIV/DeltaB670 (LeRosa et al., 1992), (Panel C) SIVsm (Hirsch et al., 1989a), and (Panel D) SIVagm TYO-1 (Fukasawa et al., 1988) have been aligned. Peaks correspond to the predicted antigenic index that was compiled from hydrophilicity, accessibility, and flexibility calculations (Parker et al., 1986). Regions with surface values over 50 are strongly predicted as B-cell epitopes. Nine peptides corresponding to regions of SIVmac251 that were predicted to have high antigenic index were synthesized; the positions of these regions in the deduced amino acid sequence of SIVmac251 are indicated numerically in the upper region of panel A. Three regions that were not predicted to be antigenic are also indicated numerically (near the middle of panel A); four peptides corresponding to these regions were also synthesized. The beginning of the TM protein is indicated by a vertical line positioned between residues 527 and 528 of the SIVmac251 envelope polyprotein in panel A, and by an arrow in panels B, C, and D.
(SAM69), 413-431 (SAM36), 452-481 (SAM52), 503-527 (RaMPS42), and 736-757 (SAM55) (Figures 11A & 12A).

Several peptides corresponding to regions of the SIV env polyprotein having low antigenic potential but known to represent antigenic regions of HIV-1 were also produced (Figures 11A & 12B). Two of these were overlapping peptides corresponding to the putative principle neutralizing domain (PND) of SIV SU (S.D. Putney, personal communication), 313-336 (RP92) and 323-345 (RP93), which span all but one residue of the PND. A peptide constituting the immunodominant TM loop (Norrby et al., 1989) denoted SIV-M (597-619), was also produced to provide a broadly reactive peptide for comparative purposes. In addition, a peptide corresponding to the extreme carboxy terminus of SIV TM designated SAM56 (852-879), which has been implicated as an important contributor to the cytopathicity of SIV (Fisher et al., 1986; Lee et al., 1989; Miller et al., 1991; Miller and Montelaro, 1991), was also included in these studies; this peptide has also been shown to have high amphipathic potential (Eisenberg and Wesson, 1990; Miller et al., 1991) and may therefore serve as a T-cell determinant since T-cell epitopes are often amphipathic structures (Margalit et al. 1987; Wahren et al., 1989).

Several peptides corresponding to regions of SIV that were predicted by the AMPHI algorithm (Margalit et al., 1987) to serve as T-cell epitopes were also synthesized (Fig. 2C). Each of these peptides, MR3 (env 81-105), MR4 (env 349-373), and MR5 (env 434-458) correspond to regions of SIV/DeltaB670 SU. The resulting panel of peptides span the entire env polyprotein and represents nearly 44% of the total amino acid sequence,
Figure 12. Primary Amino Acid Sequences of Synthetic Peptides. The primary amino acid sequences of peptides synthesized to mimic B-cell and T-cell epitope-containing regions of SIV are listed, and their position in the predicted env polyprotein sequence is indicated numerically: (A) peptides corresponding to high antigenic index regions of SIVmac251, (B) peptides corresponding to low antigenic index regions of SIVmac251, and (C) peptides corresponding to predicted T-cell epitopes of the SIV/DeltaB670 env polyprotein.
A  Predicted B-cell Epitopes

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residue #s</th>
<th>Linear Sequence</th>
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<tbody>
<tr>
<td>SAM42</td>
<td>env 46-66</td>
<td>NH₂-TKNDRTWGTTQCLPDNGDYSE-CONH₂</td>
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<tr>
<td>SAM35</td>
<td>env 112-143</td>
<td>NH₂-RCNKSQTDREGLTTSSSTTITAAPTSAPVSEK-CONH₂</td>
</tr>
<tr>
<td>SAM26</td>
<td>env 178-207</td>
<td>NH₂-KREDTKEYNETWYSTDLVCEQRNSTDNESR-CONH₂</td>
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<td>env 269-289</td>
<td>NH₂-CETQTSTWFGNGTRAENRTY-CONH₂</td>
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<td>SAM69</td>
<td>env 365-389</td>
<td>NH₂-CKHPRYTGNNMTKINLTAPGGGDPE-CONH₂</td>
</tr>
<tr>
<td>SAM36</td>
<td>env 413-431</td>
<td>NH₂-VEDKDVTTQRKHKRKNY-CONH₂</td>
</tr>
<tr>
<td>SAM52</td>
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</tr>
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<td>env 503-527</td>
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</tr>
<tr>
<td>SAM55</td>
<td>env 736-757</td>
<td>NH₂-THTQQDPALPRTREGKEGDGEG-CONH₂</td>
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</table>

B  Potential B cell Epitopes With Low Antigenic Index

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residue #s</th>
<th>Linear Sequence</th>
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<td>NH₂-CRRPNKTVLPTIMGLVHSQP-CONH₂</td>
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<tr>
<td>SAM56</td>
<td>env 852-879</td>
<td>NH₂-DLWETLRRGRWILAIOPRIRQGLET-CONH₂</td>
</tr>
</tbody>
</table>

C  Predicted T-cell Epitopes

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residue #s</th>
<th>Linear Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR3</td>
<td>env 81-105</td>
<td>NH₂-TVTEQAIEDVWNLFETSIKPCVLLT-CONH₂</td>
</tr>
<tr>
<td>MR4</td>
<td>env 349-373</td>
<td>NH₂-GGSWKAKIPEVKETIVKHPRTGNT-CONH₂</td>
</tr>
<tr>
<td>MR5</td>
<td>env 434-458</td>
<td>NH₂-CHIRQIVNTWHKVGRNVYLVPPREGD-CONH₂</td>
</tr>
</tbody>
</table>
including the TM41 region that is not present in the truncated TM which prevails in tissue culture-passaged SIV, but is contained in the TM that predominates in vivo (Hirsch et al., 1989b).

Each of the peptides produced for these studies was synthesized, characterized, and purified as described in the Materials and Methods section. Following synthesis and cleavage from support resins, peptide preparations were characterized by mass spectroscopy and the desired peptide component was purified via RP-HPLC. In each case, the desired peptide product was purified to greater than 95% homogeneity according to analytical RP-HPLC (data not shown).

Each of the peptides corresponding to potential B-cell epitopes synthesized for these studies were made according to the SIVmac251 sequence, while the putative T-cell epitopes reflect the SIV/DeltaB670 sequence. There is considerable variability of envelope sequences among SIV isolates, and since differences in peptide-specific reactivity displayed by members of the four monkey groups tested here could be due to either local sequence variations, or conformational differences between the envelope proteins of the different strains of virus with which these animals are infected, analysis of derived SIVmac251 (Hirsch et al., 1987), SIV/DeltaB670 (LeRosa et al., 1992), SIVsm (Hirsch et al., 1989a), and SIVagm TYO-1 (Fukasawa et al., 1988) sequences was performed (Figure 13). It should be noted that each of the animals involved in this study are probably infected with relatively undefined mixtures of virus, and therefore the sequences analyzed are merely representative of the
Figure 13. Sequence variations between various SIV isolates within regions corresponding to peptide panel. The sequences for SIVmac251 (Hirsch et al., 1987), SIV/DeltaB670 (LeRosa, manuscript in preparation), SIVsm (Hirsch et al., 1989a), and SIVagm TYO-1 (Fukasawa et al., 1988) within the regions defined by the synthetic peptide panel are aligned. The peptide designation and its position in the SIVmac251 envelope sequence has been noted above each alignment. Since the synthetic peptides were designed according to the SIVmac251 sequence, all residues which vary from the SIVmac251 sequence are indicated in bold print. In several instances, insertions or deletions in either the SIV/DeltaB670, SIVsm, or SIVagm TYO-1 sequences (with respect to SIVmac251) left gaps in the alignment; these gaps have been indicated using dots.
<table>
<thead>
<tr>
<th>SAM42 env 46-66</th>
<th>SAM56 env 431-431</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVmac251 NH2- TNKRTGTGQELQDDGNDDSE -CONH2</td>
<td>SIVmac251 NH2- VEDNYTTQRPKRN....AK.....NY -CONH2</td>
</tr>
<tr>
<td>SIV/DeltaB670 NH2- TNKRTGTGQELQDDGNDDSE -CONH2</td>
<td>SIV/DeltaB670 NH2- VEDNYTTQRPKRN....AK.....NY -CONH2</td>
</tr>
<tr>
<td>SIVam NH2- TNKRTGTGQELQDDGNDDSE -CONH2</td>
<td>SIVam NH2- VEDNYTTQRPKRN....AK.....NY -CONH2</td>
</tr>
<tr>
<td>SIVagm(TYO-1) NH2- TPYTVLTQAMCIDPETDLYE -CONH2</td>
<td>SIVagm(TYO-1) NH2- DAEKFGSSSKGAPAEQCV...GR.P -CONH2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SAM35 env 112-143</th>
<th>SAM52 env 452-481</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVmac251 NH2- RCKNEETDGKLSSTTPTTAAPT...SAPV.EK -CONH2</td>
<td>SIVmac251 NH2- LPPREDGTCNVTSSLANIMDGQGTS -CONH2</td>
</tr>
<tr>
<td>SIV/DeltaB670 NH2- RCKNEETDGKLSSTTPTTAAPT...SAPV.EK -CONH2</td>
<td>SIV/DeltaB670 NH2- LPPREDGTCNVTSSLANIMDGQGTS -CONH2</td>
</tr>
<tr>
<td>SIVam NH2- RCKNEETDGKLSSTTPTTAAPT...SAPV.EK -CONH2</td>
<td>SIVam NH2- LPPREDGTCNVTSSLANIMDGQGTS -CONH2</td>
</tr>
<tr>
<td>SIVagm(TYO-1) NH2- RCVEMSTRAATGTPETRDYQCV...PS.GN -CONH2</td>
<td>SIVagm(TYO-1) NH2- APEKREGLQCTYQVTGTVLQEVSKMSRS -CONH2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SAM26 env 178-207</th>
<th>RaMPS42 env 503-527</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVmac251 NH2- KRDVKTE,NETYSSDLV.CEKGSTONERS -CONH2</td>
<td>SIVmac251 NH2- EITPIGLAPTVEERTVGTGSNKR -CONH2</td>
</tr>
<tr>
<td>SIV/DeltaB670 NH2- KRDVKTE,NETYSSDLV.CEKGSTONERS -CONH2</td>
<td>SIV/DeltaB670 NH2- EITPIGLAPTVEERTVGTGSNKR -CONH2</td>
</tr>
<tr>
<td>SIVam NH2- KRDVKTE,NETYSSDLV.CEKGSTONERS -CONH2</td>
<td>SIVam NH2- EITPIGLAPTVEERTVGTGSNKR -CONH2</td>
</tr>
<tr>
<td>SIVagm(TYO-1) NH2- RCVEMSTRAATGTPETRDYQCV...PS.GN -CONH2</td>
<td>SIVagm(TYO-1) NH2- EITPIGLAPTVEERTVGTGSNKR -CONH2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SAM45 env 269-289</th>
<th>SIV-M env 597-619</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVmac251 NH2- METQSTGFQGFGNTRAENKRY -CONH2</td>
<td>SIVmac251 NH2- AIEKYLEDQALMNGCAGFPQVC -CONH2</td>
</tr>
<tr>
<td>SIV/DeltaB670 NH2- METQSTGFQGFGNTRAENKRY -CONH2</td>
<td>SIV/DeltaB670 NH2- AIEKYLEDQALMNGCAGFPQVC -CONH2</td>
</tr>
<tr>
<td>SIVam NH2- METQSTGFQGFGNTRAENKRY -CONH2</td>
<td>SIVam NH2- AIEKYLEDQALMNGCAGFPQVC -CONH2</td>
</tr>
<tr>
<td>SIVagm(TYO-1) NH2- RCVEMSTRAATGTPETRDYQCV...PS.GN -CONH2</td>
<td>SIVagm(TYO-1) NH2- AIEKYLEDQALMNGCAGFPQVC -CONH2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RP92 env 313-336</th>
<th>SAM55 env 735-757</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIV/mac251 NH2- CRGKNTVLVPITMGLFVWSQP -CONH2</td>
<td>SIV/mac251 NH2- TLIQGQ,PAFLPREGKREDGGE -CONH2</td>
</tr>
<tr>
<td>SIV/DeltaB670 NH2- CRGKNTVLVPITMGLFVWSQP -CONH2</td>
<td>SIV/DeltaB670 NH2- TLIQGQ,PAFLPREGKREDGGE -CONH2</td>
</tr>
<tr>
<td>SIVam NH2- CRGKNTVLVPITMGLFVWSQP -CONH2</td>
<td>SIVam NH2- TLIQGQ,PAFLPREGKREDGGE -CONH2</td>
</tr>
<tr>
<td>SIVagm(TYO-1) NH2- CRGKNTVLVPITMGLFVWSQP -CONH2</td>
<td>SIVagm(TYO-1) NH2- TLIQGQ,PAFLPREGKREDGGE -CONH2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RP81 env 323-345</th>
<th>SAM56 env 852-879</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIV/mac251 NH2- PVITMGLFVWSQPITDRPQAM -CONH2</td>
<td>SIV/mac251 NH2- DLMYTRGGRWALKPRIQEQGILES -CONH2</td>
</tr>
<tr>
<td>SIV/DeltaB670 NH2- PVITMGLFVWSQPITDRPQAM -CONH2</td>
<td>SIV/DeltaB670 NH2- DLMYTRGGRWALKPRIQEQGILES -CONH2</td>
</tr>
<tr>
<td>SIVam NH2- PVITMGLFVWSQPITDRPQAM -CONH2</td>
<td>SIVam NH2- DLMYTRGGRWALKPRIQEQGILES -CONH2</td>
</tr>
<tr>
<td>SIVagm(TYO-1) NH2- PVITMGLFVWSQPITDRPQAM -CONH2</td>
<td>SIVagm(TYO-1) NH2- Q1W1ACRNYTVWNPREVQGILES -CONH2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SAM69 env 365-389</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SIV/mac251 NH2- VKH....RTGGTNTKRNLTPG...GDEP -CONH2</td>
<td></td>
</tr>
<tr>
<td>SIV/DeltaB670 NH2- VKH....RTGGTNTKRNLTPG...GDEP -CONH2</td>
<td></td>
</tr>
<tr>
<td>SIVam NH2- VKH....RTGGTNTKRNLTPG...GDEP -CONH2</td>
<td></td>
</tr>
<tr>
<td>SIVagm(TYO-1) NH2- VKH....RTGGTNTKRNLTPG...GDEP -CONH2</td>
<td></td>
</tr>
</tbody>
</table>
predominant infecting strain of each panel. The results of these analyses are discussed below.

