Structural and Functional Study of the Apelin-13 Peptide, an Endogenous Ligand of the HIV-1 Coreceptor, APJ†

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ABSTRACT: The APJ receptor is widely expressed in the human central nervous system (CNS). Apelin was recently identified as the endogenous peptidic ligand for human APJ. Studies with animal models suggested that APJ and apelin play an important role in the hypothalamic regulation of water intake and the endocrine axis, in the regulation of blood pressure, and in cardiac contractility. Apelin has been found to block the activity of APJ as a human immunodeficiency virus type I (HIV-1) coreceptor. In this study, we combined chemical synthetic approaches with alanine substitution to evaluate the structural requirements for interactions with the APJ receptor. We demonstrated that apelin peptides in aqueous solution adopt a random conformation, and the positive charge and hydrophobic residues of apelin-13 play important roles in interactions with the APJ receptor. We have observed an important correlation between receptor binding affinity and cell−cell fusion inhibitory activity. The elucidation of structural requirements of apelin-13 in its interaction with the APJ receptor is critical for further investigation of apelin−APJ functions *in vivo* and in the design of small molecular inhibitors for potential treatment of HIV-1 infection in the CNS.

APJ, as a member of the G protein-coupled, seven-transmembrane receptor family, was originally identified from human genomic DNA by O'Dowd *et al*. (*1*), and subsequently was isolated from the mouse (*2*) and rat (*3*−*5*). Apelin, the endogenous ligand for the APJ receptor, was first isolated from bovine stomach extracts (*6*). Then, the protein sequences of human, rat, and mouse apelin pre-pro-proteins were deduced from the cDNAs (*3*, *6*−*8*). Although the mature apelin peptide consists of 36 amino acid residues (apelin-36) (*6*), the shorter C-terminal peptide with 13 amino acids (apelin-13) was found to be produced in bovine colostrums (*3*), and was shown to have higher activities in acidification rate promotion, cAMP production inhibition, and chemotactic action (*3*, *6*).

Northern blot analysis and *in situ* hybridization studies indicated that APJ mRNA could be detected in the central nervous system (CNS) (*1*, *9*, *10*). In addition, apelin-containing neurons were detected in the supraoptic and paraventricular nuclei in the rat brain by immunohistochemistry (*11*), suggesting that apelin plays a role as a neurotransmitter or neuromodulator. In a rat model, apelin was found to function in hypothalamic regulation of water intake and endocrine axes (*8*, *11*, *12*), in regulation of blood pressure via a nitric oxide-dependent mechanism (*8*, *13*), and in cardiac contractility (*14*, *15*). Recent studies have shown that the APJ receptor is employed by T-tropic (CXCR4), M-tropic (CCR5), and dual-tropic HIV-1 and simian immunodeficiency virus (SIV) for supporting *env*-mediated membrane fusion and viral entry *in vitro* (*10*, *16*−*18*), and apelin has been found to block the activity of APJ as an HIV-1 coreceptor (*17*, *19*, *20*).

Recent studies have investigated the physiological role of APJ and apelin, and until now, only one study reported that the arginine residues at positions 63 and 64 of apelin-15 are important for the specific ability to block HIV-1 cell entry via APJ as a coreceptor (*19*). Thus, little is known about the structural requirements for its interaction with the receptor APJ. In this study, we combined chemical synthetic approaches with alanine substitution to further explore the structural and functional determinants of apelin in critical interactions with the APJ receptor.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and G418 were purchased from Life Technologies, Inc. Rhodamine red concanavalin A (ConA), tetramethylrhodamine transferrin, LysoTracker Red, furo-2, and Pluronic F-127 were purchased from Molecular Probes Inc. (Eugene, OR). Plasmid pCDNA-APJ, recombinant vaccinia viruses encoding two envelopes of HIV-1, vBD3(89.6) and vSC60(IIIB), and vTF1.1 encoding T7RNA polymerase were generous gifts of Robert W. Doms (University of

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Pennsylvania, PA). The vector pEGFP-N1 was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). [^{125}I]-(Pyr-1)Apelin-13 was purchased from Amersham Biosciences (Piscataway, NJ), and the specific activity was 2000 Ci/mmol.

