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Effect of Amino Acid Substitutions on Calmodulin Binding and Cytolytic Properties of the LLP-1 Peptide Segment of Human Immunodeficiency Virus Type 1 Transmembrane Protein

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Previous studies have identified two highly basic amphipathic helical regions in the human immunodeficiency virus type 1 transmembrane protein that, in vitro, display both cytolytic and calmodulin-binding and -inhibitory properties that could contribute to cellular dysfunctions and cytopathogenesis during a persistent viral infection. In the current study, the structural specificity of the cytolytic and calmodulin-binding activities of the human immunodeficiency virus type 1 lentivirus lytic peptide (LLP-1) are examined with synthetic peptide homologs and analogs. The results of these studies demonstrate that even minor changes in LLP-1 amino acid content can markedly affect these properties, suggesting that sequence variation in these highly conserved LLP sequences may correlate with alterations in viral cytopathic properties.

A primary characteristic of human immunodeficiency virus type 1 (HIV-1) infection is the clinical progression from specific isolated dysfunctions in immune responses to a complete collapse of the immune system (26, 35). Many of the early immune defects observed in HIV-1-infected individuals can be attributed to disruption of normal cellular signal transduction at low levels of virus production (6, 8, 23, 35), whereas the later development of severe immune suppression appears to result from cytolytic mechanisms associated with high viral load and the emergence of cytopathic variants (10, 26, 38). Although HIV-1 cytopathic mechanisms are complex and to date ill defined, numerous studies have implicated the HIV-1 envelope glycoproteins, gp120 surface unit (SU) and gp41 transmembrane (TM) protein, as major determinants of viral cytopathicity (2, 24–26). Several potential cytopathic mechanisms of the TM protein of HIV-1 have been associated with peptide sequences near the N-terminal extracellular domain of gp41; these mechanisms include syncytium formation mediated by the hydrophobic fusion domain (1, 16, 24) and inhibition of lymphoproliferation and protein kinase C by the immunosuppressive peptide (4, 12, 33, 34). In addition to these components of the extracellular domain, we have described two potentially cytopathic regions located in the C-terminal cytoplasmic tail of the TM protein. These two segments, comprising amino acids 828 to 855 and 768 to 788, are highly positively charged because of an abundance of arginine residues and have a high probability of forming amphipathic helices (14, 40). Synthetically prepared peptide homologs of these regions are lytic when added exogenously to either prokaryotic or eukaryotic cells (30, 37); thus, they bear the name lentivirus lytic peptides, or LLP-1 and LLP-2, respectively. Membrane interactions (3, 17, 18), cytolytic activity (30, 37), and suppression of cell activation (36) by this region in vitro are well documented. In addition, the LLP region of the TM protein has been implicated in single-cell cytolysis (15, 25) and infectivity (13, 24, 25, 41), but not the syncytium-forming properties (15, 24, 39) of infectious molecular clones of HIV-1.

Recently the calmodulin (CaM)-binding and -inhibitory properties of both a synthetic peptide homolog of LLP-1 and the complete TM protein were described (31, 36). The formation of peptide-CaM complexes results in inhibition of CaM-mediated enzyme activation because of competition between peptide and enzyme for CaM binding (31, 36). HIV-1 infection can result in significant suppression of T-cell activation and subsequent cytokine production (6, 20, 21, 23, 27, 35), both of which are CaM-dependent processes (7, 11). We have proposed (31) that the LLP domains may contribute to HIV-1 pathogenesis by nonproductively sequestering CaM and interfering with CaM-dependent signal transduction and regulation of metabolic processes leading to cellular dysfunction.

To determine the contributions of specific amino acid residues to both the CaM-binding and lytic properties of HIV-1 LLP-1, a peptide analog study was undertaken. Peptide analogs of this region bearing substitutions of selected residues were synthesized as described by Miller et al. (30). Analogs 1, 2, and 3 (Table 1) each have several basic residues replaced with glutamic acid residues to alter the charge but preserve the amphipathic helical potential of the peptide. Four LLP-1 analogs, each with a single positively charged (R-841→E, R-848→E) or hydrophobic (I-840→S, I-847→S) amino acid substitution, were also synthesized and characterized. The substituted residues were chosen on the basis of homology to CaM-binding sequences (22, 28, 29) as well as their degree of conservation among HIV-1 isolates (32).