**Site-directed serology.**

Serum panels from experimentally infected macaques (infected with SIV/DeltaB670 or SIVmac) and infected but asymptomatic mangabeys and African green monkeys (AGMs) were screened for peptide-specific antibody by P-ELISA using each of the peptides as antigen substrate. Each screening was performed in quadruplicate using test serum dilutions of 1:50. A panel of 14 sera from uninfected animals were also screened for purposes of comparison. A positive reaction was defined as any absorbance value greater than three times the average of the normal serum values. The results of these screenings are summarized in Table 1 and Figure 14.

**Peptide-specific immune responses in infected monkeys.**

While all of the peptides in this panel were recognized by a number of the monkey sera tested, five broadly reactive determinants of the SIV *env* polyprotein were identified. The SIV-M, RP92/RP93, and RaMPS42 peptides, which correspond to reportedly immunodominant regions of HIV-1 and HIV-2, each reacted with a high percentage (50-90%) of the sera tested. In addition, the two regions of SIV envelope corresponding to SAM35 and SAM26 display an intermediate reactivity of about 40% with the serum panels. Most of the remaining peptides, SAM69, SAM56, SAM36, SAM42, SAM45, and SAM52 appear to represent type specific determinants as they react with a significant percentage of members of
<table>
<thead>
<tr>
<th>Serum Panela</th>
<th>SAM42</th>
<th>SAM35</th>
<th>SAM26</th>
<th>SAM45</th>
<th>RP92</th>
<th>RP93</th>
<th>SAM69</th>
<th>SAM36</th>
<th>SAM52</th>
<th>RaMPs42</th>
<th>Transmembrane Protein (TM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>macaque (21) (SIV/Delta)</td>
<td>33b</td>
<td>43</td>
<td>57</td>
<td>29</td>
<td>81</td>
<td>48</td>
<td>5</td>
<td>19</td>
<td>33</td>
<td>38</td>
<td>SIV-M 100</td>
</tr>
<tr>
<td>macaque (15) (SIVmac)</td>
<td>13</td>
<td>60</td>
<td>67</td>
<td>0</td>
<td>67</td>
<td>40</td>
<td>0</td>
<td>27</td>
<td>0</td>
<td>73</td>
<td>SIV-M 93</td>
</tr>
<tr>
<td>mangabey (26)</td>
<td>4</td>
<td>46</td>
<td>27</td>
<td>0</td>
<td>77</td>
<td>65</td>
<td>42</td>
<td>0</td>
<td>12</td>
<td>35</td>
<td>SIV-M 96</td>
</tr>
<tr>
<td>African green (48) monkey</td>
<td>15</td>
<td>33</td>
<td>25</td>
<td>4</td>
<td>85</td>
<td>52</td>
<td>8</td>
<td>17</td>
<td>25</td>
<td>23</td>
<td>SIV-M 81</td>
</tr>
<tr>
<td>Overall (110)</td>
<td>15</td>
<td>42</td>
<td>37</td>
<td>8</td>
<td>80</td>
<td>53</td>
<td>15</td>
<td>15</td>
<td>20</td>
<td>36</td>
<td>SIV-M 90</td>
</tr>
</tbody>
</table>

a Source species with number of serum samples in parentheses

b Reactivity values listed as percentage of sera yielding positive reactions
Figure 14. Reactivity of monkey serum panels against synthetic peptides corresponding to putative B-cell epitopes of SIV env proteins. Peptide-specific Ab responses produced by 5 different monkey test groups were measured via P-ELISA. $A_{570}$ values obtained from each animal screened in these studies are plotted according to the test group. Each peptide was screened against 14 non-infected control animals ○ (1st line), 21 SIV/Delta infected macaques ♦ (2nd line), 15 SIVmac infected macaques ▲ (3rd line), 26 naturally infected mangabeys + (4th line), and 48 naturally infected African green monkeys † (5th line) of each figure panel. A positive reaction is defined as any $A_{570}$ value higher than three times the average of the control sera values (this value is indicated by a straight line across each panel of the figure).
one serum panel, but a low percentage of members of the other serum panels.

Four of the broadly reactive peptides appear to represent group specific determinants of the SIV envelope protein. SIV-M, which represents the "immunodominant TM loop" of SIV (Norrby et al., 1989), was recognized by 91% of all sera tested. The intensity of anti-SIV-M responses was typically higher than that detected for any of the other peptides in this study (Figure 14). As shown in Figure 13, the primary amino acid sequence within the region corresponding to SIV-M is well conserved among various isolates of SIV. Similarly, the two overlapping peptides which span the PND of SIV SU also reacted with a high percentage of samples from each monkey sera panel, consistent with observations made for HIV-2 (Norrby et al., 1991). Most of the reactivity to this region appears to be directed toward the amino half of the PND as a higher percentage of antisera contained antibodies to RP92 (80%) than to RP93 (53%) (Table 1, Figure 14). Again, as would be expected for a group-specific determinant, the amino acid sequence of the putative PND appears to be highly conserved (Figure 13) Two peptides corresponding to regions within the amino terminal one third of SU, SAM35 and SAM26, were recognized by 42% and 37% of the sera tested, respectively. Since SAM35 and SAM26-specific Ab responses were produced by a similar percentage of animals from each test group (Table 1), and the intensities of these responses were similar within each test group (Figure 14), these two peptides appear to represent group-specific determinants of SIV.
Interestingly, both of these regions appear to have a much higher degree of overall sequence variation than observed for SIV-M or the PND.

The RaMPS42 peptide, which encompasses the carboxyl end of SIV SU, is recognized by a significant number of each sera panel, but it appears to represent a type specific antigenic determinant of SIV. This peptide was recognized by 73% of the SIVmac-infected animals, but by only 38% of SIV/Delta-infected macaques, 35% of infected sooty mangabeys, and 23% of the infected African green monkeys tested. Interestingly, the primary amino acid sequence within this region is almost identical between the representative SIV/DeltaB670 (73% reactive) and SIVsm (35% reactive) isolates (Figure 13). The results reported here appear to be consistent with observations being made elsewhere. The carboxy terminal region of HIV-1 (Palker et al., 1987), HIV-2 (Norrby et al. 1991), SIVmac (Baillou et al., 1991) and EIAV (Ball et al., 1992) have previously been reported to be broadly reactive. In addition, a recent report by Baillou et al. (1991) suggested that this region may distinguish subtypes among lentiviruses, including HIV-1, HIV-2, and SIV.

Several of the other peptides tested appeared to represent species-specific epitopes as they were recognized by a relatively high percentage of one or more of the test serum panels, but few or none of at least one panel. For instance, 42% of the sera from naturally-infected mangabeys contained SAM69-specific Ab while very few of the rhesus or African green monkeys produced SAM69-specific Ab (Table 1). It is interesting that so few of the macaques recognized this peptide as the primary sequence of SIVmac251 and SIV/DeltaB670 are very similar to SIVsm
within the SAM69 region (Figure 13). Similarly, SAM56-specific Ab was produced by 65% of the mangabey panel and 33% of the SIV/Delta-infected macaques, but by very few of the animals in the other test groups (Table 1). Again, the SIVmac251, SIV/DeltaB670, and SIVsm sequences are mostly conserved within the region defined by SAM56 (Figure 13). While relatively high titer SAM36-specific responses were produced by 27% of SIVmac251-infected macaques, only 13% of the other animals tested recognized this peptide (Figure 14; Table 1). In contrast, 33% of the SIV/Delta-infected macaques made SAM42-specific responses, but the intensity of these responses was relatively low. Only a small percentage of the other test groups produced SAM42-specific responses. Similarly, 33% of SIV/Delta-infected macaques and 25% of AGMs produced anti-SAM52 antibodies, but few of the animals from the remaining test groups made a similar response. SAM55-specific responses were made in only a low percentage of each monkey group and the intensity of these responses were relatively low.

Peptides corresponding to predicted T-cell epitopes (Figure 12C) were also employed as antigen in P-ELISA for screening of the four monkey sera panels. T-cell determinants are typically amphipathic α-helical protein segments (Margalit et al., 1987; Wahren et al., 1989) that have low surface probability and generally do not serve as B-cell epitopes (Wahren et al., 1989, Schrier et al., 1989). As expected, very few of the test animals produced Ab specific for any of these peptides (Table 2). In fact, of the 110 animal sera tested, only one recognized MR3 while two reacted with MR4 and two others reacted with MR5.
Table 2. Immunological reactivities of putative T-cell epitopes; SIV env peptide panel vs. monkey serum panels as measured by P-ELISA

<table>
<thead>
<tr>
<th>Serum Panel</th>
<th>External Envelope Protein (SU)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MR3</td>
<td>MR4</td>
<td>MR5</td>
</tr>
<tr>
<td>macaque (21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(SIV/Delta)</td>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>macaque (15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(SIVmac)</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>mangabey (26)</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>African green (48)</td>
<td></td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>monkey</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Reactivity values are listed as percentage of sera tested ( ) yielding positive reactions.
Rabbit peptide-specific antisera characterization.

Rabbit antisera were raised to each of the putative B-cell epitopes described in this study. Approximate titers of peptide-specific antisera were measured via P-ELISA and each of the titers were at least 1:10,000 (Table 3). Using standard ELISA procedures, each of the sera were also screened against whole SIV/DeltaB670, baculovirus-expressed recombinant SIV/DeltaB670 gp110 and gp140, and baculovirus-expressed recombinant SIVmac251 gp140, to determine relative titers and specificity of the antisera with respect to whole SIV proteins (Table 3). Each of the antisera specifically recognized at least one of the SIV antigens (Table 3). Results obtained from Western blotting of these sera against the same panel of antigens confirmed that the rabbit antisera specifically recognize SIV envelope proteins (data not shown).

Using standard in vitro neutralization assays and neutralization blocking assays, the ability of each of these peptides to induce neutralizing immune responses were also examined. None of these peptides, including RP92 and RP93, were able to elicit neutralizing Ab in rabbits, and none of them were able to block neutralization by a reference neutralizing macaque antisera.
Table 3. Immune Reactivities of rabbit anti-peptide antisera

<table>
<thead>
<tr>
<th>Peptide Antiserac</th>
<th>Peptide&lt;sup&gt;d&lt;/sup&gt;</th>
<th>ELISA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SIVAB670</th>
<th>SIVmac251</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM42 (env 46-66)</td>
<td>&gt; 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM35 (env 112-143)</td>
<td>&gt; 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>neg.</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM26 (env 178-207)</td>
<td>&gt; 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM45 (env 269-289)</td>
<td>&gt; 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>neg.</td>
<td>neg.</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM69 (env 365-389)</td>
<td>&gt; 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>neg.</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM36 (env 413-431)</td>
<td>&gt; 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>neg.</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM52 (env 452-481)</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>neg.</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>RaMPS42 (env 503-527)</td>
<td>&gt; 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>neg.</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>SIV-M (env 597-619)</td>
<td>&gt; 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM55 (env 736-757)</td>
<td>&gt; 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>neg.</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAM56 (env 852-879)</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>neg.</td>
<td>neg.</td>
</tr>
</tbody>
</table>

<sup>a</sup> values represent titer detected via ELISA using a 10-fold dilution series  
<sup>b</sup> rabbit anti-peptide antisera specific for indicated peptide  
<sup>c</sup> rabbit anti-peptide antibody titer; homologous peptide  
<sup>d</sup> gradient-purified SIV/DeltaB670 whole virus  
<sup>e</sup> baculovirus-expressed recombinant gp110  
<sup>f</sup> baculovirus-expressed recombinant gp140
Discussion

Serum samples from four different monkey test groups were analyzed via site-directed serology in the studies presented here. Two of these test groups were composed of symptomatic macaques infected with SIV/DeltaB670, or SIVmac251. Both of these virus isolates were originally obtained from infected macaques. The nucleotide sequence of SIVmac251 has been known for some time while the SIV/DeltaB670 sequence was obtained very recently (LeRosa, manuscript in preparation); the derived amino acid sequences of these two SIV isolates are quite similar. The mangabey monkeys used for this study were all obtained from Yerkes Regional Primate Research Center and it is reasonable to assume that they are all infected with related strains of SIV; the sequence of a virus isolate (SIVsm) isolated from one of these monkeys at Delta Regional Primate Center has been reported and shown to be very similar to SIVmac (Hirsch et al., 1989a). The African green monkeys used for this study have been obtained from many sources, both African and Asian, and may be infected with highly divergent strains of SIV (Baier et al., 1990). Infected mangabeys and AGMs are inapparent carriers of SIV and never suffer from disease symptoms. It is also important to note that serum samples were collected from animals at various points during disease progression or inapparent infection.