Peptide Synthesis. The peptides apelin-13 and apelin-36 and alanine scanning peptides were prepared by solid phase synthesis using an Fmoc strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perseptive Biosystems, Cambridge, MA). Crude peptides were purified by preparative reverse phase high-performance liquid chromatography using a Dynamax-300 Å C_{18} 25 cm × 21.4 mm (inside diameter) column with a flow rate of 9 mL/min and two solvent systems of 0.1% TFA with H_2O and 0.1% TFA with acetonitrile. Fractions containing the appropriate peptide were pooled and lyophilized. The purity of the final product was assessed by analytical reverse phase high-performance liquid chromatography, capillary electrophoresis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Circular Dichroism Spectroscopy. The circular dichroic (CD) spectra of peptides were recorded using an AVIV 62A DS spectropolarimeter (Aviv Instruments Inc., Lakewood, NJ). Solutions of peptides (50 μM) were prepared in 0.01 M sodium phosphate buffer. The spectra were measured from 280 to 180 nm every 2 nm at room temperature, with a quartz cuvette with a path length of 0.01 cm.

Cells and Cell Cultures. The human HEK293 cell line was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). HEK293 and QT6 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS).

DNA Construction. The full open reading frame of wild-type APJ was amplified using PCR and subcloned in-frame into *Hin*dIII and *Bam*HI sites of the pEGFP-N1 vector. The construct was sequenced to confirm the correct sequence and orientation. The plasmid constructs of pCDNA-APJ and pAPJEGFP were transfected into 293 cells by the calcium phosphate precipitation method. Twenty-four hours after transfection, selection for cells exhibiting stable expression was initiated by the addition of G418 (800 μg/mL). Transfected cells were evaluated for expression levels at the cell surface by flow cytometry.

Radioligand Binding Assay. The stably transfected 293APJ cells were used for binding assays. Ligand binding experiments were performed using a single concentration (0.2 nM) of [^{125}I]apelin-13 in the absence or presence of increasing concentrations of cold apelin-13 or apelin-36 in a final volume of 100 μL of binding buffer [50 mM Hepes (pH 7.4), 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% bovine serum albumin] containing 2×10^5 cells. Nonspecific binding was assessed by the addition of 2 μM unlabeled apelin-13. Samples were incubated for 60 min at room temperature. The incubation was terminated by separating the cells from the binding buffer by centrifugation and washing once with 500 μL of cold binding buffer. Amounts of bound ligands were determined by counting γ emissions. At least three independent experiments were performed.

Fluorescence Microscopy. The stable APJ-EGFP-expressing 293 cells were seeded on Nunc two-chamber slides and incubated at 37 °C overnight. After being treated with apelin peptides, cells were washed in PBS and then fixed in PBS containing 2% paraformaldehyde for 10 min. Fluorescence microscopy was performed with an Olympus System microscope, model BX60, with the BX-FLA fluorescence attachment.

Intracellular Calcium Concentration Measurements. Following a modified procedure published by others (*21*, *22*), APJ stably transfected 293 cells were trypsinized and washed twice with phosphate-buffered saline (PBS). For Ca^{2+} mobilization studies, 5×10^6 cells/mL were loaded with the fluorescent dye, fura-2 (3 μM), and 0.05% F127, in Hank's balanced salt solution [140 mM NaCl, 5 mM KCl, 10 mM Hepes (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL glucose, and 0.025% BSA], for 30 min at 37 °C. The cells were washed three times and resuspended at a density of $30-40 \times 10^6$ cells/mL, and then $1.5-2 \times 10^6$ cells were tested in the same buffer. Intracellular Ca^{2+} mobilization was assessed using excitation at 340 and 380 nm on a fluorescence spectrometer (model LS50B, Perkin-Elmer, Beaconsfield, England), upon stimulation with apelin.

