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The N-terminal domain of APJ, a CNS-based coreceptor for HIV-1, is essential for its receptor function and coreceptor activity

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Abstract

The human APJ, a G protein-coupled seven-transmembrane receptor, has been found to be dramatically expressed in the human central nervous system (CNS) and also to serve as a coreceptor for the entry of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV). Studies with animal models suggested that APJ and its natural ligand, apelin, play an important role in the central control of body fluid homeostasis, and in regulation of blood pressure and cardiac contractility. In this study, we characterize the structural and functional determinants of the N-terminal domain of APJ in interactions with its natural ligand and HIV-1 envelope glycoprotein. We demonstrate that the second 10 residues of the N-terminal domain of APJ are critical for association with apelin, while the first 20 amino acids play an important role in supporting cell–cell fusion mediated by HIV-1 gp120. With site-directed mutagenesis, we have identified that the negatively charged amino acid residues Glu20 and Asp23 are involved in receptor and coreceptor functions, but residues Tyr10 and Tyr11 substantially contribute to coreceptor function for both T-tropic (CXCR4) and dual-tropic (CXCR4 and CCR5) HIV-1 isolates. Thus, this study provides potentially important information for further characterizing APJ-apelin functions in vitro and in vivo and designing small molecules for treatment of HIV-1 infection in the CNS.

Keywords: APJ receptor; Mutagenesis; Apelin; Signaling; HIV-1; CNS

Introduction

APJ, an orphan G protein-coupled, seven-transmembrane receptor, was originally identified from human genomic DNA by O'Dowd et al. (1993) and later was also isolated from mice (Devic et al., 1999) and rats (De Mota et al., 2000; Hosoya et al., 2000; O'Carroll et al., 2000). The human APJ gene encoding a 380 amino acid resembled the angiotensin II receptor, sharing an identity of more than 30% in amino acid sequence, but angiotensin II was unable to interact with APJ (O'Dowd et al., 1993; Tatemoto et al., 1998). Recently the endogenous ligand for APJ receptor, apelin, was first isolated from bovine stomach extracts (Tatemoto et al., 1998), and the protein sequences of human, rat, and mouse apelin preproproteins were deduced from the cDNAs (Tatemoto et al., 1998; Habata et al., 1999; Hosoya et al., 2000; Lee et al., 2000).

Studies on the distribution of APJ and apelin demonstrated that both were abundantly expressed in the central nervous system (CNS) and periphery of human and rat (O'Dowd et al., 1993; Matsumoto et al., 1996; Edinger et al., 1998; Lee et al., 2000; Reaux et al., 2001, 2002). In addition, significant similarities of sequence and tissue distributions between APJ/apelin and the AT1/angiotensin II suggested that APJ and apelin would play an important physiological role as a neurotransmitter or neuromodulator (Lee et al., 2000; Reaux et al., 2001, 2002). In a rat model, APJ/apelin has been shown to play an important role in the hypothalamic regulation of water intake and the endocrine

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axes (Lee et al., 2000; Reaux et al., 2001; Taheri et al., 2002), in regulation of blood pressure via a nitric oxide -dependent mechanism (Lee et al., 2000; Tatemoto et al., 2001), and cardiac contractility (Reaux et al., 2002; Szokodi et al., 2002).

Recently the APJ receptor has been found to support env-mediated membrane fusion or viral entry as an alternative coreceptor for human and simian immunodeficiency viruses (HIV and SIV) (Choe et al., 1998; Edinger et al., 1998; Zhang et al., 1998; Puffer et al., 2000). Consistently apelin has been reported to be able to block the activity of APJ as an HIV-1 coreceptor (Cayabyab et al., 2000; Puffer et al., 2000; Zou et al., 2000). Although the absence of APJ expression in microglia and macrophages, the principal CD4-positive cell types in CNS, suggested initially that APJ is unlikely to mediate HIV-1 infection in the CNS (Choe et al., 2000), a recent study has shown that HIV-1 infection in neural cells involves a chemokine-receptor-dependent but CD4-independent entry (Alvarez Losada et al., 2002). In addition, it was reported that some viral envelope proteins could mediate fusion with APJ-positive, CD4-negative cells (Puffer et al., 2000). With the detection of abundant expression of APJ in the CNS, these observations raise the possibility that the APJ receptor might contribute to HIV-1 infection and pathogenesis in the CNS.

Although many studies have investigated the physiological role of APJ receptor, there is no report on the characterization of the structure and function of APJ as a receptor and a coreceptor. In the present study, we used deletion and site-directed mutagenesis combined with binding and functional assays to investigate structural determinants of APJ for its ligand activation and coreceptor function. Our study has revealed the residues in the N-terminal domain of APJ critical for biological function and coreceptor activity and provided information for further understanding the physiological and pathological roles of APJ and elucidating mechanisms of HIV-1 viral entry.

Results

Effects of deletion of amino acids in the N-terminal domain of APJ on functions of receptor and HIV-1 coreceptor activities

To evaluate the role of the N-terminal domain of APJ in interaction with ligand and HIV-1 envelope protein gp120, we constructed two APJ mutations, APJ10 with the deletion of the first 10 amino acids in the N-terminal domain, and APJ20 with a deletion of the first 20 amino acids (Fig. 1). Both APJ10 and APJ20 were fused to the enhanced version of the green fluorescent protein (EGFP) at its C-terminus for easy and rapid detection of cell-surface expression and receptor internalization. The cell-surface expression of APJ mutants with deletion was evaluated by flow cytometry with