These studies have identified several regions of the SIV env polyprotein that appear to contain conserved epitopes of the env glycoproteins. Similar to HIV-1 and HIV-2, SIV contains broadly reactive
epitopes near the NH$_2$-terminus of TM (SIV-M), within the PND (RP92/RP93), and at the extreme carboxy terminus of SU (RaMPS42). There are also two regions near the amino terminus of SU, defined by SAM35 and SAM26, which appear to be broadly reactive. Interestingly, none of these regions of SIV SU and TM appear to be highly conserved with respect to primary sequence (Figure 13) indicating that the overall structure of SU and TM are relatively well conserved leaving these regions accessible to the immune system despite variations in primary sequence.

These studies have also identified regions within SIV SU (defined by SAM69, SAM36, SAM42, and SAM52) and TM (defined by SAM56, and SAM55) that appear to elicit species-specific humoral responses. Interestingly, SAM69 and SAM56-specific responses were produced by a high percentage of naturally-infected asymptomatic mangabey monkeys, but were produced by only a low percentage of the remaining monkey test groups. While the SIVagm TYO-1 sequence appears to be quite different from the other SIV isolates in these regions, the sequence variation between SIVmac251, SIV/DeltaB670, and SIVsm in these regions is minimal and is probably not responsible for the differences in immune recognition by the different serum panels. Although the intensities of these particular immune responses is relatively low, these populations of Ab may play a critical role in control of SIV which prevents disease progression in mangabey monkeys infected with the virus.

One of the predicted B-cell determinants, SAM55, appears to represent a region of the TM, that is buried and inaccessible to host humoral immune responses. Only 12% of the infected monkeys made
SAM55-specific Ab, and anti-SAM55 rabbit antisera reacted poorly with SIV proteins in ELISA (Table 3) and Western analyses (data not shown). Host humoral immune responses to the region of TM defined by this peptide are probably inconsequential with respect to infection.

Recently there have been several reports describing vaccine protocols that elicit protection against lentiviral infection (Murphey-Corb et al., 1989, 1991; Desrosiers et al., 1989; Issel et al., 1992). Although it is unclear whether protection is a result of humoral and/or cellular responses, a recent study revealed that passive transfer of anti-HIV-2 or anti-SIV antisera resulted in protection of cynomolgus monkeys from HIV-2 or SIV infection, respectively (Putkonen et al., 1991). These results suggest that humoral immune responses alone may be sufficient to protect against lentivirus infections. Evaluating the kinetics of specific immune responses of SIV-infected versus protected monkeys resulting from vaccine trials may allow the identification of critical antigenic determinants of SIV. It will be interesting to evaluate the kinetics of peptide-specific Ab responses in longitudinal serum samples from protected and unprotected vaccinates (Murphey-Corb et al., 1989, 1991) to determine whether any site-specific humoral immune responses correlate with protection.

Evidence that HIV-1 and other lentiviruses cause mis-direction of humoral immune responses is mounting. The results reported here reveal that Ab specific for at least three regions of the envelope proteins (defined by SIV-M, RP92/RP93, and RaMPS42) are produced very soon after infection and persist throughout disease progression. Since animals
succumb to disease despite these responses, it appears that Ab specific for
these epitopes may not be required for an animal to mount protective
immune responses. In fact, some specific Ab populations have been
shown to be detrimental to the host. There have been several reports that
HIV-specific Ab can cause enhancement of infection (Robinson et al., 1988;
Homsy et al., 1988, 1990; Takeda et al., 1988). Zolla-Pazner and associates
have reported that Ab specific for the immunodominant TM loop region of
HIV-1 can lead to increased infectivity in vitro (Zolla-Pazner et al., 1990). It
has also been shown that viruses sometimes induce autoimmunity by
molecular mimicry (Fujinama and Oldstone, 1989). Recently it was
reported that a MAb directed toward a synthetic peptide corresponding to
the immunodominant TM loop region of HIV-1 cross reacts with
astrocytes and that antibodies specific to this region of HIV may be
responsible for the neuronal disorders experienced by some AIDS patients
(Yamada et al., 1991). Immunization of monkeys with peptide-based
vaccines may redirect immune responses resulting in a minimization of
deleterious Ab production and an altered rate of disease progression
following challenge with pathogenic virus.

These studies have identified several humoral antigenic
determinants of SIV. Three of the broadly reactive B-cell epitopes
identified during these studies are located in regions very similar to those
identified in HIV-1, HIV-2, and EIAV thus demonstrating immunological
similarities between the glycoproteins of each of these lentiviruses. These
studies have also identified type-specific humoral responses that may
correlate with protection from disease progression. The peptides used for
site-directed serology in these studies are currently being employed as subunit vaccines to evaluate their ability to modulate the kinetics of immune responses and alter the outcome of disease progression in macaques. By identifying group and type-specific antigenic determinants of the SIV env proteins, these studies have laid the groundwork for thorough evaluation of humoral immune kinetics in vaccinated animals that have been protected from SIV infection.
CHAPTER 5

CYTOPATHICITY OF LENTIVIRUS TRANSMEMBRANE PROTEINS

Introduction

The fate of any virally-infected host can be influenced by physiological and environmental conditions, but it ultimately depends on the virus-host cell combination. For example, it may be possible to attribute the differential fates of oncovirus and lentivirus infections to their respective target cells, since oncoviruses usually infect lymphocytes, while lentiviruses typically infect cells from the monocyte-macrophage lineage (Haase, 1986). However, lymphocytes are also a natural target cell for certain lentiviruses, including the immunodeficiency viruses of man (HIV-1), nonhuman primates (SIV), cattle (BIV), and cats (FIV). Therefore, the differential outcome of lentivirus and oncovirus infections of a common target cell population suggests that the cytopathology characteristics are indeed intrinsic to the virus.

Many researchers have attempted to characterize the mechanisms that are responsible for differing biological properties between oncoviruses and lentiviruses. Much of this research is based on the novel genetic content and complex patterns of gene regulation that have been observed in lentiviruses (Peterlin and Luciw, 1988; Sodroski et al., 1986). Others have concentrated their efforts on structural components of the virus that may contribute to cytopathicity since envelope proteins of several different
viruses have been shown to mediate a variety of cytotoxic effects, such as fusion, syncytium formation, and cell killing (Rasheed et al., 1986; Pinter et al., 1986; Chatterjee and Hunter, 1970; Nagy et al., 1983; Redmond et al., 1984) during infection of target cells. By measuring membrane permeability of HIV-infected cells to Ca\(^{2+}\) and glycerol, Cloyd and Lynn (1991) have shown that a primary mechanism of HIV-1 cytopathicity appears to be perturbation of host cell membranes by the insertion of virus coded envelope proteins. In addition, purified retrovirus transmembrane (TM) proteins have previously been reported to be fusogenic (Gallaher, 1987), cytopathic (Garry et al., 1988), and immunosuppressive (Cianciolo et al., 1985; Cianciolo et al., 1986; Mathes et al., 1978) to cultured cells. Due to the documented abilities of retrovirus TM proteins to alter critical membrane properties and the obvious requirement for intimate interaction between TM proteins and host cell membranes, many investigators are concentrating their studies on retrovirus TM proteins.

Detailed comparative analyses of retrovirus TM proteins have revealed striking structural similarities, although there are only negligible degrees of sequence identity reported for the different viruses. According to secondary structure predictions, Gallaher and associates have proposed a common structural motif for the amino terminal extracellular portion of lentivirus and oncovirus TM proteins; this structure appears to resemble the structure of the influenza HA\(_2\) protein that has been determined by x-ray crystallography (Gallaher et al., 1989). This model can be used to identify several important correlations between
TM structures and functions, e.g., antigenicity, membrane anchoring, and binding to the viral surface glycoprotein (SU).

In contrast to the structural similarities observed in the amino terminal portion of the various TM proteins (Gallaher et al., 1989), careful examination of the carboxyl half of the TM molecules failed to reveal a common structural motif for oncoviruses and lentiviruses. However, one distinct structural domain was observed in lentivirus TM proteins that was absent in oncovirus TM molecules. This characteristic lentivirus TM domain consisted of strong amphipathic helices with the special feature of an unusually high and distinctly organized clustering of positively charged residues, predominantly arginines. This chapter deals with the properties of amphipathic helices and the work that we have done suggesting an important role for the amphipathic sequence near the carboxyl terminus of lentivirus TM proteins.

The amphipathic helix motif.

X-ray crystallography, nuclear magnetic resonance (NMR), and circular dichroism studies of soluble proteins remain the only reliable methods for determining the exact secondary structure conformations formed within polypeptides. Results from these studies have yielded a large database of information regarding the propensity of different amino acids for residing in certain secondary structures that allow the prediction of secondary structure from primary amino acid sequences. Due to the difficulties involved in obtaining quantities of purified membrane-associated proteins, the physical properties of membrane proteins that prevent crystallization, and the difficulties in working with these proteins
outside of an aqueous milieu, few of these proteins have been characterized at the level of atomic structure. Since the most reliable method currently available for predicting secondary structures within proteins is based on comparisons drawn from high-resolution structural determinations of homologous proteins, to date there is no dependable method for predicting membrane protein structures from primary sequence data. However, several basic structural themes that can be applied to protein structural models have emerged through membrane studies. The first is that any segment of a protein consisting of approximately 20 residues and having a high average hydrophobicity is likely to form a transmembrane α-helix (Eisenberg, 1984; Engelman et al., 1986; Kaiser and Kezdy, 1987); however, three dimensional electron microscopy image reconstruction analyses of E. coli porin proteins have revealed that hydrophilic regions can also traverse lipid bilayers (Paul and Rosenbusch, 1985). Interestingly, there appears to be a bias toward having a proline residue within membrane-spanning regions of transmembrane proteins even though proline is known to destabilize α-helical structures folding in aqueous environments (Chou and Fasman, 1974). Since the cyclic side chain of proline cannot form a hydrogen bond with the residue in the preceding turn, it is postulated that these residues are left available for hydrogen bonding with other monomers of the protein or with other domains of the same protein molecule; this type interaction would favor the formation of oligomers or stable tertiary structures, respectively (Deber et al., 1986; Brandl and Deber, 1986). The second basic theme concerns any α-helical region in which the hydrophobic side chains partition on one
face of the helix while the hydrophilic side chains segregate on the opposite helical face; this type of structure is designated an amphipathic \(\alpha\)-helix. These amphipathic helical structures tend to seek membrane interactions and are frequently membrane associated (Kaiser and Kezdy, 1987; Eisenberg et al., 1984). In addition, there is a large amount of indirect evidence that suggests that amphipathic helices often span membranes as aggregates in which the hydrophobic faces of the multimers interact with the lipid bilayer while the hydrophilic faces form a stable pore (Figure 15) that may allow flow of water and electrolytes across the cytoplasmic membrane (Kaiser and Kezdy, 1987; Eisenberg et al., 1984; Lear et al., 1988). Therefore, in the absence of methodology that enables definitive structural characterization of integral membrane proteins, there is an abundance of indirect evidence that allows investigators to apply these general themes to primary amino acid sequences, resulting in theoretical approximations of secondary structure. These proposed secondary structure models can then be related to specific biological consequences, thus facilitating experimental design that can be used to test these models.

**Methods for predicting amphipathic helical structures.**

With the advent of computer algorithms which require only primary sequence data as input, the identification of regions within a protein that have high potential to form amphipathic \(\alpha\)-helical secondary structures has become a rapid process. Amphipathic helical structures are among the most conspicuous of secondary structural elements as they are largely composed of a hydrophobic face and a primarily hydrophilic face due to a characteristic periodicity of hydrophobic and hydrophilic
Figure 15. Model of a transmembrane pore formed by a multimer of an α-helical amphipathic peptide. Two different views of a multimer of an amphipathic peptide as it forms a transmembrane pore. Each peptide monomer is represented as a cylinder; the hydrophilic amino acid side chains are segregated to one side of the helix (shaded) while the hydrophobic amino acid side-chains are segregated on the opposite face of the helix (unshaded). (A) Cross-sectional view. (B) Top-view.
residues. The amphipathic property of helical structures can be graphically demonstrated using "helical wheel" diagrams (Schiffer and Edmondson, 1967) or the helical net or grid representation (Lim, 1978). Preparing these type diagrams is a simple method for visualization of amphipathic potential, but they are limited in that they often over-estimate amphipathicity (Flinta et al., 1983) and they are too tedious for application to long sequences. A more quantitative method for identification of amphipathic helical protein segments, designated the "helical hydrophobic moment" (Eisenberg et al., 1982), has been developed as a computer algorithm that expresses amphipathicity either numerically or graphically. The "Eisenberg plot" not only identifies amphipathic segments, but additionally it has limited ability to differentiate between different types of amphipathic structures (i.e. transmembrane helices, lipid associated helices, receptor-binding helices, etc.) according to characteristic hydrophobicity and hydrophobic moment values common to these type structures. Another computer algorithm based on similar parameters, designated "Amphi", scores the potential of protein segments to form amphipathic helical structures; this program was initially designed to identify immunodominant T cell epitopes since there is a good correlation between amphipathicity and T-cell immunogenicity (Margalit et al., 1987). While new algorithms for identification of amphipathic peptide segments within proteins are still being developed, there is a full range of methods currently in use that enable investigators to detect amphipathic helical domains from primary sequences.
Biological significance of amphipathic helices.