Gene Reporter Fusion Assay. A gene reporter fusion assay was used to determine the activities of apelin-13 and its alanine scanning peptides in inhibition of cell−cell fusion via the APJ coreceptor, following a modified procedure published by others (*23*−*25*). Briefly, the effector HEK293 cells were infected with recombinant vaccinia virus with HIV-1 Env proteins and T7 RNA polymerase for 2 h. The target QT6 were cotransfected in six-well plates with plasmids encoding CD4, wild-type APJ, and luciferase under control of the T7 promoter, using the calcium phosphate precipitation method. Four hours after transfection, cells were lifted, seeded in 24-well plates, and incubated at 37 °C overnight. Before fusion took place, the target cells were incubated in the presence or absence of the test apelin for 30 min. To initiate fusion, 10^5 effector cells were added to each well and incubated at 37 °C in the presence or absence of the test apelin. After fusion for 5 h, cells were lysed in 150 μL of reporter lysis buffer (Pharmingen) and assayed for luciferase activity by using commercially available reagents (Pharmingen) with an FB12 luminometer (Zylux Corp., Maryville, TN).

RESULTS

Secondary Structure of Synthetic Apelins. On the basis of the amino acid sequences of apelins (*6*), we chemically synthesized apelin-36 and apelin-13. As shown in previous studies (*26*), these synthetic peptides could bind to the APJ receptor and induce intracellular Ca^{2+} mobilization and receptor internalization in APJ stably expressing 293 cells. To determine the secondary structures of apelin-13 and apelin-36, CD spectroscopy was used to study the solution conformation of apelin-13 and apelin-36. As shown in Figure 1, both the apelin-13 and apelin-36 peptides have a strong negative band in the vicinity of 200 nm, which is characteristic of an unordered structure for both peptides in aqueous solution (*27*).

Properties of the Apelin-13 Mutants in Binding to APJ. Previous studies have indicated that apelin-13 exhibits a

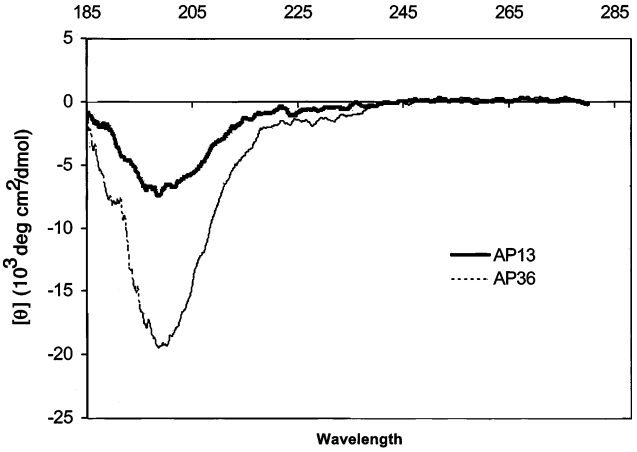

FIGURE 1: Circular dichroism spectra of apelin-13 and apelin-36. Peptides (50 μM) were dissolved in 0.01 M sodium phosphate buffer. All spectra were recorded at room temperature using an AVIV 62A DS spectropolarimeter.

Table 1: Sequence of Apelin-13 and Alanine Scanning Apelin-13

sequence	name of peptide	APJ internalization	signaling
Q-R-P-R-L-S-H-K-G-P-M-P-F	apelin 13	+++++	+++++
A-R-P-R-L-S-H-K-G-P-M-P-F	Q1A	+++++	+++++
Q-**A**-P-R-L-S-H-K-G-P-M-P-F	R2A	+	++++
Q-R-**A**-R-L-S-H-K-G-P-M-P-F	P3A	+	++
Q-R-P-**A**-L-S-H-K-G-P-M-P-F	R4A	+++	+++++
Q-R-P-R-**A**-S-H-K-G-P-M-P-F	L5A	−	++
Q-R-P-R-L-**A**-H-K-G-P-M-P-F	S6A	++	++++
Q-R-P-R-L-S-**A**-K-G-P-M-P-F	H7A	+++++	+++++
Q-R-P-R-L-S-H-**A**-G-P-M-P-F	K8A	+	++++
Q-R-P-R-L-S-H-K-**A**-P-M-P-F	G9A	++	++++
Q-R-P-R-L-S-H-K-G-**A**-M-P-F	P10A	−	++
Q-R-P-R-L-S-H-K-G-P-**A**-P-F	M11A	+	++
Q-R-P-R-L-S-H-K-G-P-M-**A**-F	P12A	+++++	+++++
Q-R-P-R-L-S-H-K-G-P-M-P-**A**	F13A	+++++	+++++