The ability of the LLP-1 and analog peptides to bind CaM was evaluated by gel mobility shift analysis (19, 31). In this assay, CaM is incubated with various peptides in the presence of Ca^{2+} and then subjected to electrophoresis under nondenaturing gel conditions to qualitatively assess complex formation. In this gel system, free CaM and CaM-peptide complexes differ in their migration rates because, in part, of differences in net charge. Complex formation is judged by a reduction in intensity of the free CaM band as well as the appearance of a shifted band corresponding to the CaM-peptide complex. Figure 1A shows the results of a gel shift assay in which CaM was

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TABLE 1. Sequences of synthetic LLP-1 and analog peptides

Peptide	Residue no.[a]																											
	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855
LLP-1	R	V	I	E	V	V	Q	G	A	C	R	A	I	R	H	I	P	R	R	I	R	Q	G	L	E	R	I	L
Analog																												
1	–	–	–	–	–	–	–	–	–	–	E	–	–	–	E	–	–	E	–	–	–	–	–	–	–	–	–	–
2	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	–	–	–	–	–	E	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	–	–	–	–	–	E	–	–	–	–	–	–	–
R-841→E	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R-848→E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	–	–	–	–	–	–
I-840→S	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
I-847→S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–

[a] Residue numbers correspond to HIV-1 HX2BR *env* gp160. Identical residues are represented by dashes, and substitutions are indicated by the single-letter code of the replacement amino acid.

incubated with LLP-1 and several analog peptides. LLP-1 binds CaM, resulting in a complex which migrates at a slower rate (lane 2). Under these experimental conditions, none of the peptides with multiple arginine→glutamic acid substitutions (analog 1, 2, or 3) is able to bind CaM at detectable levels, even when a 10-fold molar excess of peptide is used (lanes 3 to 5). The few slowly migrating bands observed in the upper regions of the analog-containing lanes are due to peptide alone, as evidenced by their presence when peptide is run in the absence of CaM (Fig. 1A, lanes 8 and 9). These data demonstrate that substitution of two to three basic residues in LLP-1 completely abrogates CaM-binding properties, reflecting strict criteria for positively charged residues in peptide-CaM interactions.

Similarly, two LLP-1 analogs, each containing only a single arginine→glutamic acid substitution, were evaluated in the gel mobility shift assay (Fig. 1B). These data demonstrate that both the R-841→E (lanes 5 and 6) and R-848→E (lanes 7 and 8) peptide analogs display a substantial impairment of CaM-binding capacity compared with LLP-1: very weak binding is observed by a slight reduction of the free CaM band only at a peptide/CaM molar ratio 10-fold higher than that required to visualize a CaM–LLP-1 complex. These results show that even a single charge change in the peptide sequence can effectively inhibit the CaM-binding ability of LLP-1. To examine the relative contributions of specific hydrophobic residues to the interaction between LLP-1 and CaM, the two peptide analogs bearing single isoleucine→serine substitutions were tested. As shown in Fig. 1B, both the I-840→S (lanes 9 and 10) and I-847→S (lanes 11 and 12) peptides bind CaM; however, approximately fivefold more analog peptide than LLP-1 is required to form a complex with CaM (as indicated by a shift in the CaM band), indicating a lower affinity of these peptides for CaM compared with LLP-1. In contrast to the arginine-substituted LLP-1 analogs, neither hydrophobic residue substitution completely destroys the peptide-CaM interaction, suggesting that the requirements for this type of residue are somewhat less stringent for CaM binding. The affinity of a peptide for CaM can be correlated with its ability to inhibit CaM activation of CaM-dependent enzymes (9, 31).