A large body of evidence suggests that the amphipathic α-helical secondary structure motif is critical for cellular immune recognition. There have been several reports that the antigenic entity for T-cell recognition is a peptide fragment derived from the whole protein (Berzofsky, 1980; Benacerraf, 1978; Berzofsky, 1987); it has also been reported that T-cell antigenic determinants involve local features of protein structure such as sequence properties involving charge and hydrophilicity, and certain types of secondary structure, such as helicity, that do not depend on the tertiary folding of the entire protein (Margalit et al., 1987). More specifically, Delisi and Berzofsky (1985) showed that short peptides recognized by helper T-cells are usually amphipathic protein segments. It has been proposed that the hydrophobic face of an amphipathic peptide interacts with the MHC molecule while the hydrophilic face interacts with the T-cell receptor (Pincus et al., 1983; Delisi and Berzofsky, 1985). This hypothesis was experimentally supported by structural analyses of 12 immunodominant T-cell epitopes of sperm whale myoglobin (Berkower et al., 1986; Cease et al., 1986) and was further supported by Spouge and co-workers (Spouge et al., 1987) who analyzed 23 immunodominant class II restricted T-cell antigenic sites from 12 proteins and showed that helical amphipathicity and α-helicity are both statistically significant correlates of T-cell antigenicity, independently of each other. These results together suggest that following antigen processing, amphipathic α-helical segments of the processed protein are the basis of cellular immune recognition.
The amphipathic helical motif has also been found in several membrane-spanning helical regions of proteins that form membrane pores. The most well established is bacteriorhodopsin, an integral membrane protein of a marine archaebacterium, *Halobacterium halobium*. Bacteriorhodopsin functions as a pump producing a proton electrochemical potential across the cellular membrane. This protein contains seven transmembrane helical regions that are thought to associate through hydrophobic interactions between the apolar faces of their amphipathic helical transmembrane regions and lipids within the membrane bilayer. This interaction allows the polar faces of the helices to form a hydrophilic pore spanning the membrane (Engelman and Zaccai, 1980).

In addition, amphipathic helical structures are also characteristic of naturally occurring membrane-disruptive peptides such as alamethicin (Engel et al., 1985), melittin (Terwilliger et al., 1982; Terwilliger and Eisenberg, 1982a & b), magainins (Zasloff, 1987), and cecropins (Andreu et al., 1985). Alamethicin is a 20 amino acid long peptide antibiotic that oligomerizes (6-11 molecules) to form voltage-gated channels in bacterial membranes; it is proposed that conformational changes in the structure during oligomerization allow a voltage across the bilayer that stabilizes the "open" form of the channel (Fox and Richards, 1982). Melittin is a well studied peptide component of bee venom which also perturbs membranes by forming voltage-gated channels (Kempf et al., 1982). The atomic structure of melittin tetramers has been determined and refined to <2.5 Å. The crystal structure reveals that each melittin monomer exists as an
amphipathic α-helical structure (Terwilliger et al., 1982; Terwilliger and Eisenberg, 1982a & b). Magainins and cecropins are antibacterial peptides found in the skin of frogs (Zasloff, 1987; Zasloff et al., 1988), and in the hemolymph of Cecropia moths (Hultmark et al., 1980; Hultmark et al., 1983; Bowman and Steiner, 1981; Bowman et al., 1985; van Hofsten et al., 1985), respectively. These peptides are similar to melittin in that they form amphipathic helical structures that have a high positive charge density, and they are believed to form multimeric structures that insert into cellular membranes forming ion channels. In each of these cases, ion channels formed by multimers of amphipathic helical peptide structures allow flow of H$_2$O and electrolytes across cellular membranes resulting in a loss of osmotic integrity and eventual lysis of the cell (Jaynes et al., 1986). These cytolytic peptides can inhibit both prokaryotic and eukaryotic cells (Zasloff et al., 1988; Jaynes et al., 1988; Jaynes et al., 1989). It should be emphasized that natural cytolytic peptides are heterogeneous at the primary amino acid sequence level, but all of them have the potential to form amphipathic secondary structures (Figure 16). It appears that the amphipathic helical secondary structural motif is commonly exploited to facilitate specific pathologic or protective ends in nature, and thus may even be utilized by viruses to cause cytopathic effects in host cells.
Figure 16. **Primary amino acid sequences and helical wheel diagrams of the HIV-1 TM amphipathic helical region, Melittin, and Magainin-2.**

Primary amino acid sequences (A) and helical wheel diagrams (B) of HIV-1 (env 828-855), melittin, and magainin-2 showing non-homology of sequences, but similar amphipathic structure. Each residue has been coded by differential shading. Residues within non-shaded boxes are hydrophobic while those within shaded boxes are hydrophilic. To further detail side chain characteristics, three different box-fills have been employed: ⬠ represents basic, ⬡ acidic, and ⬢ polar non-charged residues. These assignments have been made according to the polarity scale of transmembrane helices (Engelman *et al*., 1986).
A

HIV-1  

Melittin  

Magainin  

B

HIV-1 (env 828-855)  

Magainin-2  

Melittin
Results

Amphipathic helices of HIV-1 and SIV TM protein.

We have recently reported that the extreme carboxy-terminus of HIV-1 TM protein contains a region of high amphipathic potential that is absent from the TM protein of oncoviruses (Miller et al., 1991; Miller and Montelaro, 1991). Sequence analysis of various isolates of HIV-1 has revealed that this amphipathic motif is highly conserved between different strains of HIV (Myers et al., 1988). The apparent conservation of these amphipathic regions within the HIV TM protein may imply an important biological role. Figure 17 summarizes representative segments of helices identified in the carboxyl portion of lentivirus TM proteins and the linear distribution of the charged residues. In Figure 18, selected TM helices and their charge distribution are presented in computer-derived models to illustrate the amphipathic nature of the lentivirus helical segments. For purposes of comparison, representative oncovirus TM protein segments are included in each figure. The data presented in these two figures clearly demonstrate the characteristic arginine-rich, positively charged amphipathic helix present in all lentivirus TM proteins examined. For example, HIV-1 (Ratner et al., 1985) contains a 28 residue amphipathic helix that contains a total of 7 arginines for a net positive charge of 5 across the amphipathic helical segment (Figure 17). Similar structures and charge distribution are readily evident in other lentiviruses such as HIV-2 (Guyader et al., 1987), SIV (Hirsch et al., 1987), and EIAV (Rushlow et al., 1986).
Figure 17. **Primary amino acid sequences of proposed lentiviral lytic peptide segments.** The primary amino acid sequences of potentially amphipathic α-helical structures from each of these viruses are listed, and their position in the predicted env polyprotein sequence is indicated numerically. Each residue has been coded by differential shading to show the structural similarities between these sequences. Residues within non-shaded boxes are hydrophobic while those within shaded boxes are hydrophilic. To further detail side chain characteristics, three different box-fills have been employed: ■ represents basic, □ acidic, and △ polar non-charged residues. These assignments have been made according to the polarity scale of transmembrane helices (Engleman *et al.*, 1986). For comparative purposes, the sequences of Magainin-2, a natural cytolytic peptide produced by amphibians, and corresponding segments from several oncoviruses have been included.
HIV-1 828-855  RVILLEQAGRAAIHIEERRICGELRIL
HIV-2 829-857  RGLWRERICGRGILAVERRICGASIAL
SIV 852-879  DLWETLRRGWRILAIEERRICGELTL
EIAV 808-836  RIAYGLRGLAVIIRICIRGILNLFZIIR
HIV-1 465-488  RQLRHLPSRVRVYPLSLKPESSL
RSV 575-602  OFVESIRFMNSSINYHTXREMCGA
MuLV 625-649  LLFCIEILKRLVFVRIRISVVDAL
Figure 18. Computer-generated models of amphipathic α-helical segments from lentiviral transmembrane proteins. Each panel contains an amino acid chain diagram (left), and a space-fill model (right). Each residue has been color-coded to show structural characteristics; hydrophobic residues are colored blue, basic residues are red, acidic residues are yellow, and polar non-charged residues are green. Included in the chain diagrams is a white ribbon used to trace the backbone of the helix; any distortion of this ribbon is due to the presence of proline in the sequence. The chain diagrams are viewed down the barrel of the helix from the carboxy terminal to the amino terminal end of each structure. The space-fill models are viewed laterally with the carboxy terminal end toward the lower right side of the structure. The identity of each of the peptide segments are as follows: (A) HIV-1 (Ratner et al., 1985), (B) HIV-2 (Guyader et al., 1987), (C) SIV (Hirsch et al., 1987), (D) EIAV (Rushlow et al., 1986), (E) Magainin-2, (F) HTLV-1 (Seiki et al., 1983), (G) RSV (Schwartz et al., 1983), and (H) MuLV (Shinnick et al., 1981).
In contrast, examination of the oncovirus TM proteins fails to reveal a comparable structural motif (Figures 17 & 18). In certain oncoviruses, there does appear to be some potential for the formation of helical segments at the carboxyl portions of the TM molecules, however, none of these helical segments display the strong amphipathic properties evident in lentivirus TM proteins (Figure 18). It is important to note that oncoviruses usually also lack the high concentration of arginine residues observed in lentiviruses (Figure 17), even in those TM proteins that have weak potential to form an amphipathic helix. Thus, it appears that the amphipathic helix containing a high density of positive charges is a structural motif unique to lentiviruses and absent from oncoviruses. This fundamental difference in TM structure may correlate with the differences in cytopathicity exhibited by oncoviruses and lentiviruses.

**Bioactivity of lentivirus lytic peptides (LLPs).**

To test this hypothesis directly, we synthesized peptides corresponding to the HIV-1 (HIV-L) and SIV (SIV-L) LLP sequences shown in Figure 1, as well as a peptide of similar size corresponding to a region of SIV gp120 (residues 112-143, control-pep) that does not share the potential structural motif or high positive charge density described for the proposed lentiviral lytic peptides; the primary amino acid sequence of this peptide is NH$_2$-RCNKSQTDRWGLTKSSTTTAAAPTSAPVSEK-CONH$_2$.

For comparative purposes, peptides corresponding to this region of two oncoviruses, RSV (RSV-pep) and HTLV-1 (HTLV-1-pep, Figure 17), were also synthesized. Since synthetic peptides were produced to be employed in biological assays, rigorous purification and characterization procedures
Figure 19. **Characterization of synthetic peptides corresponding to the proposed cytolytic segment of lentiviral TM proteins.** Analytical HPLC profiles of purified (A) HIV-L, (C) SIV-L, and (E) control-pep reveal greater than 95% homogeneity of each peptide preparation. Plasma desorption mass spectrometry data from (B) HIV-L (3309.2 amu), (D) SIV-L (3390.5 amu), and (F) control-pep (3425.1 amu) reveal that the desired peptide product was obtained in each case. Similar data for RSV-pep and HTLV-1-pep is not shown.
were performed to insure that any bioactivity detected was inherent to the desired peptide product (Fontenot et al., 1991). Representative HPLC and mass spectrometry profiles of purified peptide preparations are shown in Figure 19. Following purification and characterization, synthetic peptide preparations were employed in standard assays designed to measure the ability of cytolytic peptides to kill prokaryotic cells (Lehrer et al., 1983).

The results summarized in Figure 20 reveal that both HIV-L and SIV-L have the ability to kill significant numbers of *Staphylococcus aureus* (gram-positive) at 10 μM and 100 μM peptide concentration, respectively, while concentrated RSV-Pep, HTLV-1-Pep, and control-pep are unable to kill these bacteria. Assays in which 1 X 10^4, 1 X 10^5 and 1 X 10^6 *Staphylococcus aureus* were treated yielded very similar results (Figure 20) and thus, demonstrated that cell killing was independent of the number of bacteria treated. Similar assays using approximately 1 X 10^4, 1 X 10^5 and 1 X 10^6 *Pseudomonas aeruginosa* (gram-negative) resulted in total killing by HIV-L and SIV-L at 1 μM and 10 μM peptide concentration, respectively (data not shown). As expected, *S. aureus* is less sensitive than *P. aeruginosa* to treatment with the LLPs, probably due to differences in glycolipid and phospholipid content of bacterial cell membranes and the peptidoglycan content of bacterial cell walls. Clearly, HIV-L is more active against both species of bacteria than SIV-L, presumably because of the higher positive charge density and greater degree of overall amphipathy across HIV-L (Figures 17 & 18).