higher activity in acidification rate promotion, cAMP production inhibition, and chemotactic action than apelin-36 (*3*, *6*). To determine the structural requirements of apelin for binding and activation of the APJ receptor, as illustrated in Table 1, 13 alanine scanning apelin-13 peptides were chemically synthesized. In our competitive binding assays, we found that [^{125}I]apelin-13 bound to the APJ receptor with high affinity, and cold apelin-13 (IC_{50} = 0.7 nM) competed more efficiently with [^{125}I]apelin-13 than cold apelin-36 (IC_{50} = 2.3 nM) (Figure 2a). We then tested these 13 alanine scanning peptides for their potential to compete with the binding of [^{125}I]apelin-13 to APJ. The binding results demonstrated that substitutions of alanine for residues R2, P2, R4, L5, S6, K8, G9, P10, and M11 within the sequence of apelin-13 decrease the binding activity, whereas peptide analogues with alanine replacements for residues Q1, H7, P12, and F13 were comparable to wild-type apelin-13 (Figure 2b).

Biological Activities of Alanine Scanning Apelin-13 Peptides. Next, the alanine scanning peptides were further investigated for their abilities to induce signaling and receptor internalization. The treatment of the 293 cells stably expressing APJ with alanine scanning peptides using final concentrations of 3 μM caused intracellular Ca^{2+} mobilization, but compared to those with wild-type apelin-13, mutant peptides P3A, L5A, P10A, and M11A yielded weaker responses (Figure 3a and Table 1).

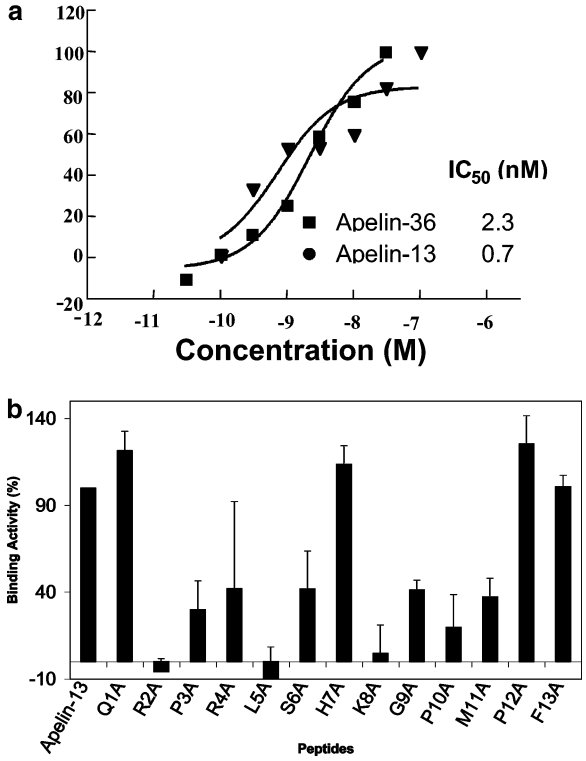

FIGURE 2: Receptor binding activity of alanine scanning apelin-13 peptides. (a) Competitive binding of apelin-13 and apelin-36 with [^{125}I]apelin-13 to APJ. (b) Binding activity of alanine scanning apelin-13 peptides. The APJ stably expressing 293 cells were incubated with [^{125}I]apelin-13 (0.2 nM) in the presence of increasing concentrations of cold apelin-13 or apelin-36, and cell-associated radioactivity was counted with γ emissions. The bars represent the mean values of three independent assays, whereas the error bars are the mean ± the standard error of the mean.

Interestingly, mutant peptide R2A, which demonstrated low levels of induced APJ internalization (see below), was shown to have increased potency in intracellular Ca^{2+} internalization (Figure 3b). This effect was shown to be APJ-dependent, as it was not detected in cells lacking all chemokine receptors or cells expressing CXCR4 alone (Figure 3b).