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          1          11         21  25
wtAPJ     MEEGGDFDNY YGADNQSECE YTDWK SSGALIPAIY
APJ10           M  YGADNQSECE YTDWK SSGALIPAIY
APJ20                      M  YTDWK SSGALIPAIY
Y10A      MEEGGDFDNA YGADNQSECE YTDWK SSGALIPAIY
Y11A      MEEGGDFDNY AGADNQSECE YTDWK SSGALIPAIY
D14A      MEEGGDFDNY YGAANQSECE YTDWK SSGALIPAIY
Q16A      MEEGGDFDNY YGADNASECE YTDWK SSGALIPAIY
S17A      MEEGGDFDNY YGADNQAECE YTDWK SSGALIPAIY
E18A      MEEGGDFDNY YGADNQSACE YTDWK SSGALIPAIY
E20A      MEEGGDFDNY YGADNQSECA YTDWK SSGALIPAIY
Y21A      MEEGGDFDNY YGADNQSECE ATDWK SSGALIPAIY
D23A      MEEGGDFDNY YGADNQSECE YTAWK SSGALIPAIY
W24A      MEEGGDFDNY YGADNQSECE YTDAK SSGALIPAIY
K25A      MEEGGDFDNY YGADNQSECE YTDWA SSGALIPAIY
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Fig. 1. Amino acid sequence of the N-terminal domain of wild-type and mutated APJ. Residues substituted with alanine are shown in bold. The underlined sequence is the predicted transmembrane domain.

GFP and anti-human APJ monoclonal antibody. As shown in Fig. 2A, FACS analysis with GFP indicated that deletion of the first 10 and 20 amino acids in the N-terminal domain resulted in about a 20 to 35% decrease in cell-surface expression, but the expression of both APJ10 and APJ20 was not detectable with monoclonal antibody (mAb)MAB856, suggesting that mAb MAB856 recognizes the N-terminal first 10 amino acids of APJ. APJ10 and APJ20 mutants were further tested by binding and functional assay. The activities of APJ10 in ligand binding, signaling, and internalization remained comparable to wild-type, whereas deletion of 20 residues from the N-terminal domain resulted in complete loss of binding activity and biological functions (Figs. 2B, C, and D). We also examined the coreceptor activity using a well-characterized luciferase-based cell fusion assay. The results indicated that APJ10 and APJ20 exhibited lose of >60 and $>80\%$ of coreceptor activity, respectively, as compared to wild-type APJ (Fig. 2E).

Cell-surface expression of APJ site-directed mutants

As shown in the APJ mutations with deletion of amino acids in the N-terminal domain, the second 10 amino acids of the N-terminus of APJ plays an important role in ligand binding and function. To further identify amino acid side chains that are essential for ligand binding and function, 11 residues located within the second 10 amino acids of the N-terminal domain were selected for site-directed mutagenesis studies, and all of the selected residues were individually substituted with alanine (Fig. 1). All alanine-scanning mutants were stably transfected into 293 cells, and cell-surface expression was analyzed by flow cytometry with both GFP and mAb MAB865. As illustrated in Fig. 3, the mutant Y10A showed a higher expression level with detection of GFP, but similar expression levels to wild-type APJ with mAb MAB865, while all other mutants exhibited expression levels comparable to wild-type APJ in detection by both GFP and mAb MAB865.

