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Human immunodeficiency virus gp120-induced apoptosis of human neuroblastoma cells in the absence of CXCR4 internalization

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The chemokine receptor CXCR4 functions as human immunodeficiency virus (HIV)-1 coreceptor and is involved in acquired immunodeficiency virus (AIDS) neuropathogenesis. CXCR4 is expressed by most cell types in the brain, including microglia, astrocytes, and neurons. Studies have shown that the HIV envelope protein gp120 binds to neuronal CXCR4 and activates signal transduction pathways leading to apoptosis. However, the natural CXCR4 ligand (CXCL12) has been referred to induce both neuronal survival and death. Here the authors used flow cytometry to determine whether gp120 and CXCL12 differ in their ability to induce CXCR4 internalization in the human neuroblastoma cells SH-SY5Y, which constitutively express CXCR4. As expected, increasing concentration of CXCL12 reduced surface expression of CXCR4 in a time- and concentration-dependent manner. Conversely, $gp120_{IIIB}$ (monomeric or oligomeric, in presence or absence of soluble CD4) did not change CXCR4 membrane levels. Similar results were obtained in a murine lymphocyte cell line (300-19) stably expressing human CXCR4. Nevertheless, $gp120_{IIIB}$ was still able to activate intracellular signaling and proapoptotic pathways, via CXCR4. These results show that $gp120_{IIIB}$ toxicity and signaling do not require CXCR4 internalization in SH-SY5Y cells, and suggest that the viral protein may alter normal CXCR4 trafficking thus, interfering with activation of prosurvival pathways. *Journal of NeuroVirology* (2006) **12**, 211–218.

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Introduction

The chemokine receptor CXCR4 is involved in the neurological complications of acquired immunodeficiency syndrome (AIDS) (Kaul *et al*, 2001; Zhang

et al, 2003b). CXCR4 is the specific receptor for the chemokine CXCL12 (previously known as SDF-1), which orchestrates the development of many areas of the brain as well as other tissues (Lazarini *et al*, 2003; Tran and Miller, 2003; Zou *et al*, 1998). CXCR4 can also bind the human immunodeficiency virus (HIV) envelope protein gp120 that induces apoptotic cell death in primary neurons and cell lines (Bachis and Mocchetti, 2004; Catani *et al*, 2000; Hesselgesser *et al*, 1997; Kaul and Lipton, 1999; Mattson *et al*, 2005; Meucci and Miller, 1996). Despite a convincing role of gp120 in the apoptosis of neurons, the effects of CXCL12 on neuronal survival remain controversial.

In vivo studies indicate that the CXCR4/CXCL12 pair is crucial to neuronal development and proliferation, and a number of *in vitro* evidence favors a prosurvival action of this chemokine (Chalasani *et al*, 2003; Khan *et al*, 2003; Meucci *et al*, 1998). However, studies from other authors suggest that CXCL12

can also be neurotoxic (Kaul and Lipton, 1999). A recent report demonstrated that only a cleaved form of the chemokine—produced by the action of matrix metalloproteinases under pathological conditions, namely HIV encephalitis—is responsible for cell death (Zhang *et al*, 2003a). Interestingly, cleaved CXCL12 does not bind to CXCR4 as well as the full-length protein. This is in agreement with our previous studies showing that recombinant CXCL12 promotes neuronal survival and that differences in the intracellular signaling activated by CXCL12 and gp120 exist. Specifically, our data show that, although gp120 is able to stimulate mitogen-activated protein (MAP) kinases, and to induce Ca^{2+} rise in neurons, it is unable to activate prosurvival pathways, such as the phosphoinositide-3 kinase/(PI3K)/AKT pathway, which is necessary for chemokine neurotrophic action (Khan *et al*, 2004; Meucci *et al*, 1998). Moreover, gp120 and CXCL12 also differ in their regulation of specific cell cycle proteins involved in neuronal survival, namely p53, Rb, and E2F (Khan *et al*, 2003, 2005), which are directly and indirectly modulated by AKT. This could depend on differences in the intrinsic efficacies of the two CXCR4 ligands (Khan *et al*, 2004) and/or the presence of multiple receptor conformations (Baribaud *et al*, 2001; Zhou *et al*, 2002). Furthermore, based on recent evidence suggesting that receptor internalization may regulate G protein–coupled receptor (GPCR)-mediated signaling in various ways (Lefkowitz and Shenoy, 2005), we hypothesized that gp120 and CXCL12 may also diverge in their ability to induce CXCR4 internalization. Indeed, studies on alpha-thrombin receptor (a member of the GPCR family) have shown that the expression of a dominant negative beta-arrestin 1 (a group of intracellular proteins that promote receptor endocytosis and recycling) inhibits the rapid activation of the PI3K/AKT pathway without affecting the extra cellular signal-regulated kinase (ERK) pathway (Goel and Baldassare, 2002; Goel *et al*, 2002; Lefkowitz and Shenoy, 2005).