We then used stable APJ-EGFP-expressing 293 cells to examine receptor internalization upon administration of alanine scanning peptides. Figure 4 and Table 1 demonstrate that the peptides mutated at residues Q1, H7, P12, and F13 were capable of inducing receptor internalization as potently as wild-type apelin-13; however, R4A, S6A, and G9A had a weakened ability in inducing APJ internalization, and R2A, P3A, L5A, K8A, P10A, and M11A lost the potential for inducing receptor internalization.

Antiretroviral Activity of Alanine-Substituted Apelin-13 Peptides. The alanine scanning peptides were further examined for their ability to block HIV-1 entry via the APJ coreceptor and gp120 of HIV-1 isolate IIIB using a cell fusion reporter assay. As indicated in Figure 5a, the peptides with lower binding activity showed lower activity in blocking cell−cell fusion, and alanine replacement of the first and last amino acid preserved the same activity in inhibition of HIV-1 fusion exhibited by apelin-13. The substitution of alanine at sites H7 and P12 showed greater activity in blocking cell−cell fusion than apelin-13. We further tested these two peptides for their ability to inhibit cell−cell fusion via APJ and the 89.6 isolate gp120. As shown in previous

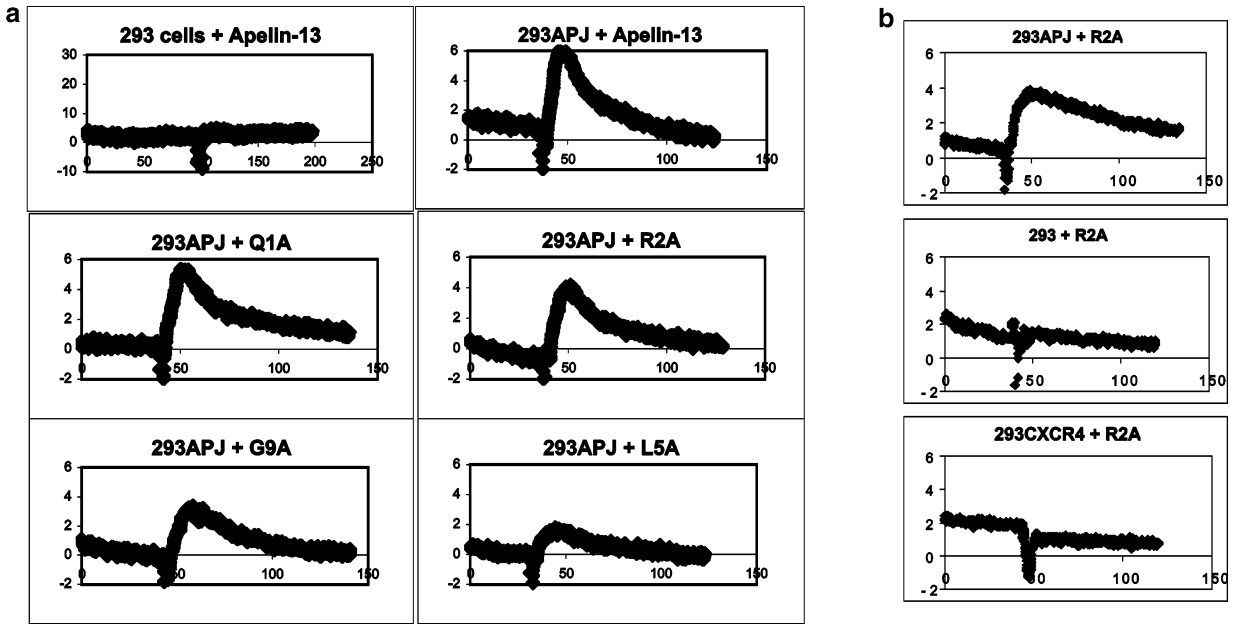

FIGURE 3: Signaling activities of alanine scanning apelin-13 peptides. (a) Induction of intracellular Ca^{2+} mobilization. The intracellular Ca^{2+} concentration in 293 cells, stably expressing APJ, was measured in response to the indicated amount of peptides. (b) Induction of intracellular Ca^{2+} mobilization by the R2A peptide. Intracellular Ca^{2+} concentrations in 293 cells or 293 cells stably expressing APJ or CXCR4 were measured in response to the indicated peptides.