These results demonstrate that LLPs, like natural lytic peptides, are able to specifically kill prokaryotic cells. In fact, the specific activity of the
Figure 20. Lentivirus TM lytic peptides mediate killing of prokaryotic cells. One ml suspensions of *Staphylococcus aureus* in 10 mM K$_2$HPO$_4$ buffer were incubated for 1 hour at 37°C with SIV-L, HIV-L, RSV-pep, HTLV-1-pep, or Control-Pep at 1 μM, 10 μM, and 100 μM concentration; approximately (A) 1 X 10$^4$, (B) 1 X 10$^5$, and (C) 1 X 10$^6$ bacteria were treated in separate assays. Following incubation, the bacterial suspensions were serially diluted (10-fold) and 100 μl of each dilution was spread evenly over the surface of a TSA plate and then incubated overnight at 37°C. Colonies were then counted to determine the number of survivors. Results shown represent the average of duplicate plating and are presented as percentage of cells surviving incubation with peptide. All assays were performed at least two times to insure reproducibility.
HIV-1 LLP appears to be higher than that observed for synthetic magainin-2; in these assays, lytic activity of magainin-2 was very similar to that observed for SIV-L (data not shown).

As lentiviruses infect eukaryotic rather than prokaryotic cells, we next examined the cytopathic properties of the synthetic LLPs using the RH9 subclone of HuT78, a CD4+ T-lymphocytic cell line susceptible to HIV and SIV cytopathic effects (Garry et al., 1988; Figure 21). It is clear from comparison of these results with those in Figure 20, that the concentrations of HIV-L and SIV-L required to reduce viable cell numbers of the eukaryotic cells by 1-2 logs were similar to the concentrations required to kill like numbers of prokaryotic cells. Assays with RSV-pep, HTLV-1-pep, and Control-pep revealed that treatment with these peptides resulted in no reduction in numbers of viable cells. We have begun to investigate the actual mechanism of synthetic LLP-mediated cell inhibition by performing ⁵¹chromium (⁵¹Cr)-release assays on fresh human peripheral blood leukocytes (PBLs). Preliminary results indicate that synthetic LLPs do, in fact, perturb plasma membranes as ⁵¹Cr is released by ⁵¹Cr-loaded cells following treatment with either HIV-L or SIV-L (Figure 22). Addition of as little as 100 μM of either LLP to resting PBLs loaded with ⁵¹Cr results in release of 70-75% of the total ⁵¹Cr content and in death of all cells treated. Addition of Control-pep, RSV-Pep, or HTLV-1-Pep resulted in only background levels of ⁵¹Cr release and cell inhibition. Similar results were obtained from assays using H9 cells and mitogen-stimulated PBLs. Since LLP-mediated ⁵¹Cr release appears to
Figure 21. **Lentivirus** TM lytic peptides mediate killing of eukaryotic cells. One ml cultures (5 x 10^5 cells/ml) of the RH9 subclone of HuT78 cells were incubated with HIV-L, SIV-L, RSV-pep, or Control-pep at the indicated concentrations. Following incubation for 24 hours at 37°C in RPMI 1640 medium with 10% serum supplement, the cells were stained with the vital exclusion dye trypan blue and the numbers of viable cells were determined by counting in a hemocytometer. The values represent the average of quadruplicate determinations and the S.E.M. was smaller in each case than the size of the symbols as drawn. The results are representative of at least three independent experiments with each peptide.
Figure 22. LLP-mediated $^{51}$Cr release from PBLs. Unstimulated (Panel A) and mitogen-stimulated (Panel B) human peripheral blood leukocytes (PBLs), and unstimulated H9 cells (Panel C) loaded with $^{51}$Cr were treated with 10 and 100 μM concentrations of HIV-L, SIV-L, Control-Pep, RSV-Pep, and HTLV-1-Pep. Following a 24 hour incubation period, cell supernatants were assayed for $^{51}$Cr content and viable cells were counted in a hemacytometer via trypan blue dye exclusion. All values were corrected for spontaneous $^{51}$Cr release and cell death as measured for untreated cell cultures.
correlate with cell death, it appears that a loss of osmotic integrity leads to death of these cells.

To further investigate the membrane perturbing properties of HIV-1 and SIV LLP, these peptides were employed in assays in which the influx of several radioactive molecules into CEM cells were measured following exogenous addition of LLP in vitro. Results obtained from these experiments were very similar to those reported for HIV-infected cells (Cloyd and Lynn, 1991). Treatment of CEM cells with HIV-L resulted in influx of significant levels of $^{45}$Ca$^{2+}$ and $^{14}$C-sucrose, and only a small influx of $^{14}$C-inulin (Figure 23A). Influxes of $^{45}$Ca$^{2+}$ and $^{14}$C-sucrose were also measured in cells treated with SIV-L, at lower levels, while no influx of $^{14}$C-inulin was detected (data not shown). Similar assays employing Control-Pep (data not shown), and MR-7 (randomized HIV-L peptide; discussed further in Chapter 6) revealed no influx of any of these compounds (Figure 23B). These data suggest that small pores are rapidly formed in the cytoplasmic membrane of the LLP treated cells allowing the passage of small molecules across the membranes. These results are consistent with those obtained from $^{51}$Cr-release assays, and are also consistent with our original hypothesis that the LLPs oligomerize, insert into biological membranes, and disrupt the osmotic integrity of the cell.

Overall, these results indicate that HIV-L and SIV-L are capable of killing both eukaryotic and prokaryotic cells. While the exact mechanism of either bacterial or eukaryotic cell killing mediated by the lentivirus TM lytic peptides cannot be determined from these results, preliminary studies reveal that LLPs do perturb cellular membranes and therefore
share functional as well as structural characteristics of natural cytolytic peptides.
Approximately $5 \times 10^6$ CEM cells were treated with 10 $\mu M$, 30 $\mu M$, or 100 $\mu M$ HIV-L, SIV-L, Control-pep, or MR7 (negative control). Influx of $^{45}$Ca$^{2+}$ (Panel A), $^{14}$C-sucrose (Panel B), and $^{14}$C-inulin (Panel C) into peptide-treated CEM cells was then measured. Samples of $5 \times 10^5$ cells were removed 30, 60, 90, and 120 seconds after addition of peptide and radiolabel, centrifuged through mineral oil to separate the cells from the supernatant, and the cell pellets were assayed for radioactivity. The MR7 peptide has the same amino acid composition as HIV-L, but the sequence has been scrambled so that the peptide has no amphipathic potential. This peptide is further described in Chapter 6.
A

**Graphs showing time post peptide addition in seconds and CPM 45-Ca+ levels for different peptides and concentrations.**

- **Graph 1:**
  - No Peptide
  - HIV-L (10µM)
  - HIV-L (25µM)
  - HIV-L (50µM)

- **Graph 2:**
  - No Peptide
  - SIV-L (10µM)
  - SIV-L (25µM)
  - SIV-L (50µM)

- **Graph 3:**
  - No Peptide
  - Control Pcp (15µM)
  - Control Pcp (25µM)
  - Control Pcp (10µM)

- **Graph 4:**
  - No Peptide
  - MR7 (1µM)
  - MR7 (10µM)
  - MR7 (100µM)
Discussion

The assignment of cytopathic properties to the indicated carboxyl amphipathic helices in lentivirus TM proteins may explain several observations being made with lentiviruses. For example, a variant of HIV-1 designated X10-1 has been described that replicates in normal human T cells in vitro, but does not kill the infected cell (Fisher et al., 1986). Sequence analysis of this variant HIV-1 genome revealed a replacement of the four carboxy-terminal amino acids with a 14 residue segment that does not appear to have amphipathic character. Using a panel of mutants containing deletions from the carboxyl end of HIV TM protein, Lee et al. (1989) have shown that deletions as small as 12-15 residues are sufficient to impair infectivity and cytopathicity of some clones. In further support of this concept is the report that isolates of HIV differing in their relative cytopathicity display amino acid sequence variations in the env gene sequences encoding the proposed cytopathic helices described here (Kowalski et al., 1987; Sakai et al., 1988). It also has been reported that propagation of SIV in cultured cells results in the production of truncated TM proteins, the result of termination codons introduced into the env gene during replication in vitro (Hirsch et al., 1989b; Kodama et al., 1989). These env termination codons are always upstream from the predicted cytopathic peptide segments. Thus, passage of SIV in cell cultures may be selecting for noncytopathic variants produced by the loss of cytopathic env sequences in the TM proteins.
The lytic properties of natural cytolytic peptides require the formation of peptide multimers (Jaynes et al., 1986; Jaynes et al., 1988; Jaynes et al., 1989; Zasloff et al., 1988). For lentivirus TM amphipathic helices to affect host cell membranes, it is reasonable to assume a similar requirement for TM multimer formation. In this regard, Özel et al. (1988) have reported that the envelope proteins of HIV appear to exist as trimers of gp120-gp41, while Schawaller et al. (1989) have concluded a tetrameric arrangement. Moreover, Haffar et al. (1991) have shown that the cytoplasmic tail of HIV TM forms a stable association with the lipid bilayer of host cells, while Venable et al. (1989) have used extensive computer modelling and energy calculations of the HIV TM protein to predict an aggregation of the carboxyl amphipathic helices which could span the lipid bilayer of a cell membrane. Results of bacterial-inhibition assays with the HIV and SIV amphipathic peptides suggested a requirement for multimer formation since inhibition was dependent on peptide concentration but was independent of the number of bacteria used in each assay (Miller et al., 1991). Taken together, these observations suggest that lentivirus TM multimers should be able to form, to insert into cell membranes, and to alter membrane properties as described for natural cytolytic peptides.

It is difficult to correlate the concentration of exogenous LLP required to produce cytopathology with a particular level of virus envelope protein expression in infected target cells. In the case of lentivirus infected cells, the TM proteins rapidly form oligomeric structures that are always intimately associated with internal and plasma membranes of the
cell (Özel et al., 1988; Schawaller et al., 1989). Thus there is no concentration threshold required to facilitate multimerization and cytopathic effects can result from chronic exposure to even low levels of membrane altering TM proteins. In contrast, exogenous amphipathic peptides require critical concentrations for assembly into multimers that can interact only with the plasma membrane of target cells, where cytopathic effects are relatively rapid. The role of LLP concentration is demonstrated by the observation that bacterial inhibition was dependent on peptide concentration, but independent of bacterial cell concentrations. In addition, the specific cytolytic activity of the LLPs appeared to correlate with the specific amphipathic potential and, thus, propensity to form oligomeric structures. The greater amphipathic potential of the HIV-L peptide compared to SIV-L may explain the higher cytopathic activity of the former in certain assays.

Several groups have reported that the carboxy-terminal end of HIV-1 and HIV-2 gp41 contains two domains (residues 768-788 and 826-854, and 757-779 and 828-854, respectively) that have a very high hydrophobic moment indicating that these regions may form amphipathic α-helices (Eisenberg and Wesson, 1990; Eisenberg et al., 1988; Fuji et al., 1988; Haffar et al., 1987). The second of these domains in both HIV-1 and HIV-2 correspond closely to the LLPs described in the present study. Recently, Segrest et al. (1990) devised a classification scheme in which amphipathic α-helical domain-containing proteins and peptides where placed into one of seven groups according to their physiochemical and structural properties. According to this classification scheme, the HIV-1 and HIV-2
amphipathic domains, and thus the LLPs described here, most closely resemble the calmodulin-binding amphipathic helices (class K), while natural cytolytic peptides constitute class L. Members of class K and class L characteristically have both a very high mean hydrophobic moment and a high positive charge density, but they differ primarily in their lysine to arginine ratio, as members of class K typically have a significantly lower lysyl to arginyl ratio than do members of class L. Since calmodulin (CaM) is a ubiquitous intracellular protein that is important for regulation of the activity of many cellular enzymes, any interference with CaM "activity" could produce a variety of alterations in individual cells. Preliminary investigations regarding the CaM-perturbation potential of the LLPs are underway, and results from these studies are discussed in Chapter 6.

Implication of the LLP domain of lentivirus TM proteins as a modulator of CaM cascade kinetics, along with the demonstrated lytic activity of this structure, suggests that this amphipathic helical structure could play a very important role in lentivirus-induced cytopathology.

Previous studies with a variety of retroviruses have identified fusogenic peptides (Garry et al., 1988) and immunosuppressive peptides (Mathis et al., 1978; Cianciolo et al., 1985; Cianciolo et al., 1986; Ruegg et al., 1989a; Ruegg et al., 1989b) in the TM proteins of both oncoviruses and lentiviruses. Recently, Kowalski and coworkers demonstrated that mutations in the fusion domain of HIV-1 greatly reduced cytopathicity as well as syncytia formation (Kowalski et al., 1991). The lytic peptides described here are distinct from these other cytopathic sequences in their location and in their biochemical properties. For example, the HIV fusion
peptide is a highly hydrophobic sequence located at the amino terminus of the TM protein (Garry et al., 1988), while the HIV immunosuppressive peptide is a hydrophilic peptide located toward the middle of the TM polypeptide chain (Ruegg et al., 1989b). Thus, it appears that lentivirus TM proteins have integrated a repertoire of different membrane altering protein segments that all contribute to cell cytopathicity and presumably to the development of disease.

Although the proposed LLPs may traverse the cell membrane, they are evidently nonessential and not required for assembly of the viral envelope. Strains of SIV encoding truncated TM proteins produce infectious virus particles containing the TM protein lacking the proposed cytopathic peptide segments (Chakrabarti et al., 1989). Thus, other hydrophobic sequences in the SIV TM protein must serve to anchor the envelope complex to the viral lipid bilayer as proposed by Gallaher et al. (1989).