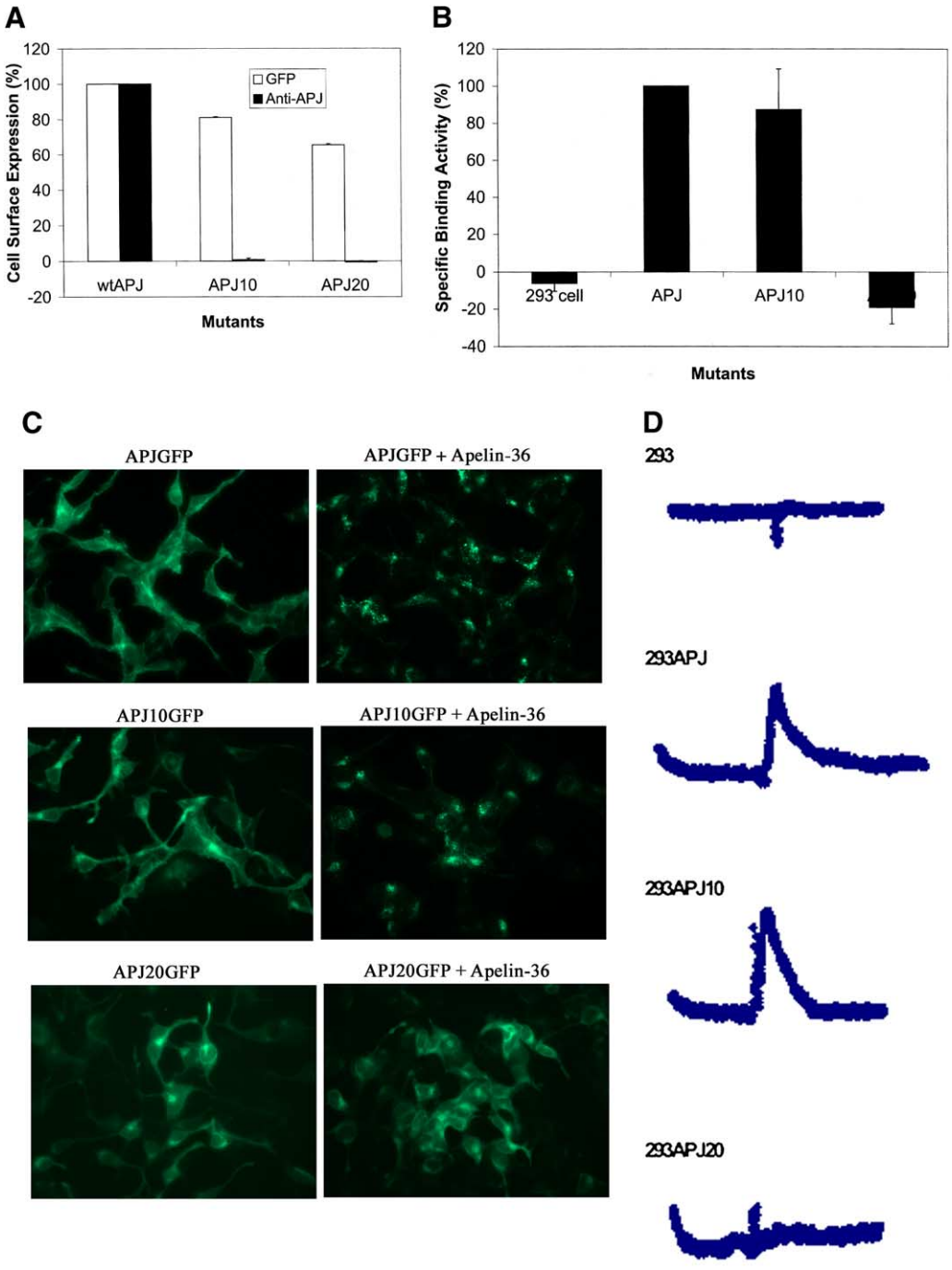

Fig. 2. Expression and activities of APJ mutations with deletions. (A) Cell-surface expression of APJ mutants. 293 cells, stably transfected with wild-type and mutant APJ, were analyzed with GFP and anti-APJ MAB856 by flow cytometry. (B) Binding activity. The 293 cells stably expressing wild-type and mutant APJ were incubated with ^{125}I-apelin-13 (0.2 nM) in the presence of 200 nM cold apelin-13, and cell-associated radioactivity was counted via gamma emissions. The 293 cells were used as negative control. Error bars represent the standard error of the mean derived from three independent experiments. (C) Receptor internalization. 293 cells stably expressing APJ-EGFP treated with apelin-36 (2 μM) at 37°C for 40 min. (D) Intracellular Ca^{2+} mobilization. Intracellular Ca^{2+} concentration in nontransduced 293 cells, or 293 cells stably expressing wild-type or mutant APJ, were measured in response to apelin-36 (3 μM). The data shown are representative of at least three independent experiments. (E) Coreceptor activity of APJ. Target cells were cotransfected with plasmids encoding coreceptors, CD4, and luciferase under the control of the T7 promoter. 293 effector cells were infected with a vaccinia virus encoding T7 polymerase and a vaccinia virus encoding the specific HIV-1 IIIB envelope protein. Cell mixtures were maintained at 37°C for 4 to 6 h. Fusion was determined by a luciferase assay. The target cells were cotransfected with APJ/CD4, but without CD4 as a negative control. Values are expressed as luciferase activity (RLU/s). Error bars represent the standard error of the mean derived from three independent experiments.

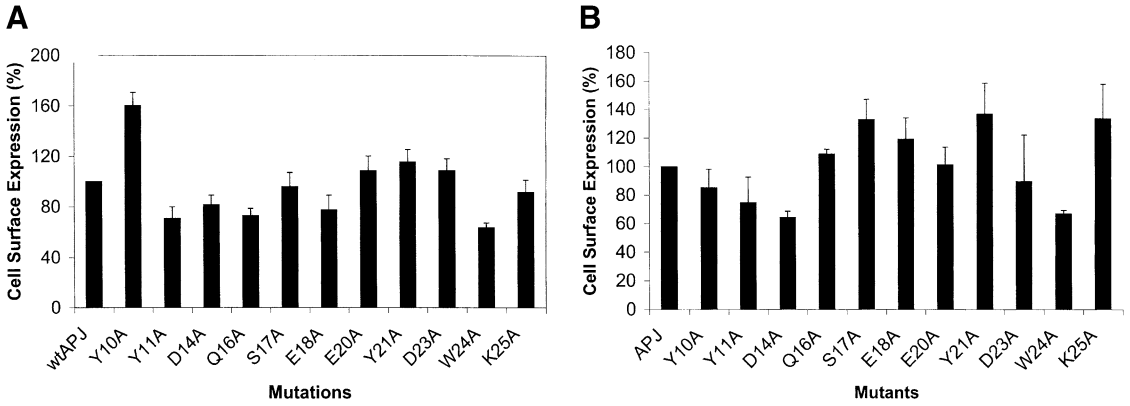

Fig. 3. Cell-surface expression of alanine-scanning APJ mutants. 293 cells, stably transfected with wild-type and alanine-scanning mutated APJ, were analyzed with GFP (A) and anti-APJ MAB856 (B) by flow cytometry. Experiments were repeated at least three times, and results are expressed as the mean value ± SE.

Ligand binding activities of alanine scanning mutants

In our previous study, ^{125}I-apelin-13 was found to bind to APJ receptor with high affinity (Zhou et al., 2002). In this study, we used ^{125}I-apelin-13 to examine our alanine-scanning of APJ mutants for their ligand binding activity. As shown in Fig. 4, replacement of Glu20 and Asp23 with alanine resulted in a dramatic drop in ligand binding activity, and the E18A mutation exhibited a slight drop, whereas mutant Y11A yielded an increase in binding activity.

Biological functions of alanine scanning mutants

APJ receptor undergoes rapid internalization upon exposure to apelin (Reaux et al., 2001; Zhou et al., 2002, 2003). We used an approach expressing an APJ–GFP fusion protein to visualize the receptor internalization for characterizing activation of mutated APJ by apelin ligand. After incubation with 2 μM apelin-13 or apelin-36 at 37°C for 40 min, the stably expressing cells were observed using fluorescence microscopy. Our analyses indicated that alanine substitution of Asp23 led to complete loss of the potential for receptor internalization induced by apelin (Fig. 5A). The E20A mutant was found to be partially internalized from cell surface upon exposure to apelin. Other mutations showed no effects on receptor internalization in response to ligand.