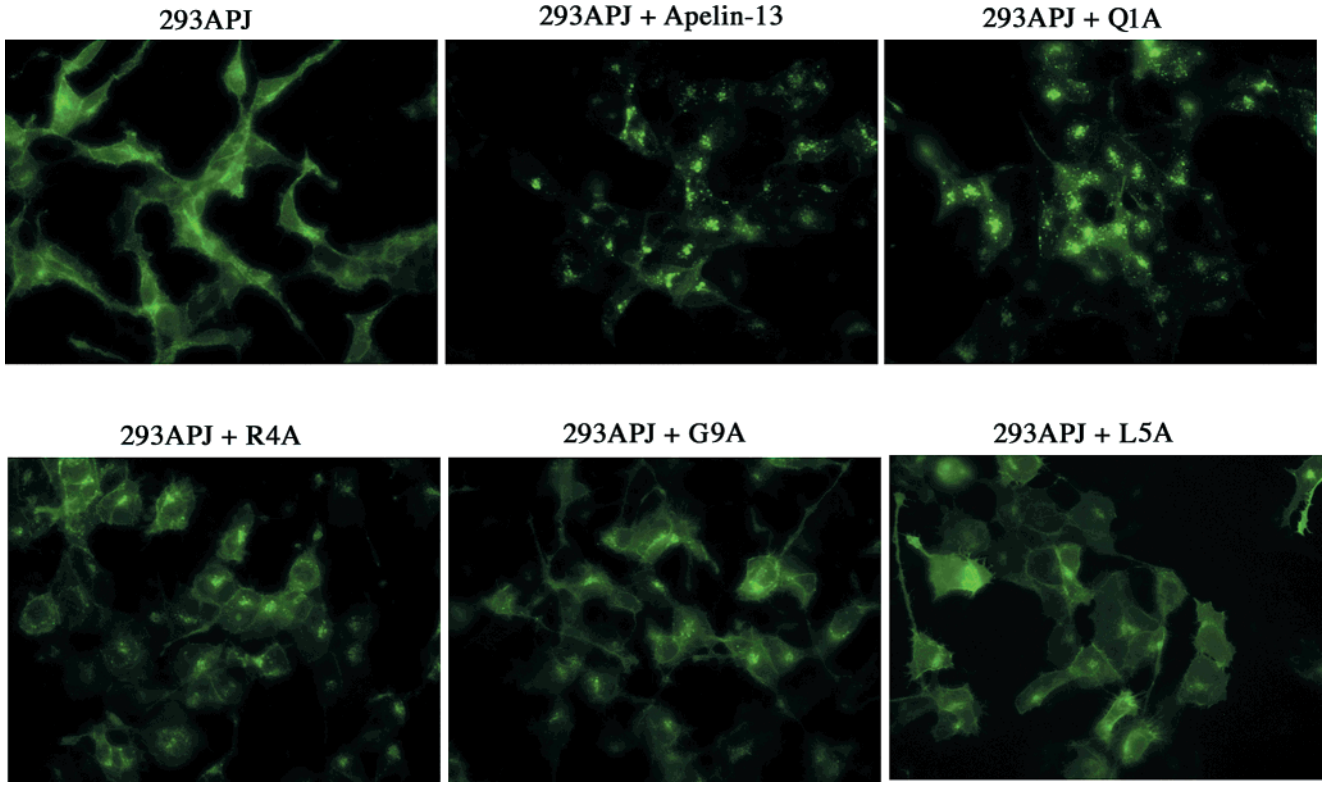

FIGURE 4: Induction of receptor internalization by alaning scanning apelin-13 peptides. The 293 cells stably expressing APJ-EGFP were treated with peptides at 37 °C for 40 min and evaluated by fluorescence microscopy. Internalization was demonstrated by primarily cytoplasmic as opposed to cell surface staining. The data are representative of at least three independent experiments.

studies (*19*, *26*), apelin-13 was unable to block 89.6 gp120-induced cell−cell fusion, but peptide analogues with alanine substituted for residues 7 and 12 showed some reduced inhibitory activity (Figure 5b).