In summary, we have reported a variety of observations and experimental evidence that make a interesting case for a correlation of lentivirus cytopathicity with unique structural domains in the viral TM protein, specifically the highly positively charged amphipathic helices at the carboxyl end of the TM protein. Although the model described here is somewhat hypothetical, it does provide an important framework for experiments that can directly test the relationship of these LLPs with cytopathogenesis. These include more intensive functional and structural analyses of lentiviruses containing altered transmembrane proteins produced naturally or by in vitro mutagenesis procedures.
CHAPTER 6

ALTERATIONS IN CALMODULIN ACTIVITIES BY LLPS

Introduction

One of the most perplexing problems in current AIDS research is defining the molecular determinants and mechanisms of HIV-1 pathogenesis. HIV-1-associated disease is characterized by a variety of distinct pathologies, ranging from depletion of specific cell populations, to severe immunosuppression, to CNS disease, to the development of Kaposi's sarcoma. At this time it is uncertain whether the diversity of disease symptoms is the result of a single mechanism that produces a remarkably pleiotropic disease pattern or if distinct pathologies result from different aspects of virus gene expression and replication processes. To unravel the genetic determinants of HIV-1 pathogenesis, a number of laboratories have utilized site directed mutagenesis to analyze the role of specific genes (structural and nonstructural) in virus mediated cytopathicity in vitro. Other laboratories have attempted to correlate natural variations in HIV-1 isolates with specific pathogenic properties in vivo. A conclusion of both approaches is that the envelope gene is a major, but not exclusive, determinant of critical biological and pathogenic characteristics of different virus strains.

Complementary to these genetic studies have been a number of reports demonstrating that viral replication can produce deleterious modifications in cell membrane properties that may contribute to
cytopathogenesis and cell death (Cloyd and Lynn, 1991; Lynn et al., 1988; Garry et al., 1988). Moreover, studies on purified HIV-1 envelope proteins and peptide segments reveal a variety of intrinsic membrane perturbations, including alterations in permeability, fusion of membranes, and suppression of lymphoproliferation (Garry et al., 1988; Gallaher 1987; Cianciolo et al., 1985). Interestingly, most of these cytopathic effects are associated with the HIV-1 transmembrane protein (TM) which is most intimately involved with the cell membrane and which is in the unique orientation of accessing both the exterior and cytoplasmic sides of the plasma cell membrane. In light of the numerous regulatory processes that are cell membrane associated, it is not surprising that viral envelope protein insertion into the organized structure of the plasma membrane should result in significant perturbations in membrane structure and function.

While depletion of CD4+ cells is a hallmark of HIV-1 infection (Fauci, 1988; Kopelman and Zolla-Pazner, 1988; Yarchoan and Broder, 1989; Spickett and Dalgleish, 1988), parameters of immune responses such as T-cell activation (reviewed in Shearer and Clerici, 1991), IL-2 production, and interferon-γ production have also been reported to be altered by HIV-1 infection (Ciobanu et al., 1983; Lane et al., 1985; Kirkpatrick et al., 1985; Murray et al., 1984; Notka and Pollard, 1989; Notka and Pollard, 1990). Several studies have shown that T-helper functions can be defective in asymptomatic HIV-seropositive individuals although levels of CD4+ cells have not been significantly reduced (Peterson et al., 1989; Shearer et al., 1986; Clerici et al., 1989) suggesting that HIV infection
results in alterations of normal lymphocyte functions that are independent of cellular depletion. Activation of T-cells is initiated through presentation of ligand by MHC to the T-cell antigen receptor complex (Haskins et al., 1983; Meuer et al., 1983, Weiss et al., 1986) which leads to the mobilization of Ca\textsuperscript{2+} (Finkel et al., 1987); the sudden increase in free cytosolic Ca\textsuperscript{2+} is considered to be a universal signal for cell activation (Durham and Walton, 1982) because enzyme systems required for T-cell activation are either directly, or indirectly dependent on intracellular [Ca\textsuperscript{2+}]\textsubscript{i} fluxes. Thus, perturbation of the Ca\textsuperscript{2+} second messenger system in these lymphocyte populations could lead to unresponsiveness of these cells.

Calmodulin (CaM), a ubiquitous 16,700 Da protein that is found in all eukaryotic cells (Asselin et al., 1989), regulates a wide range of cellular and enzymatic functions in response to intracellular [Ca\textsuperscript{2+}]\textsubscript{i} (Figure 24; Cheung, 1980; Cheung, 1985; Feinberg et al., 1987) and is believed to be the primary intracellular receptor for Ca\textsuperscript{2+} (Levin and Weiss, 1978). CaM contains four Ca\textsuperscript{2+}-binding sites which are unoccupied at normal intracellular [Ca\textsuperscript{2+}]\textsubscript{i} within unstimulated T-cells. Upon Ca\textsuperscript{2+} influx, due to mitogenic or receptor-mediated activation of T-cells, the four Ca\textsuperscript{2+}-binding sites of CaM become fully saturated resulting in a conformational alteration which increases the affinity of CaM for target enzymes and proteins by several orders of magnitude (Klee, 1988). Previous studies revealed that shortly following mitogenic stimulation, cytoskeletal contractile proteins of human T lymphocytes undergo a dynamic reorganization by a poorly understood calmodulin-dependent mechanism.
Figure 24. **Schematic diagram of calmodulin-dependent processes.** At normal resting intracellular [Ca$^{2+}$], the four Ca$^{2+}$-binding sites of CaM are empty (CaM, Ca$^{2+}$-unbound) and CaM has low affinity for its effector enzymes. Upon cellular stimulation, which begins as an influx of Ca$^{2+}$ across the plasma membrane and/or release of Ca$^{2+}$ from intracellular stores, all four of the Ca$^{2+}$-binding sites of CaM become filled and the CaM undergoes a conformational shift (CaM-Ca$^{2+}$) that results in a tremendous increase in its affinity for its effector enzymes. These enzymes, which are inactive when not bound by CaM (effector enzymes, inactive), become activated when bound by CaM (effector enzyme-CaM complex, active). This diagram depicts a non-exhaustive list of many of the CaM-dependent enzymes and the processes they are involved in; in several cases, CaM is known to be involved in the process that is indicated, yet the effector enzyme has not yet been identified.
(Bachvaroff et al., 1980; Bachvaroff et al., 1984). It has been postulated that mitogen-induced Ca\(^{2+}\) influx results in the formation of Ca\(^{2+}\)-CaM complexes which activate vital enzyme systems and mediate cytoskeletal alterations that are essential to the transmission of the mitogenic signal (Cheung et al., 1983; Mookerjee and Jung, 1990). Mitogenic activation of T-lymphocytes results in proliferation of cells in the presence of interleukin-2 (IL-2; Figure 25; Nisbet-Brown et al., 1985; Crispe et al., 1985; Ledbetter et al., 1987). Since IL-2 is an essential signal for immune cell proliferation, and since CaM has been shown to participate in IL-2 secretion (Colombani et al., 1985; Kronke et al., 1984), CaM has a pivotal role in T-cell proliferation. In fact, the results of several studies suggest that extracellular CaM stimulates DNA synthesis and is therefore intimately involved in cell proliferation (Crocker et al., 1988; Gorbacherskaya et al., 1983; Boynton et al., 1980).

In addition to its role(s) in activation and proliferation of T-cells, CaM plays a critical role in many other cellular processes. For instance, evidence suggests that CaM is essential for interferon-\(\gamma\) (IFN-\(\gamma\))-induced HLA class II expression on many cell types. Upon binding to its receptor, IFN-\(\gamma\) mediates Ca\(^{2+}\) influx and activation of the CaM second messenger cascade which in turn induces class II expression (Koide et al., 1988).

Following cellular activation, CaM also plays an integral role in regulation of intracellular Ca\(^{2+}\) by binding to and activating the plasma membrane Ca\(^{2+}\)-pump in a Ca\(^{2+}\)-dependent manner (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977; James et al., 1988), thereby stimulating the pump to extrude Ca\(^{2+}\), returning the cell to homeostasis.
Figure 25. **Schematic diagram showing interleukin-2 (IL-2) involvement in lymphocyte expansion cascade.** IL-2 acts on lymphocytes that have already been activated (*) by antigen. IL-2 signals activated cells to expand, and drives their differentiation into effector cells. Acronyms used are: CTL, cytotoxic T lymphocyte; IL, interleukin; NK, natural killer; Th, T helper; Bp, B lymphocyte. From Swain, 1991.
Requirement for CaM has also been implicated in the process of exocytosis of granular compartments that contain cytolytic proteins and enzymes necessary for cytotoxic T-lymphocyte-mediated lysis of target cells (Takayama and Sitkovsky, 1987). Studies using the CaM antagonist, cyclosporin A, suggest that CaM may also play an integral role in the incorporation of fatty acids into membrane phospholipids (Szamel et al., 1986; Szamel et al., 1985). The results of one study even suggests that extracellular CaM accumulates during the S phase of the cell cycle and then activates several intracellular processes leading to cell division (Tomlinson et al., 1984). In addition to these processes that depend on CaM, there are many other enzymes that perform a variety of cellular functions that require functional CaM to relay the Ca$^{2+}$-activation message (Figure 24).

**Potential calmodulin-binding regions of lentivirus transmembrane (TM) proteins.**

As described in Chapter 5, we have recently reported the identification of a region near the carboxyl terminus of the TM protein of several lentiviruses which structurally and functionally resembles natural cytolytic peptides. We have provisionally designated this region of lentivirus TM protein as a lentivirus lytic peptide (LLP) and have demonstrated that synthetic peptide analogs indeed are functionally cytopathic (Miller et al., 1991; Miller and Montelaro, 1991). These initial studies and the reports of others describing a correlation between natural and experimental genetic variations in the LLP env gene sequences and viral cytopathicity suggest that this unique structural domain
significantly contributes to HIV-1 pathogenesis through cell membrane perturbation. As was also mentioned in Chapter 5, this LLP structural motif is very similar to the CaM-binding regions of many calmodulin-dependent enzymes. CaM has been shown to bind tightly to peptides which have the properties of forming amphipathic α-helices and which have a high positive charge density (Cox et al., 1985; DeGrado, 1988; Persechini and Kretsinger, 1988; O’Neil and DeGrado, 1989). The described LLPs also have an unusually high hydrophobic moment (Eisenberg and Wesson, 1990; Eisenberg et al., 1988; Haffar et al., 1987) and a high arginine to lysine ratio, which are also characteristics of CaM-binding peptides (Segrest et al., 1990). As CaM is an essential component of activation and proliferation pathways of T-cell populations and plays an important role in many other cellular processes, any virus-specific subversion of CaM activity could be detrimental to the host cell.
Results

Gel mobility shift analyses.

To determine whether the amphipathic helical motifs common to lentiviral TM proteins bind to CaM and thereby interrupt the Ca\(^{2+}\) second messenger cascade within infected cells, we first examined the CaM-binding potential of synthetic peptide analogues of these amphipathic sequences. The peptides HIV-L and SIV-L, which correspond to the LLP of HIV-1 and SIVmac251 respectively (as described in Chapter 5), as well as several control peptides, where tested for CaM binding activity in PAGE gel-mobility shift assays as described by Head and Perry (1974).

Since amphipathity and high positive charge density are the structural features reported to be characteristic of CaM-binding peptides, we worked with several peptides, in addition to HIV-L and SIV-L, which have one, neither, or both of these features. The primary amino acid sequence and the amphipathic potential of each peptide tested are shown in Figure 26. As previously discussed, both HIV-L and SIV-L have high amphipathic potential and high positive charge density. The synthetic peptide used as a positive control, designated MR9, was previously reported to bind CaM with an affinity within the nanomolar range (Erickson-Viitanen and DeGrado, 1987); this peptide has both high positive charge density and high amphipathic potential. Two negative control peptides were also evaluated for their CaM-binding activities (Figure 26). One of these peptides, designated DG-A, has potential to form an amphipathic α-helix, but lacks the high positive charge that appears to be
Figure 26. Primary amino acid sequences and helical wheel diagrams of the synthetic peptides employed in CaM-binding assays. Primary amino acid sequences (Panel A) and helical wheel diagrams (Panel B) of HIV-L, SIV-L, MR9 (positive control), DG-A (negative control), and MR7 (negative control) showing the amphipathic potential and positive charge density of each peptide. Each residue has been coded by differential shading. Residues within non-shaded boxes are hydrophobic while those within shaded boxes are hydrophilic. To further detail side chain characteristics, three different box-fills have been employed: □ represents basic, □ acidic, and □ polar non-charged residues. These assignments have been made according to the polarity scale of transmembrane helices (Engelman et al., 1986). The tryptophan residue of MR9 has been acetylated (Ac).
a requirement for CaM binding capacity. The negative control peptide designated MR7 is a derivative of the HIV-L peptide; it has the exact amino acid composition of HIV-L, however, the sequence has been engineered in an effort to eliminate all amphipathic potential of the peptide. The MR7 peptide therefore has high positive charge density, but it has little amphipathic potential.