To evaluate the effects of alanine substitution in the N-terminal domain of APJ on signal transduction in response to apelin, changes in intracellular Ca^{2+} were monitored. The results are illustrated in Fig. 5B. Consistent with binding activity and receptor internalization, the D23A mutant showed no response in intracellular Ca^{2+} upon stimulation with 3 μM apelin, and E20A exhibited a low-intensity Ca^{2+} alteration.

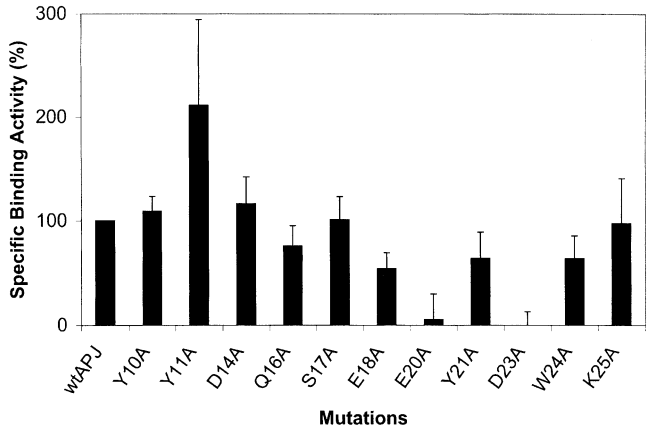

Fig. 4. Binding activity of alanine-scanning APJ mutants. The APJ stably expressing 293 cells were incubated with ^{125}I-apelin-13 (0.2 nM) in the presence of 200 nM cold apelin-13, and cell-associated radioactivity was counted by gamma emissions. The bars represent the mean values of three independent assays, whereas the error bars are the mean ± SEM.

Coreceptor activities of APJ site-directed mutations

To further examine the role of APJ as a coreceptor to mediate HIV-1 infection, in preliminary experiments, human HEK-293 cells expressing CD4 and APJ were infected with the HIV-1 89.6 isolate, and the efficiency of infection was determined by detection of HIV-1 p24 antigen 3 days after infection. The results, in Fig. 6A, showed that the 293 cells expressing CD4 and APJ could be infected by HIV-1 89.6, and the infection by 89.6 viruses was inhibited in a dose-dependent manner by the synthetic ligand apelin-36, but not by SDF-1α, the ligand of human CXCR4.

The alanine scanning APJ mutants were further examined for their ability to support HIV-1 entry as a coreceptor using a well-characterized cell-fusion reporter assay. QT6 target cells were cotransfected with plasmid vectors expressing wild-type APJ or APJ mutants, CD4, and luciferase under the control of T7 promoter. 293 effector cells were coinfected with vaccinia viruses encoding HIV-1 envelope proteins and T7 RNA polymerase, respectively. After 5 h of fusion, cells were lysed and quantitatively analyzed by