We have previously shown that the analog peptides containing multiple substitutions are not cytolytic in standard in vitro assays (30), whereas the parent LLP-1 peptide is cytolytic at concentrations of 10 μM and greater (30, 37). To ascertain the effect of amino acid substitutions on the ability of the peptide to lyse eukaryotic cells, the entire panel of LLP-1 analogs was tested in an erythrocyte lysis assay, in which erythrocytes are incubated with various concentrations of peptide and the amount of cell lysis is determined by measuring the amount of hemoglobin released by treated cells (37). Figure 2 shows the cytolytic activity of LLP-1 and analog peptides in this assay. Addition of 100 μM LLP-1 results in 100% lysis of erythrocytes, with a 50% lytic dose calculated to be 22 μM. However, analogs 1, 2, and 3, with multiple arginine→glutamic acid substitutions, show no lytic activity at concentrations as high as 100 μM (only analog 3 is shown). To determine the fewest number of substitutions which cause reduced cytolytic ability and to learn whether the lytic and CaM-binding properties of the peptide could be uncoupled, the single-substituted analogs were tested in the erythrocyte lysis assay. Each of these peptides displayed a markedly reduced cytolytic ability. None of the analogs was able to achieve 50% lysis at the highest concentrations tested; addition of 100 μM R-841→E peptide results in 30% cell lysis, and a 100 μM concentration of R-848→E causes only 10% lysis (Fig. 2). Even more striking is the fact that both of the hydrophobic analogs, I-840→S and I-847→S, cause less than 10% lysis at 100 μM peptide. It is clear that each of these residues is important for the cytolytic properties of LLP-1.

It is interesting that for the LLP-1 analogs possessing a single charge substitution, the losses of lytic and CaM-binding properties are very closely linked; that is, the single arginine substitution had a significant effect on both CaM-binding and lytic properties of LLP-1. However, in the hydrophobic analogs, cytolytic activity overall is more affected than CaM affinity, suggesting that the hydrophobic residues are particularly important for membrane interactions. Crystallographic and nuclear magnetic resonance structural data (22, 28, 29) indicate that CaM is rather flexible in its orientation towards CaM-binding peptides, accommodating slightly different spacing and/or orientation between hydrophobic residues. Electrostatic interactions are believed to contribute significantly to the formation of the CaM-peptide complex, and Meador and coworkers (28) have shown that a specific arginine residue is important for binding of the CaM-binding MLCK peptide to CaM. The nuclear magnetic resonance and crystallographic data are therefore in agreement with our observations that the CaM-binding properties of the LLP-1 analogs are related to the total number of positive charges as well as the particular