As expected, the HIV-L, SIV-L, and the positive control peptide (MR9) caused a total shift of CaM toward the cathode (Figure 27A). Each of these peptides elicited unique shift patterns, possibly due to their net positive charge. The shift of CaM migration toward the cathode by the binding peptides was as follows: HIV-L > SIV-L > MR9. To determine whether the altered migration of CaM was due in part to multimerization of peptide, binding reactions were carried out in binding buffer containing either 2 M or 4 M urea to prevent peptide aggregation. Since this effect was repeatable when using either 2 M or 4 M urea, alterations in CaM migration appeared to be the result of the net positive charge of the binding peptide and not due to binding of CaM by different sized peptide multimers. The exact concentration of each peptide stock solution and the CaM stock solution used for these assays was determined by amino acid composition analysis. The results of these quantitative assays indicated that a 15:1 molar ratio of HIV-L and SIV-L to CaM was used in these initial studies while a 3:1 molar ratio of MR9 to peptide was used. Neither the DG-A (Figure 27A) or the MR7 peptide (data not shown) elicited a shift in CaM mobility. These results demonstrate that synthetic peptides corresponding to the LLP region of HIV and SIV have the ability to bind to
Figure 27. Gel mobility shift assay demonstrating binding of CaM by synthetic peptides. Synthetic peptides corresponding to amphipathic segments of HIV-1 and SIVmac251 TM proteins as well as several control peptides were evaluated for their ability to bind CaM in the presence and absence of calcium. Synthetic peptide was incubated in the presence of CaM at a molar ratio of approximately 15:1 (for HIV-L, SIV-L, and DG-A) or 5:1 (MR9) for 1 hour in binding buffer containing 4 M or 2 M urea and either 0.1 mM CaCl$_2$ (Panel A) or 2.0 mM EGTA (Panel B). The synthetic peptide/CaM mixtures were then subjected to non-denaturing PAGE on a 12.5% gel containing 4 M urea and either 0.1 mM CaCl$_2$ (Panel A) or 2.0 mM EGTA (Panel B). The sample compositions (Panels A and B) were as follows: A) CaM alone, B) CaM + DG-A (negative control), C) CaM + MR9 (positive control), D) CaM + HIV-L, E) CaM + SIV-L. Panel A also contains samples that were incubated in binding buffer containing 2 M urea. The composition of these samples is as follows: F) CaM alone, G) CaM + DG-A (negative control), H) CaM + MR9 (positive control), I) CaM + HIV-L, J) CaM + SIV-L.
CaM in the presence of Ca\(^{2+}\) in a manner which is similar to a well-described CaM-binding peptide.

To determine whether binding of these peptides to CaM was a Ca\(^{2+}\)-dependent interaction, similar assays were performed in the absence of Ca\(^{2+}\). To accomplish this the specific Ca\(^{2+}\) chelator EGTA was substituted in the binding buffer and in each component of the resolving gel and resolving buffer to maintain CaM in its unsaturated state. The removal of Ca\(^{2+}\) resulted in unaltered migration of CaM following incubation with CaM-binding peptides (Figure 27B). These studies show that binding of CaM by the LLPs requires that CaM exists in its Ca\(^{2+}\)-bound conformation. Since CaM-dependent enzymes also bind CaM in a Ca\(^{2+}\)-dependent manner, these results suggest that the LLPs may bind in the same CaM site as do CaM-binding proteins.

To evaluate further the CaM binding activities of HIV-L and SIV-L, the relative binding affinity of each peptide was measured for by performing 2-fold dilutions of each peptide, prior to addition of CaM, followed by gel mobility shift analysis. The results of this experiment reveal that the HIV-L, SIV-L and MR9 peptides each are able to shift the migration of CaM at a molar ratio of peptide to CaM as low as 1:1 for MR9 and 2:1 for HIV-L and SIV-L (Figure 28). In addition, the shift pattern characteristic of each CaM-binding peptide was not altered when molar ratios of peptide to CaM were lowered, further demonstrating that multimers of peptide are probably not required to elicit a shift in PAGE migration. The negative control MR7 peptide was unable to cause a shift in CaM migration even at 15:1 molar ratio.
Figure 28. Gel mobility shift assay to determine the minimum molar ratio of LLP to CaM required to induce altered migration of CaM. Each synthetic peptide was serially diluted 2-fold prior to addition of CaM and then incubated in the presence of CaM for 1 hour in binding buffer containing 4 M urea and 0.1 mM CaCl$_2$. The beginning peptide concentration resulted in a molar ratio of approximately 15:1 for HIV-L, SIV-L, MR9, and MR7 while the starting molar ratio of DG-A to CaM was 5:1. The synthetic peptide/CaM mixtures were then subjected to non-denaturing PAGE on a 12.5% gel containing 4 M urea and 0.1 mM CaCl$_2$. The sample compositions were as follows: Panel A: A) CaM alone, B) CaM + DG-A (negative control), C) CaM + HIV-L (15:1), D) CaM + HIV-L (7.5:1), E) CaM + HIV-L (4:1), F) CaM + HIV-L (2:1), G) CaM + HIV-L (1:1), H) CaM + SIV-L (15:1), I) CaM + SIV-L (7.5:1), J) CaM + SIV-L (4:1), K) CaM + SIV-L (2:1), L) CaM + SIV-L (1:1). Panel B: A) CaM alone, B) CaM + DG-A (negative control), C) CaM + MR9 (positive control) (5:1), D) CaM + MR9 (2.5:1), E) CaM + MR9 (1.25:1), F) CaM + MR9 (0.75:1), G) CaM + MR9 (0.38:1), H) CaM + MR9 (0.19:1), I) CaM + MR7 (randomized HIV-L) (15:1), J) CaM + MR7 (7.5:1), K) CaM + MR7 (4:1), L) CaM + MR7 (2:1), M) CaM + MR7 (1:1).
Phosphodiesterase competition assays.

To quantitate the extent to which the HIV-L and SIV-L peptides bind CaM, a standard competition assay was employed as previously described (Schiefer, 1986). In this assay, the activity of the CaM-dependent enzyme phosphodiesterase (PDE) was monitored as a measure of CaM-binding. The dependence of PDE activity on CaM concentration is demonstrated in Figure 29. From this PDE-CaM calibration curve, the concentration of CaM required to stimulate 50% of CaM-dependent PDE activity was derived. While holding the stoichiometries between CaM and PDE constant, increasing quantities of MR9 (a known CaM-binding peptide which has a $K_D$ for CaM of approximately $1 \times 10^{-9}$ M), HIV-L, SIV-L, and the negative control peptide MR7 were added to the reaction mixture and PDE activity was monitored. The data demonstrate almost identical enzyme inhibition kinetics for MR9, HIV-L, and SIV-L while two orders of magnitude higher concentrations of the negative control peptide MR7 were required to inhibit 50% PDE activity (Figure 30). The amount of each peptide required to inhibit PDE activity in this assay by 50% is listed in Table 4. Applying the standard mathematical model for determining relative affinities using competition assays, and taking the affinity of the MR9 peptide as $1 \times 10^{-9}$ M (Erickson-Viitanen and DeGrado, 1987; Table 4), the results of these assays suggest that the relative affinity of HIV-L and SIV-L for CaM is within the nanomolar range.

Lentivirus TM binding to CaM.

Although the gel mobility shift assays described above conclusively show that synthetic peptides corresponding to the carboxy terminal region
Figure 29. **Stimulation of phosphodiesterase activity by calmodulin.** The stimulatory effect of calmodulin (CaM) on phosphodiesterase (PDE) was measured in a standard PDE assay as previously described (Schiefer, 1986). While holding the PDE concentration constant, varying amounts of CaM were added to a series of PDE assay mixtures, and PDE activity was measured by monitoring absorbance over a 12 minute period. The amount of CaM required to induce approximately 50% activation of PDE was determined for use in subsequent experiments.
Figure 30. Inhibition of phosphodiesterase activity by synthetic LLPs. The relative affinity of HIV and SIV LLPs for CaM were measured in standard PDE inhibition assays (Schiefer, 1986). The amount of CaM required to induce 50% maximal PDE activity, as determined previously (Figure 29), was added to each reaction mixture. Varying quantities of HIV-L (Panel A), SIV-L (Panel B), or the negative control peptide MR7 (Panel C) were added to a series of assay mixtures. The absorbance (265 nm) of each reaction mixture was monitored over a 12 minute period to determine the PDE activity. Results of similar experiments using a known CaM-binding peptide, designated MR9, are shown in each panel for comparative purposes.
Table 4. Inhibition of calmodulin-dependent phosphodiesterase activity by calmodulin-binding peptides

<table>
<thead>
<tr>
<th>Peptide Inhibitor</th>
<th>HIV-L</th>
<th>SIV-L</th>
<th>MR9</th>
<th>MR7</th>
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*a* enough CaM was added to each assay mixture to induce 50% stimulation of PDE activity

*b* pmoles peptide added to reaction mixture

> indicates the approximate point of 50% PDE inhibition
of HIV-1 and SIV are able to bind to CaM, they provide no evidence that this region of lentiviral TM proteins are able to bind CaM while in the context of the entire TM protein. To this end, we attempted to identify virus-specific CaM-binding proteins from HIV-1 or SIV-infected cell extracts as well as from gradient-purified virus preparations.

First, we employed a previously described method for detecting CaM-binding proteins (Billingsley et al., 1990) which is modified from typical Western blot analysis. Cellular extracts from HIV-IIIB, HIV-RF, and SIV/DeltaB670 infected CEM cells, as well as gradient-purified HIV-1 and SIV/DeltaB670 were resolved via SDS-PAGE on 12.5% gels, resolved proteins were electrophoretically transferred to nitrocellulose membrane, and CaM-binding proteins were detected as described in Materials and Methods (Chapter 2). As expected, many CaM-binding proteins, corresponding to cellurally-derived CaM-binding proteins, were detected in each of the cell extracts (Figure 31). No bands corresponding to the TM protein of HIV-1 or SIV/DeltaB670 (41-kDa) were detected in any of the virus containing lanes with the CaM probe. However, one band corresponding to a protein of approximately 160-kDa, which is the size of the envelope protein precursor of SIV, was detected in the SIV-infected CEM cell extract. A similar band was not identified in the uninfected CEM cell extract suggesting that this 160-kDa protein was virus-coded. In addition, faint bands corresponding to proteins of approximately 65-kDa and 28-kDa were detected in the gradient purified HIV-1 and SIV/DeltaB670 preparations; similar proteins were not detected in uninfected CEM cell extracts. Although these results are not definitive,
Figure 31. Detection of CaM-binding proteins on blots using biotinylated CaM as a probe. HIV-1 and SIV/DeltaB670-infected cell extracts and gradient-purified virions were subjected to SDS-PAGE and the resolved proteins were electrophoretically transferred to nitrocellulose membranes. Biotinylated CaM was then used as a probe to identify CaM-binding proteins in each preparation. The contents of each lane probed are as follows: (A) HIV-IIIB-infected CEM cell extract; (B) HIV-RF-infected CEM cell extract; (C) HIV-1 gradient-purified virions; (D) uninfected CEM cells; (E) SIV/DeltaB670-infected CEM cell extract; and (F) gradient-purified SIV/DeltaB670 virions.
they suggest that HIV-1 and/or SIV/DeltaB670-encoded proteins are able to bind to CaM.

To further address this question, we performed calmodulin precipitation assays using each of the HIV-1 and SIV protein preparations described above. Following precipitation with CaM sepharose, CaM-binding proteins from each of these preparations were resolved via SDS-PAGE and then electrophoretically transferred to nitrocellulose membrane. HIV-1 and SIV-encoded proteins were then identified by blotting with sera from HIV-1-infected humans and SIV/DeltaB670-infected macaques, respectively. A preliminary experiment revealed that several SIV-specific proteins bound to the CaM sepharose (data not shown). SIV TM-specific antisera will be employed to definitively show whether either of these SIV encoded CaM-binding proteins are the TM protein. Similar experiments with HIV protein preparations are currently underway.
Discussion

These studies have shown that synthetic peptide analogues corresponding to the carboxy terminus of HIV-1 and SIV are able to bind to CaM and interfere with CaM-dependent second messenger processes. It is widely known that HIV-infected individuals suffer from comprehensive immune dysfunction, much of which is the result of the inability of immune cells to become activated and to receive proliferative signals. As the primary vehicle for Ca\textsuperscript{2+}-mediated signal transduction, CaM plays an essential role in these processes. The interaction of the carboxy terminus of lentivirus TM proteins with CaM explain many of the cellular dysfunctions that have been observed by AIDS researchers.

Several studies have demonstrated defects in lymphocyte signal transduction that are induced by HIV-1 infection (Nye and Pinching, 1990; Gupta and Vayuvegula, 1987; Hofman et al., 1990). These studies have shown that intracellular [Ca\textsuperscript{2+}] in HIV-1 infected cells is elevated and that inositol polyphosphate metabolism is abnormal. Each of these studies reported that inositol triphosphate (Ins[1,4,5]P\textsubscript{3}) and tetrakisphosphate (Ins[1,3,4,5]P\textsubscript{4}) levels in resting T-lymphocytes were chronically elevated in HIV-1 infected cells. Both Ins[1,4,5]P\textsubscript{3} and Ins[1,3,4,5]P\textsubscript{4} are key second messengers that are important for protein kinase C activity and therefore are involved in activation pathways. It is possible that interruption of CaM interactions could be involved since CaM-dependent enzymes are involved in the metabolism of Ins[1,4,5]P\textsubscript{3}. Several reports have shown that synthetic peptides homologous to immunosuppressive regions of HIV and
other retroviral TM proteins (Ruegg et al., 1989b; Cianciolo et al., 1985), when conjugated to bovine serum albumin and added exogenously to lymphocyte cultures, are able to inhibit lymphocyte activation by interfering with PKC activation pathways (Ruegg and Strand, 1991; Kadota et al., 1991). To date, there have been no reports of unconjugated synthetic peptide analogues of HIV-1 envelope proteins that alter cellular processes upon exogenous addition to cells in vitro.