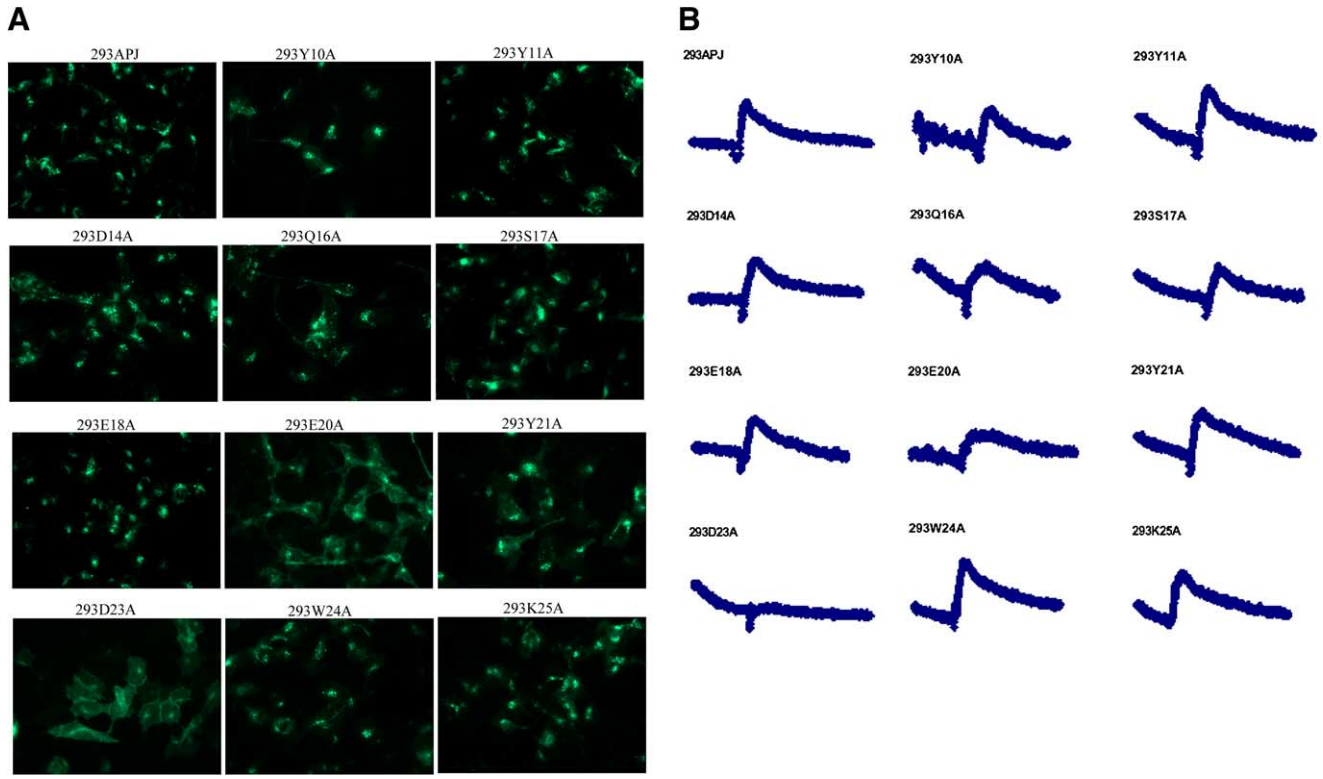

Fig. 5. Biological functions of alanine-scanning APJ mutations. (A) Induction of receptor internalization by apelin-36 peptides. 293 cells stably expressing wild type and mutated APJ treated with 2 μM apelin-36 at 37°C for 40 min. (B) Induction of intracellular Ca^{2+} mobilization. Intracellular Ca^{2+} concentration in 293 cells stably expressing wild-type and mutated APJ was measured in response to 3 μM apelin-36. The data shown are representative of three independent experiments.

luciferase assay. The results are shown in Fig. 6B and presented as an average of the percentages of wild-type APJ. Replacement of Tyr10, Tyr11, and Asp23 with alanine resulted in a dramatic decrease (>80%) in coreceptor activity for both the T-tropic (X4) IIIB and the dual-tropic (R5X4) 89.6. Relatively lower reductions (<50%) in potency toward inducing cell–cell fusion mediated by T-tropic IIIB gp120, but not by dual-tropic 89.6 gp120, were observed when Asp14, Glu18, Glu20, and Trp24 were individually substituted with alanine.

For further confirmation of coreceptor activity, Y10A, Y11A, Y21A, and D23A mutants were tested at the same time for coreceptor activity and transient cell-surface expression. As shown in Fig. 6C and D, although the cell-surface expression of APJ mutants in transiently transfected QT6 cells was comparable to wild-type, Y10A, Y11A, and D23A mutants showed a significant decrease in the ability to support cell–cell fusion.

Discussion

APJ and its natural ligand apelin were found to distribute in the CNS and periphery of the human and rat (O'Dowd et al., 1993; Matsumoto et al., 1996; Edinger et al., 1998; Lee et al., 2000; Reaux et al., 2001, 2002). Recent studies suggested that APJ plays an important physiological role (Lee et al., 2000; Tatemoto et al., 2001; Reaux et al., 2001, 2002; Szokodi et al., 2002; Taheri et al., 2002). In addition, APJ receptor has been found to support *env*-mediated membrane fusion or viral entry as a coreceptor for HIV and SIV (Choe et al., 1998; Edinger et al., 1998; Zhang et al., 1998; Puffer et al., 2000). The characterization of structural and functional determinants of APJ for its ligand activation and coreceptor function is essential toward better understanding the physiological and pathological roles of APJ and elucidating mechanisms of HIV-1 viral entry plus developing novel anti-HIV-1 agents. In this study, we used deletion and site-directed mutagenesis combined with binding and function assays to investigate the role of the N-terminal domain of APJ in ligand activation and coreceptor function.

Our previous study had demonstrated that fusing APJ with the enhanced version of the EGFP at the C-terminus maintained its full biological activities in binding, signaling, and HIV-1 gp 120-induced cell membrane fusion (Zhou et al., 2002). This is in agreement with observations in CCKAR (Tarasova et al., 1997), the β_2-adrenergic receptor (Barak et al., 1997), and CXCR4 (Tarasova et al., 1998). In the present study, we used GFP-fused expression of wild-type and mutant APJs for efficiently detecting cell-surface expression and receptor internalization. By FACS analysis with GFP and monoclonal antibody MAB856, the results