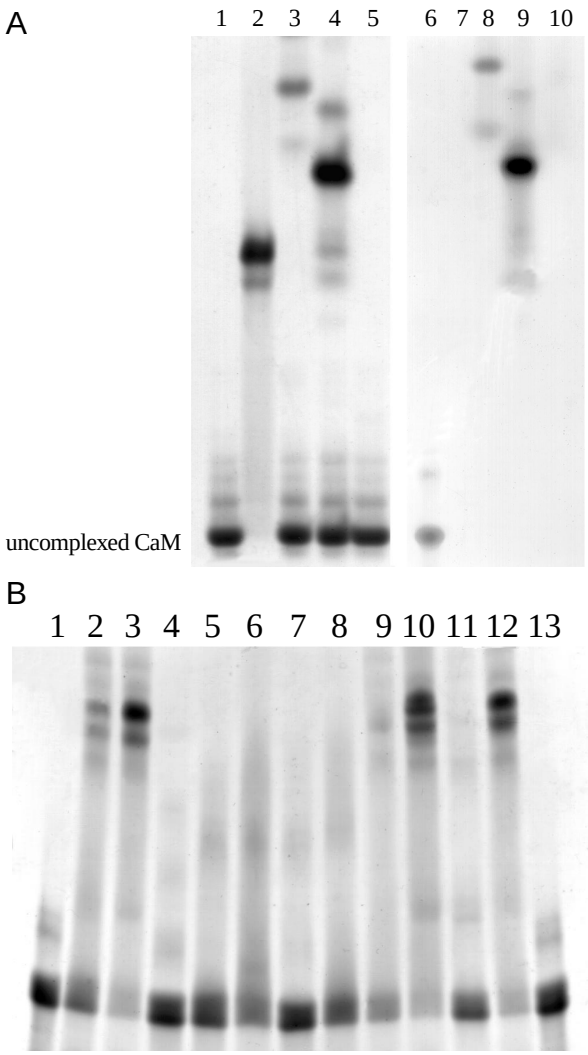

FIG. 1. Gel mobility shift assay of CaM-peptide complexes of LLP-1. CaM (40 μg) was incubated alone or with peptides in the presence of Ca^{2+} prior to electrophoresis in a nondenaturing gel. The gels were scanned and processed with Adobe Photoshop. (A) Gel mobility shift assay of LLP-1 and multiply substituted analogs in the presence or absence of CaM (10:1 peptide/CaM ratio). Lanes: 1 and 6, free CaM; 2, CaM plus LLP-1; 3, CaM plus analog 1; 4, CaM plus analog 2; 5, CaM plus analog 3; 7, LLP-1 alone; 8, analog 1 alone; 9, analog 2 alone; 10, analog 3 alone. None of the substituted analogs binds CaM in this gel system. The bands appearing in the upper regions of lanes 3 and 4 are due to peptide alone, as evidenced by the dark nonshifted CaM bands in these lanes as well as the presence of the upper bands when peptide is run alone (lanes 8 and 9). (B) Gel mobility shift assay of singly substituted LLP-1 analogs. Each singly substituted peptide was tested at peptide/CaM ratios of 1:1 and 10:1. Lanes: 1 and 13, CaM alone; 2, CaM plus LLP-1 (1:1 peptide/CaM ratio); 3, CaM plus LLP-1 (2:1); 4, CaM plus analog 3 (10:1); 5 and 6, CaM plus R-841→E (1:1 and 10:1, respectively); 7 and 8, CaM plus R-848→E (1:1 and 10:1, respectively); 9 and 10, CaM plus I-840→S (1:1 and 10:1, respectively); 11 and 12, CaM plus I-847→S (1:1 and 10:1, respectively). Only the hydrophobic residue-substituted analogs show appreciable complexation with CaM.

placement of these residues in the sequence. Our observation that CaM is much more accommodating of peptides with hydrophobic substitutions is remarkably consistent with the documented flexibility of CaM binding to peptide analogs of CaM-dependent enzymes (22, 28, 29).

The results of the CaM-binding and cytolytic assays utilizing a panel of LLP analogs reveal strict structural requirements for the peptide's functional properties. The data reported here are

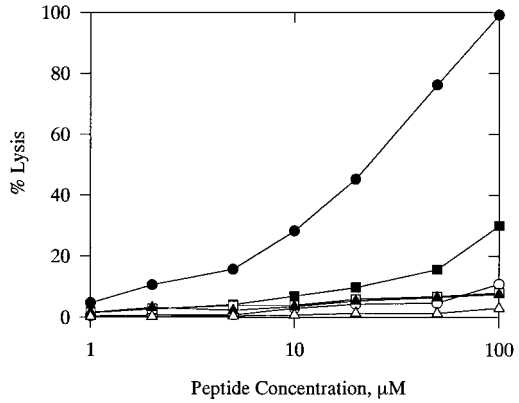

FIG. 2. Lysis of erythrocytes as a function of peptide concentration. Erythrocytes were incubated with various concentrations of peptides, and the degree of lysis was determined by the amount of hemoglobin released from the cells. Peptide concentrations were determined by a quantitative ninhydrin assay (5) with a leucine standard. The multiply substituted analog 3 is unable to lyse the cells to any degree, whereas LLP-1 is increasingly lytic. The singly substituted analogs show a 3- to 10-fold reduction in lytic activity compared with the parent peptide. ●, LLP-1; ■, R-841→E; ○, R-848→E; □, I-840→S; ▲, I-847→S; △, analog 3.

consistent with our hypothesis that the LLP segments constitute highly specific structural domains that could contribute to cellular dysfunction and cytopathicity by perturbing membrane integrity and/or interrupting CaM-dependent regulatory processes in HIV-1-infected cells. In light of the high degree of conservation in LLP sequences among diverse isolates of HIV-1, it would appear that these regions provide functions important to the virus. The structural studies described here suggest that even minor variations in LLP sequences could affect viral cytopathic properties by markedly altering the lytic and CaM-binding potential of the TM protein. Studies to date with specific deletion mutants to assess the function of these regions of TM protein have demonstrated a correlation with viral infectivity and cytopathic properties (13, 15, 25). However, the interpretation of the effects observed in these studies has been somewhat complicated by the reduced levels of TM protein production caused by the relatively large deletions made in the *env* gene. The present studies form an important foundation for the creation of minimal site-directed mutations in the context of the full-length TM protein and molecular clones of HIV-1 in order to evaluate, in well-defined experimental systems, the significance of the LLP regions to HIV-1 cytopathogenesis.

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