DISCUSSION

The APJ receptor is widely expressed in the human CNS (*1*, *9*, *10*, *28*). APJ and its natural ligand apelin may play an important role in the hypothalamic regulation of water intake and the endocrine axis (*8*, *11*, *12*) and in lowering blood pressure via a nitric oxide-dependent mechanism (*8*, *13*). Recently, the APJ receptor has been identified as a coreceptor for HIV-1 and SIV for supporting *env*-mediated membrane fusion and viral entry *in vitro* (*10*, *16*−*18*, *29*). As shown in previous studies, although a mature apelin peptide consisting of 36 amino acid residues dominantly exists in bovine milk

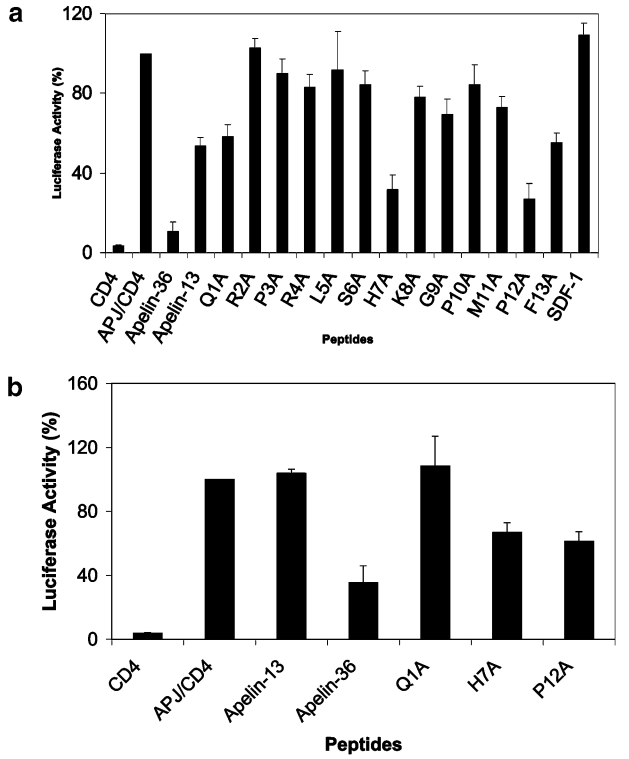

FIGURE 5: Inhibition of cell−cell fusion by alanine scanning apelin-13 peptides. (a) Activity of alanine scanning apelin-13 peptides in blocking cell−cell fusion supported by T-tropic IIIB gp120. (b) Effect of alanine scanning apelin-13 peptides on inhibition of cell fusion induced by dual-tropic 89.6 gp120. Target cells were cotransfected with plasmids encoding wild-type APJ or mutant APJ329, CD4, and luciferase under the control of the T7 promoter. The 293 effector cells were infected with a vaccinia virus encoding T7 polymerase and a vaccinia virus encoding the HIV-1 envelope protein. Before fusion took place, the target cells were incubated in the presence or absence of the test apelin for 30 min. Cell mixtures were kept at 37 °C for 4−6 h. The extent of fusion was determined by a luciferase assay. Values are expressed as percentages of luciferase activity. All experiments were repeated at least three times, and results are expressed as the mean value ± standard error of the mean.

(*7*), the shorter form of the apelin peptide, apelin-13, is also produced *in vivo* (*3*, *7*). In addition, the synthetic apelin-13 was found to exhibit higher activities in acidification rate promotion, cAMP production, inhibition, and chemotaxis than apelin-36 (*3*, *6*). In the study presented here, on the basis of the structure of apelin-13, we used a combination of chemical synthetic approaches with alanine substitution to evaluate the structural requirements for apelin interactions with the APJ receptor. We observed that apelin peptides in aqueous solution adopt a random conformation, and substitution of alanine in most sites of apelin-13, besides Q1, H7, P12, and F13, impaired its binding activity and biological function via APJ. In this study, we further identified the residues of apelin-13 for blocking APJ function as a HIV-1 coreceptor, and provide new reagents for the investigation of the significance of APJ in HIV-1 infection and pathogenesis.