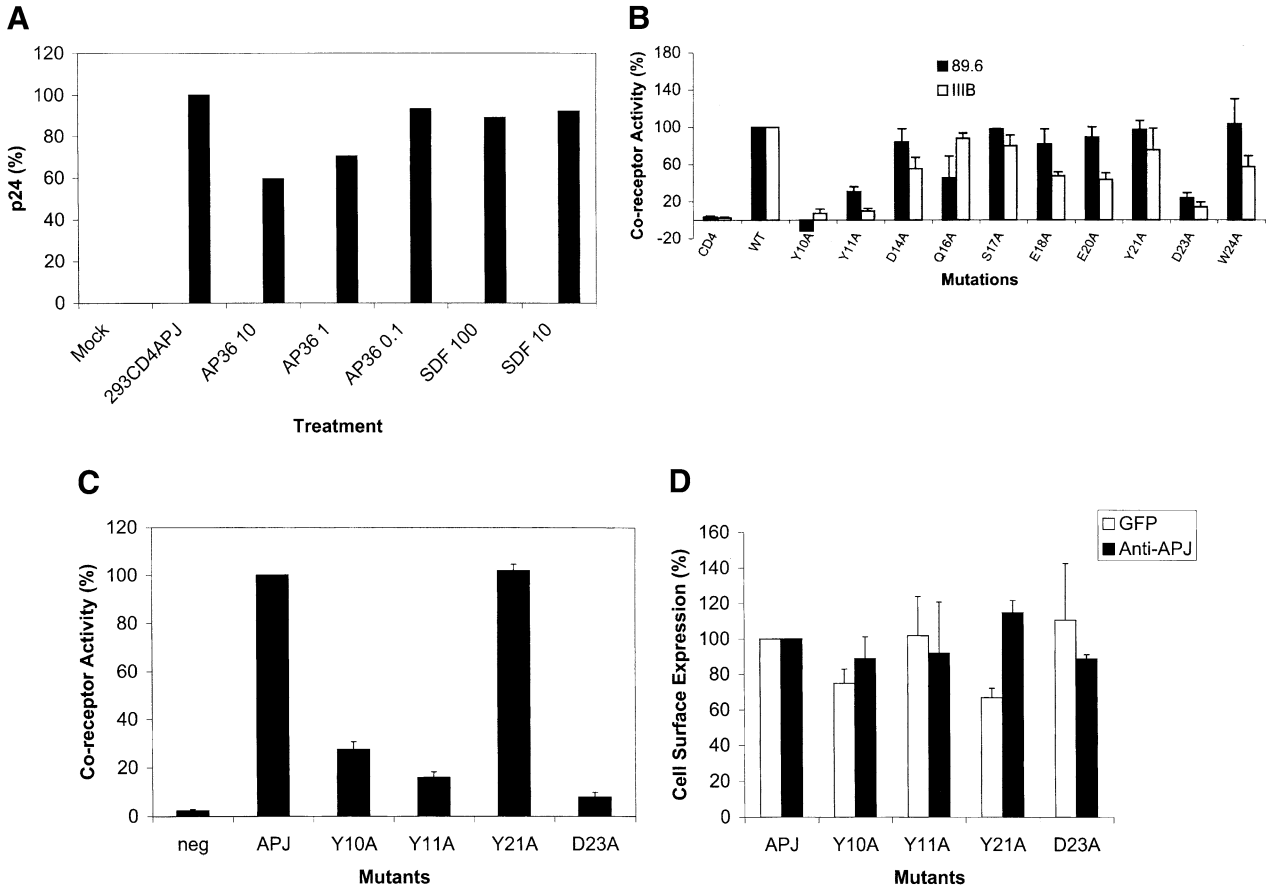

Fig. 6. Coreceptor activity of alanine-scanning mutated APJ. (A) HIV-1 cell-free virus infection assay. The human 293 cells expressing CD4 and APJ were incubated with apelin-36 (μM) and SDF-1α (nM) for 15 min prior to addition of 10 ng of p24 of HIV-1 89.6 viruses. The HIV-1 p24 antigen levels were measured 72 h after infection, using enzyme-linked immunosorbent assays. Similar results were obtained in additional independent experiments. (B) Coreceptor activity of alanine scanning mutant APJ receptors in mediating cell–cell fusion by the T-tropic IIIB and dual-tropic 89.6 gp120s is illustrated. Target cells were cotransfected with plasmids encoding wild-type APJ or APJ mutants, CD4, and luciferase under the control of the T7 promoter. 293 effector cells were infected with a vaccinia virus encoding the T7 polymerase and a vaccinia virus encoding a strain-specific HIV-1 envelope protein. Cell mixtures were maintained at 37°C for 4 to 6 h. Fusion was determined by a luciferase assay. The target cells were cotransfected with APJ/CD4, and without APJ as negative control. The values were normalized by setting the extent of luciferase activity obtained when CD4 and wild-type APJ were coexpressed at 100%. All experiments were repeated at least three times, and results are expressed as the mean value ± SEM. (C) Coreceptor activities of APJ mutants and (D) cell–surface expression of APJ mutants were determined in the same transient transfection, by cell–cell fusion assay with HIV-1 89.6 gp120 and FACS analysis with GFP and MAB856. All experiments were repeated at least three times, and results are expressed as the mean value ± SEM.

showed no difference in cell-surface expression of wild-type and mutated APJ. In addition, observation with fluorescence microscopy demonstrated that the GFP-fused wild-type APJ and APJ mutants were mainly distributed evenly along the cell membrane. Our results also showed that alanine substitution of Asp23 resulted in substantial loss of binding activity, signaling, and receptor internalization, indicating that receptor internalization was well-correlated with binding activity and intracellular Ca^{2+} mobilization. The assay of receptor internalization was a sensitive, simple, and useful means to characterize G-protein coupled receptors (GPCR).

Previous studies have demonstrated that the N-terminal domain plays a critical role in the ability of ligands to associate with G-protein coupled chemotactic receptors: Duffy antigen, a receptor that binds to both CC and CXC chemokines (Lu et al., 1995), IL-8 receptor (Hèbert et al., 1993; Horuk, 1994; Ahuja et al., 1996), C5a receptor (Farzan et al., 2001), CCR2b (Preobrazhensky et al., 2000), CCR5 (Zhou et al., 2000), and CXCR4 (Doranz et al., 1999; Zhou et al., 2001). In this study, the second 10 residues within the N-terminal domain of the APJ receptor were found to be essential for interaction with its ligand. Furthermore, the alanine substitution of residues Asp23 and Glu20 impaired ligand binding activity and key biological functions, suggesting the negatively charged residues in the N-terminal domain are critical for the APJ receptor to interact with apelin. Cayabyab et al. (2000) have reported that the positively charged arginine residues at positions of 63 and 64 in the apelin-15 peptide play an important role for the specific ability to block HIV-1 entry via the APJ coreceptor. Our previous study (Zhou et al., 2002) showed that substitution of alanine at sites R66, R68, and K72 resulted in decreased receptor binding and internalization (and un-

published data). It is noteworthy that the net charge of the N-terminal domain of APJ is −7. We hypothesized that the negatively charged N-terminal domain of APJ is critical for forming favorable electrostatic interactions with the positively charged residues in apelin.

HIV-1 infection of human cells requires the receptor CD4 and chemokine receptors as coreceptors. The major coreceptors used by HIV-1 viruses are CCR5 and CXCR4 (Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). Recently, the APJ receptor has been identified as an alternative coreceptor for HIV-1 and SIV to support *env*-mediated membrane fusion or viral entry in vitro (Choe et al., 1998; Edinger et al., 1998; Zhang et al., 1998; Puffer et al., 2000). In our preliminary infection assay, the human 293 cells expressing CD4 and APJ were infected with HIV-1 89.6 viruses, and the infection of 89.6 virues could be inhibited by apelin-36, the natural ligand of APJ, by not by SDF-1α, ligand of CXCR4, suggesting that APJ receptor functioned as a coreceptor of HIV-1. Although some reports argue for the significance of APJ as a coreceptor during in vivo infection with HIV-1 (Choe et al., 2000; Puffer et al., 2000), identification of structural determinants of APJ in mediation of HIV-1 entry is helpful for fuller understanding of the interactions of HIV-1 *env* glycoprotein with CD4 and coreceptor toward developing novel anti-HIV-1 agents.