HIV-1 infection has been shown to result in a variety of other dysfunctions of host cells that can be traced to an uncoupling of enzymatic processes. For instance, lymphokines such as IL-2 and IFN-γ are produced at greatly reduced levels in HIV-1 infected individuals. Expression of IL-2, which is the primary proliferative signal for many lymphocyte subtypes (Figure 25), has been shown to be a Ca²⁺-CaM-dependent process; the CaM antagonists cyclosporin A (CsA) and FK506 block transcription of the IL-2 gene (Crabtree, 1989; Liu et al., 1991), suggesting a direct role for CaM in the transcription of IL-2 mRNA. HIV-1 infection is also associated with defects in IFN-γ which is another lymphokine essential to immune proliferative cascades (Nokta and Pollard, 1990). IFN-γ induces the expression of MHC class-II markers (Basham and Merigan, 1983; Collins et al., 1984; Pober et al., 1983), which are essential for restriction of immune responses (Koide et al., 1982), and it mediates Ca²⁺ influx by a receptor-mediated calcium channel (Yasutaka et al., 1987; Koide et al., 1988). Studies with the calmodulin inhibitor W7 have revealed that CaM and CaM-dependent enzyme systems are involved in IFN-γ production (Antonelli et al., 1988) and class-II marker expression
In vitro studies suggest that HIV-infection is also associated with an increase in intracellular cAMP and cGMP levels. In HIV-1 infected MT-4 cells, cAMP and cGMP levels were elevated 40-fold by day 8 PI and 4-fold by 4 days PI, respectively (Nokta and Pollard, 1991). One of the possible mechanisms of elevation of these cyclic nucleotides is the inhibition of the CaM-dependent phosphodiesterases which degrade cyclic nucleotides. Interestingly, the resulting rise in cAMP levels may play an important role in HIV replication as an artificial increase of cAMP (by addition of forskolin, an adenylate cyclase activator) results in enhancement of HIV-1 replication in a dose dependent manner (Nokta and Pollard, 1991). Finally, there is evidence that HIV-1 infection is also associated with altered phospholipid synthesis, a process that is CaM-dependent (Szamel et al., 1986; Szamel et al., 1985). Cloyd and Lynn (1991) reported that HIV-1 infected cells grew normally for 24-48 hours post-infection. After 48 hours however, the process of cell division was slowed although host cell DNA, RNA, and protein synthesis remained unaltered. The only detectable manifestation of cell injury was perturbation of lipid synthesis. Levels of phospholipid synthesis was decreased while neutral lipid synthesis was elevated, resulting in leaky membranes that became more and more leaky until cell lysis. The mechanism of perturbation of phospholipid synthesis was not clear from these experiments. Clearly, HIV-1 infection can result in the perturbation of many enzymatic processes of host cells.

The mechanism of HIV-1-induced enzymatic dysfunction leading to lymphocyte unresponsiveness may be similar to that exhibited by known
CaM antagonists. Several studies have shown that lymphocytes treated with CaM antagonists are left unable to become activated and to proliferate in response to mitogenic stimulation (Hidaka et al., 1981; Stavitsky et al., 1984; Wright and Hoffman, 1986; Britton and Palacios, 1982). Moreover, each of the HIV-1 related cellular alterations described above could be caused by subversion of CaM activity. The studies described here suggest that the carboxyl terminus of HIV-1 and SIV TM may mimic known CaM antagonists by competing with CaM-dependent enzymes for CaM-binding. Synthetic peptides corresponding to this potential CaM-binding segment of TM (designated LLP, Chapter 5), which share structural homology with known CaM-binding peptides, bind CaM in a calcium-dependent manner and with relatively high affinity. Addition of these peptides to standard PDE assays resulted in inhibition of enzyme activity similar to that seen when no CaM was added to the reaction mixture. Overall, these results suggest that the LLP of the TM protein of HIV and other lentiviruses should be able to bind to CaM within the host cell, thus preventing CaM from performing its second messenger functions that are critical for cellular activation, lymphokine production, phospholipid synthesis, and many other normal cellular processes.

Due to its centralized role in a large number of cellular processes, especially its many functions integral to activation of immune cells, CaM seems to be a logical target for a virus that induces immunosupression. It appears that the envelope proteins of lentiviruses, which are the primary cytopathic determinants of lentiviruses, may also have targeted this essential Ca^{2+}-second messenger. It has been previously reported that
CaM, in its Ca\(^{2+}\)-unbound state, is a substrate for the proteases of HIV-1 and HIV-2 (Tomasselli et al., 1991), suggesting an additional means by which these viruses may subvert CaM-dependent processes.

Although the experiments described here do not definitively show that the enzyme dysfunctions resulting from lentivirus infections are mediated by the highly positively charged, amphipathic helical region near the carboxyl end of the TM protein, they do suggest that this region of these viruses could at least play a contributing role in lentivirus pathology. Together, the potential CaM modulation and membrane perturbation properties (discussed in Chapter 5) of this region of HIV-1 and other lentiviruses may play a pivotal role in lentivirus-induced disease. This possibility is very interesting and certainly merits further study as it would represent a novel mode of viral and/or microbial pathogenesis.
Acquired immunodeficiency syndrome (AIDS), and its etiological agent HIV-1, has rapidly become one of the most intensively investigated diseases known to man. Due to the incredibly debilitating disease syndrome that results from HIV-1 infection, and because of its 100% mortality rate, the HIV virus is also one of the most feared pathogens in history. Many researchers are currently attempting to develop appropriate animal models of AIDS in order to provide avenues for investigating the pathogenesis of HIV-1, development and testing of candidate vaccine strategies, and the evaluation of new antiviral therapies. As was previously discussed in Chapter 1, SIV-infected rhesus macaques continue to be the most appropriate animal model for AIDS.

Biochemical and serological analysis of SIV/Delta B670 proteins

The studies reported here were initiated soon after SIV had been isolated and described as the etiological agent of an immunosuppressive AIDS-like syndrome of rhesus monkeys. Our first efforts were directed toward the biochemical and immunological characterization of SIV/DeltaB670. Using standard biochemical procedures we were able to identify the SU, TM, CA, and MA proteins of SIV/Delta B670. Our results, described in Chapter 3, along with the observations of several other groups have led to a putative virion model that is very similar to that of HIV-1. These studies formed the foundation for the first successful SIV vaccine
trial (Murphey-Corb et al., 1989) as well as one of the first reported protective SIV subunit vaccine trials (Murphey-Corb et al., 1991).

In addition to these biochemical studies, we utilized several computer algorithms and manual procedures designed to identify secondary structure from primary amino acid sequences of SU and TM of SIV. The resulting secondary structure models (Figure 10) are very similar to those predicted for the SU and TM of other oncoviruses, including HIV-1. Although these structural models are highly speculative, they may serve as a useful tool for experimental design. In fact, structural modeling of the TM protein led to the experiments described in Chapters 5 and 6 herein.

**Site-directed serological studies**

Due to increasing interest in HIV vaccine development, and due to our initial success with both whole virus and subunit vaccine approaches, our emphasis, and that of our collaborators at the Tulane Regional Primate Center (Michael Murphey-Corb and associates) shifted toward the intensive evaluation of protective immune responses. Using synthetic peptide methodologies, we employed site-directed serological techniques in an effort to identify the viral correlates of protection against SIV infection. Since the results of our subunit vaccine trials suggested that the presentation of envelope proteins of SIV was essential for eliciting a protective immune response in vaccinates (Murphey-Corb et al., 1991), we concentrated our efforts on the envelope proteins of SIV. While screening serum panels from asymptomatic mangabey and African green monkeys as well as experimentally-infected and diseased rhesus macaques
(discussed in Chapter 4) we were able to identify several type-specific epitopes which are immunogenic in asymptomatic mangabeys but do not elicit humoral responses in macaques. We have also identified potential T-cell determinants of SIV envelope proteins that are currently being assayed for reactivity with T-cells from SIV-infected monkeys. Synthetic peptides corresponding to these epitopes are currently being evaluated as subunit vaccine candidates to determine whether they elicit protective antibody populations. In addition, we are currently employing synthetic peptide methodologies to identify cellular epitopes of SIV envelope proteins.

Membrane perturbation and CaM binding properties of LLP

The secondary structural characterizations described in Chapter 3 led to the identification of a structural feature within the carboxyl terminus of the TM protein of several lentiviruses that was not identified in any of the oncoviruses surveyed. As discussed in Chapters 5 and 6, the structural characteristics of this TM domain were very similar to those of natural cytolytic peptides and to calmodulin-binding peptides. Our studies have shown that synthetic peptides corresponding to this carboxy terminal domain of HIV-1 and SIV (provisionally termed the lentivirus lytic peptide, or LLP) also share functional characteristics with both natural cytolytic peptides and with calmodulin-binding proteins. We have hypothesized that the LLP region of the TM protein contributes to viral cytopathogenesis by interfering with the cellular calmodulin-dependent second messenger response and, as the infection progresses, contributes to membrane perturbation of infected host cells contributing to cell death.
The studies described in Chapter 5 have shown that this amphipathic TM domain of lentiviruses shares functional as well as structural characteristics of natural cytolytic peptides. We have shown that μM concentrations of HIV or SIV LLP are sufficient to inhibit prokaryotic and eukaryotic cells. Membrane permeability studies and ⁵¹Cr-release assays with these peptides have further suggested that the mechanism of cell perturbation is similar to that utilized by natural cytolytic peptides. The results reported in Chapter 5 make an interesting case for a correlation of lentivirus cytopathicity and this positively charged, amphipathic TM segment.

The results reported in Chapter 6 suggest that in addition to its potential role in cytopathicity, the LLP may also interfere with the second messenger signalling mechanisms of infected cells. We have shown that the HIV and SIV LLPs bind to calmodulin with affinities in the nanomolar range. Since calmodulin is the primary cellular sensor of Ca²⁺, and since Ca²⁺ is the primary signal for activation of many enzyme systems, alterations in calmodulin cascade kinetics could result in the uncoupling of many normal cellular enzymatic processes. As was discussed in Chapter 6, many of the manifestations of HIV infection that lead to AIDS could result from subversion of the calmodulin second messenger system.

Since the possibility that a defined region of lentivirus TM proteins may contribute to viral cytopathicity by disturbing host cell enzymatic processes, by competitively inhibiting normal second messenger signalling and by perturbing critical membrane properties, and since this
would represent a novel pathogenic mechanism, we have determined that further study of the LLP is warranted. We have recently begun more intensive study of this region with the ultimate goal of answering the following questions:

1) **Are there critical residues essential to these CaM-binding and membrane perturbative properties of LLPs?** One component of this question is the analysis of the mechanisms responsible for these cytopathic activities. We will attempt to identify specific residues within the LLP sequence responsible for these biologic activities using synthetic peptide analogues. These studies will be invaluable for directing future site-directed mutagenesis strategies required to test our hypothesis with isogenic infectious HIV-1 isolates.

2) **Does this LLP, when presented in the context of the folded TM protein, have analogous biologic properties?** For this analysis, HIV-1 TM protein in infected cells, purified virus, or purified to homogeneity will be applied to similar biologic assays as described in question 1.

Results of these studies will define the extent to which the HIV-1 LLP perturbs membrane properties and binds calmodulin (CaM), thereby inhibiting CaM-regulated enzymatic processes. The data obtained will also identify the critical amino acid residues required for these potential cytopathic functions. Thus, the experiments proposed here will produce standard assays and structural information that will prove useful in the design of site-directed mutagenesis studies of LLP functions in infectious molecular clones of HIV-1.
In summary, the studies presented here make a substantial contribution to the biological and immunological characterization of SIV/DeltaB670. The initial modeling studies presented here led to the identification of a structural motif within the carboxyl terminus of lentivirus TM proteins that may be involved in the cytopathicity of lentiviruses. Our results show that synthetic peptides homologous to this region of HIV and SIV TM proteins have membrane pertubative and CaM-binding properties, and that this amphipathic segment of lentivirus TM may participate in a novel method of viral/microbial pathogenesis.
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VITA

Mark Miller was born in Denton, Texas on the twenty-fifth day of March in 1962. He attended first and second grades at University Terrace Elementary School in Baton Rouge, Louisiana between the years 1969 and 1970. He then attended third through sixth grades between the years 1971 and 1974 at Oscar Hinger Grammer School in Canyon, Texas. Mark then completed seventh, eighth, and ninth grades at Canyon Jr. High School in Canyon, Texas between the years 1975 and 1977. From 1978 to 1980 Mark attended and graduated from Canyon High School in Canyon, Texas. From 1981 to 1984 he attended West Texas State University in Canyon, Texas. Following the spring semester of 1984, Mark received a B.S. in Biology and Chemistry. Mark has been attending Louisiana State University in Baton Rouge, Louisiana since the fall semester of 1985 as a graduate student in the Microbiology department. Mark completed the requirements for a doctorate degree in Microbiology on the thirtieth day of January, 1992.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate:  Mark Anton Miller

Major Field:  Microbiology

Title of Dissertation:  Structural, Immunological, and Functional Characterization of Simian Immunodeficiency Virus Proteins

Approved:

Ronald C. Montelaro
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:  1/30/92