In our competitive binding assay, we found that [^{125}I]-apelin-13 bound to the APJ receptor with high affinity, and cold apelin-13 (IC_{50} = 0.7 nM) competed more efficiently with [^{125}I]apelin-13 than cold apelin-36 (IC_{50} = 2.3 nM). This result is consistent with a previous study (*3*). Alanine replacement of residues 2−6 and 8−11 resulted in a decrease in the receptor binding activity and blocking of coreceptor activity, indicating a good correlation between APJ receptor binding affinity and cell−cell fusion inhibitory activity. Two mutated peptides, H7A and P12A, showed binding activities comparable to that of wild-type apelin-13, but an enhanced ability to block cell−cell fusion via IIIB gp120 and even the dual-tropic 89.6 gp120. Previous studies indicated that apelin-13 did not inhibit entry of the HIV-1 isolate 89.6 (*19*) and cell fusion via 89.6 gp120 (*26*). Although apelin-13 was more efficiently associated with APJ than apelin-36, bound apelin-36 was found to be more difficult to dissociate from APJ than apelin-13 (*3*). It is possible that rates of dissociation of the apelin peptide bound to APJ play the critical role in blocking HIV-1 entry via the APJ coreceptor. Alanine replacement at residues H7 and P12 increased the rate of dissociation of the apelin peptide bound to APJ and enhanced its ability to block cell fusion via 89.6 gp120.

In this study, both receptor internalization and intracellular Ca^{2+} mobilization were employed for further evaluation of biological activities of mutated apelin-13 peptides. We demonstrated that receptor internalization induced by alanine scanning peptides was consistent with binding affinity. Although we observed a correlation between binding activity and signaling among most alanine scanning peptides, and peptides R2A, S6A, K8A, and G9A exhibited lower binding activity and a decreased level of internalization, these mutated peptides kept the ability to induce signaling, like wild-type apelin-13. It is likely that some alanine scanning peptides with a lower binding activity can associate with APJ (e.g., R2A), but more easily dissociate from APJ than wild-type apelin-13. These peptides, therefore, still are able to induce Ca^{2+} mobilization, but do not cause receptor internalization and block cell fusion. Of note, these data are consistent with sudies in a recent article by Medhurst *et al*. (*31*). In this study, an alanine substitution of arginine 2 resulted in a substantial drop in binding affinity but a much smaller change in effects on intracellular Ca^{2+} levels. In this regard, further investigations are necessary to confirm the role of dissociation of the apelin-13 peptide in receptor internalization, signaling, and anti-HIV-1 activity.

Cayabyab *et al*. (*19*) have demonstrated that the positively charged arginine residues at positions 63 and 64 in the apelin-15 peptide (i.e., the two amino acids before the start of apelin-13) play an important role in the specific ability to block HIV-1 entry via the APJ coreceptor. Our previous study has shown that ALX40-4C (*N*-α-acetylnona-*d*-arginine amide) with a positive charge, an antagonist of HIV-1 coreceptor CXCR4, directly binds to APJ and blocks cell fusion via APJ, suggesting that APJ has a negatively charged surface similar to CXCR4 (*30*). The study presented here demonstrates that substitution of alanine for R2, R4, and K8 resulted in a weakened ability to bind to the receptor, to be internalized, and to block cell−cell fusion, and changing Q1, H7, P12, and F13 individually to alanine increased the APJ receptor binding activity. Taken together, these results reveal that the positive charge and the hydrophobic residues of apelin-13 play an important role in interactions with APJ.

In this study, we demonstrate that apelin peptides in aqueous solution adopt a random conformation, and the positive charge and the hydrophobic residues of apelin-13 play an important role in the interaction with the APJ receptor. We have observed a good correlation between

receptor binding affinity and cell−cell fusion inhibitory activity. The characterization of the structure and function of apelin-13 will likely contribute to elucidation of the *in vivo* physiological role of apelin-APJ and the design of novel anti-HIV-1 reagents.

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