In the present study, we use a well-characterized cell–cell fusion assay to evaluate the coreceptor activities of APJ mutations. The data showed that deletion of the first 10 residues and 20 residues in the N-terminal domain resulted in 60 and 80% reduction of coreceptor activity, respectively, indicating that the N-terminal domain was required for interactions with HIV-1 gp120. Further site-directed mutagenesis analysis demonstrated that residues Tyr10, Tyr11, and Asp23 were critical in supporting *env* glycoprotein-mediated membrane fusion for both T-tropic and dual-tropic viruses. It was interesting to note that replacement of negatively charged residues Asp20, Glu18, and Glu20 with alanine impaired cell–cell fusion activity mediated by T-tropic viral *env* glycoprotein. APJ has a negatively charged N-terminal domain, similar to CXCR4. Mutagenesis studies also indicated that the negatively charged residues in the N-terminal domain were critical determinants for CXCR4 coreceptor activity (Chabot et al., 1999; Brelot et al., 2000). In addition, the V3 loop of gp120 from T-tropic isolates has been found to have a higher net positive charge than the V3 loop from M-tropic strains (O'Brien et al., 1996). It is likely that the electrostatic interactions of coreceptors with HIV-1 gp120 play significant roles in CXCR4 and APJ coordinated cell entry by T-tropic viruses. Our previous study demonstrated that ALX40-4C, initially identified as a small-molecule antagonist of the chemokine receptor CXCR4, directly bound to APJ and prevented use of APJ as a HIV-1 coreceptor (Zhou et al., 2003). Taken together, APJ as a receptor and coreceptor, in structure and function, bears a strong resemblance to CXCR4, although APJ contains an aminoterminal NYYG sequence, and a similar motif, NYYT, has been identified at the N-terminal domain of CCR5, that is particularly important for coreceptor function (Farzan et al., 1998).

There are three tyrosine residues, Tyr10, Tyr11, and Tyr21, within the N-terminal domain of APJ. The same tyrosine-rich N-terminal domain exists in most or all chemokine receptors and all HIV-1 coreceptors (Farzan et al., 1999). CCR5 contains at least three sulfated tyrosines within the N-terminal domain that contribute to both receptor functions and coreceptor activity (Farzan et al., 1999; Bannert et al., 2001). The tyrosine sulfate moieties on CCR2b and the chemotactic receptor for C5a also have been observed to play an important role in association with ligand (Bannert et al., 2001; Farzan et al., 2001). In the case of CXCR4, the sulfate group at tyrosine 21 makes a substantial contribution to its interaction with ligand SDF-1α, but plays a less significant role in CXCR4-mediated HIV-1 entry than in CCR5-dependent HIV-1 entry (Farzan et al., 2002). However, in the present study, we observed that Tyr10 and Tyr11 of APJ receptor were critical for the ability to support cell–cell fusion mediated by both T-tropic and dual-tropic HIV-1 isolates, but none of the three tyrosine residues contributed to receptor binding and function. We observed that the deletion mutant APJ10 still showed some activity, although quite less, in support of IIIB gp120 induced cell–cell fusion. Nonetheless, the site-directed mutant Y10A maintained little coreceptor activity for both 89.6 and IIIB gp120. This discrepancy may be accounted for by the conformational change induced by alanine substitution at Tyr10.

We have characterized the role of the N-terminal domain of APJ receptor in association with ligand and HIV-1 *env* glycoprotein. Our results have revealed the residues in the N-terminal domain of APJ critical for biological function and coreceptor activity. In the case of most G-protein coupled receptors, the N-terminal domain, together with two or three extracellular loops, are involved in interactions with ligand, and also with HIV-1 envelope glycoproteins. However, further studies are necessary to examine the role of the extracellular loops of APJ in ligand binding functions and coreceptor activity.

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). Anti-human APJ monoclonal antibody (MAB856) was obtained from R&D Systems, Inc. Plasmid pCDNA-APJ, recombinant vaccinia viruses encoding two

envelopes of HIV-1 vSC60 (IIIB) and vBD3 (89.6), and vTF1.1 encoding T7 RNA polymerase were generous gifts from Dr. Robert W. Doms, University of Pennsylvania. The vector pEGFP-N1 was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

Cell and cell culture

The HEK-293 cell line was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). The Japanese quail fibrosarcoma QT6 cell line was kindly provided by Robert W. Doms (University of Pennsylvania, Philadelphia, PA). HEK-293 and QT6 cells were maintained in DMEM plus 10% fetal bovine serum.

DNA construction and mutagenesis

Plasmid pCDNA-APJ containing the human APJ gene was kindly provided by Robert W. Doms (University of Pennsylvania, Philadelphia, PA). The full open reading frame (*ORF*) of wild-type and N-terminally deleted APJ were amplified using polymerase chain reaction (PCR) and subcloned in-frame into *Hin*dIII and *Bam*HI sites of the pEGFP-N1 vector. All constructs were sequenced to confirm the correct sequence and orientation.

Site-directed mutagenesis was performed on pAPJ-EGFP vector and prepared with QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The mutations were confirmed by sequencing. The plasmid constructs of wild-type APJ and mutants were transfected into 293 cells by the calcium-phosphate precipitation method. Twenty-four hours after transfection, selection for 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 radioiodinated human apelin-13 was purchased from Amersham Biosciences, Inc. (Piscataway, NJ). The specific activity of human ^{125}I-apelin-13 was 2000 Ci/mmol. The binding assay was carried out as described previously (Zhou et al., 2002). The wild-type APJ and mutated APJ stably expressing 293 cells were harvested in phosphate-buffered saline (PBS) (Ca^{2+} and Mg^{2+} free) plus 0.5 mM EDTA and washed twice with PBS. Ligand binding experiments were performed using a single concentration (0.2 nM) of ^{125}I-apelin-13 in the absence or presence of 200 nM cold apelin-13 in a final volume of 100 μl of binding buffer (50 nM HEPES pH 7.4, 1 nM $CaCl_2$, 5 nM $MgCl_2$, 0.1% bovine serum albumin) containing 5×10^5 cells. 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. Bound ligands were determined by counting gamma emissions. At least three independent experiments were performed.

Flow cytometry

APJ-EGFP and APJ mutant stably transfected 293 cells (2×10^5) were washed with fluorescence-activated cell sorting (FACS) buffer (0.3% bovine serum albumin, 0.05% sodium azide in PBS) and fixed in fixing buffer (2% paraformaldehyde in PBS). For detection of APJ expression using anti-APJ antibody, stably or transiently APJ expressing cells were harvested with PBS containing 5 mM EDTA. After washing with FACS buffer, cells were incubated with an anti-APJ mAb (10 μg/ml) for 30 min at 4°C. After washing with FACS buffer, cells were incubated with 10 μg/ml PE-conjugated goat anti-mouse IgG (PharMingen) for 30 min at 4°C. After washing twice with FACS buffer, cells were fixed in fixing buffer (2% paraformaldehyde in PBS) and then analyzed on a FACScan flow cytometer (Coulter EPICS Elite, Coolten Corp., Hialeah, FL).

Receptor internalization and fluorescence microscopy

The stable APJ or mutated APJ expressing 293 cells were seeded on Nunc two-chamber slides and incubated at 37°C overnight. After treatment with apelin at 37°C for 40 min, cells were washed in PBS and then fixed in PBS containing 2% paraformaldehyde for 10 min. Fluorescence microscopy was performed on an Olympus System microscope, model BX60, with fluorescence attachment BX-FLA.

Intracellular calcium measurements

Intracellular Ca^{2+} was monitored following a modified procedure, as published by others (Donnadieu et al., 1994; Heveker et al., 1998). Briefly, untransduced 293 cells and wild-type APJ or APJ mutant stably transfected 293 cells were cultured in the DMEM medium containing 10% FBS. Cells were harvested with PBS containing 5 mM EDTA and washed twice with PBS. For Ca^{2+} mobilization studies, 5×10^6/ml cells were loaded with the fluorescent dye, fura-2 (3 μM), and 0.05% F172, 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 concentration of 30 to 40 $\times 10^6$/ml, then 1.5 to 2 $\times 10^6$ cells were tested in the same buffer. Intracellular Ca^{2+} mobilization was measured using excitation at 340 and 380 nm in a fluorescence spectrometer (Perkin–Elmer LS50B, Beaconsfield, UK), upon stimulation with apelin. Intracellular Ca^{2+} concentrations were calculated using a fluorescence spectrometer measurement program.

HIV-1 infection assay

To determine coreceptor activity of APJ receptor in mediating HIV-1 infection, the human HEK-293 cells were transfected with CD4 and APJ. The 293 cells expressing CD4/APJ were challenged with 10 ng of HIV-1 p24 antigen, isolate 89.6, for 6 h, and then washed three times. For inhibition with ligand, the cells were incubated with indicated amounts of apelin-36 and SDF-1α for 15 min prior to addition of HIV-1. Supernatants were collect for p24 antigen measurement by ELISA, 72 h after infection. The HIV-1 p24 antigen levels were measured using enzyme-linked immunosorbent assays (ELISA; Zeptometrix).

Gene reporter fusion assay

A gene reporter fusion assay was used to determine the coreceptor activity of APJ in mediating HIV-1 viral entry following a modified procedure, as published by others (Nussbaum et al., 1994; Doranz et al., 1996; Rucker et al., 1997). Briefly, the effector 293 cells were infected with recombinant vaccinia virus with diverse HIV-1 Env proteins and T7 RNA polymerase for 2 h. Infected cells were then trypsinized, washed with PBS, resuspended in medium, and incubated overnight at 32°C in the presence of rifampicin (100 μg/ml). Target QT6 cells were cotransfected in six-well plates with plasmids encoding CD4, wild-type, or mutant APJs and luciferase under control of the T7 promotor, using the calcium-phosphate precipitation method. Four hours after transfection, cells were lifted, seeded in 24-well plates, and incubated at 37°C overnight. To initiate fusion, 10^5 effector cells were added to each well and incubated at 37°C. After 5 h of fusion, cells were lysed in 100 μl of reporter lysis buffer (Pharmingen) and assayed for luciferase activity by using commercially available reagents (Pharmingen) with FB12 Luminometer (Zylux Corp., Maryville, TN).

Peptide synthesis

Peptide synthesis was carried out as described previously (Zhou et al., 2002). Briefly, apelin-13 and apelin-36 were prepared by solid-phase synthesis using *Fmoc*-strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perceptive Biosystems, Cambridge, MA). Crude peptides were purified by preparative reverse-phase high-performance liquid chromatography using a Dynamax-300 Å C_{18} 25 cm $\times$ 21.4 mm ID column with flow rate of 9 ml/min and two solvent systems of 0.1% TFA/H_2O and 0.1% TFA/acetonitrile. Fractions containing the appropriate peptide were pooled together 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.

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