In order to determine whether differences in intracellular signaling and biological outcomes between CXCL12 and gp120 are associated with alterations in receptor trafficking, we studied the effect of the viral protein on CXCR4 internalization in the human neuroblastoma cell line SH-SY5Y by flow cytometry. Though other studies have previously reported the effect of gp120 on CXCR4 internalization, the data are quite controversial (Badr *et al*, 2005; Bodner *et al*, 2003; Fernandis *et al*, 2002, 2003; Tarasova *et al*, 1998), and the correlation between internalization, signaling, and toxicity have not been addressed. Furthermore, most of the previous studies were performed on cells transfected with CXCR4, whereas SH-SY5Y cells constitutively express CXCR4. This is a crucial aspect, considering that the signal transduction machinery may be quite different in cells that do not normally express CXCR4. The results reported here show that, despite the effect of the viral protein

on cell signaling and survival, treatment of cells with gp120$_{IIIB}$ (alone or in complex with CD4) did not affect surface expression of CXCR4. These findings suggest that gp120-induced signaling and neurotoxicity do not require receptor internalization.

Results

Several studies have reported the neurotoxic effect of gp120 *in vitro* and *in vivo* and the involvement of chemokine receptors in this process (Catani *et al*, 2000; Hesselgesser *et al*, 1998; Kaul and Lipton, 1999; Meucci and Miller, 1996; Pandey and Bolsover, 2000; Zheng *et al*, 1999). In line with these reports, treatment of SH-SY5Y cells with gp120$_{IIIB}$ (200 pM) resulted in significant, albeit moderate, cell death (Figure 1), which was blocked by pretreatment with AMD3100 (100 ng/ml). This result confirms the involvement of CXCR4 in gp120$_{IIIB}$-induced death of SH-SY5Y cells.

As the gp120 is expressed on the HIV virus as a trimeric complex, we asked whether gp120-induced toxicity was higher in cultures treated with an oligomeric form of the protein. Furthermore, as binding of HIV to the chemokine receptor is regulated by CD4, we tested the action of gp120$_{IIIB}$/CD4 complexes on the neuroblastoma cells. However, comparable levels of cell death were induced by monomeric and oligomeric gp120$_{IIIB}$ either in the presence or absence of CD4 (Figure 1B). Of note, dependence of gp120$_{IIIB}$ on CD4 has been reported to be a cell type–specific event (Bodner *et al*, 2003). Treatment of SH-SY5Y cells with CXCL12 (20 nM) or AMD3100 (100 ng/ml) alone did not affect basal cell survival ($P > 0.05$ versus control), as expected (Figure 1B).

Changes in cell surface expression of CXCR4 induced by gp120$_{IIIB}$ (or CXCL12) were studied by flow cytometry. SH-SY5Y cells were incubated for 60 min at 37°C with increasing concentrations of either ligand, ranging from 20 pM to 20 nM (Figure 2). The samples were stained with a phycoerythrin-conjugated mouse monoclonal antibody as described in the methods section, and analyzed by fluorescence-activated cell sorting (FACS). To quantify differences in receptor expression, we measured the mean fluorescence intensity after every treatment and evaluated the percentage of CXCR4 on the cell surface of treated cells in comparison to the untreated cells. As expected, CXCL12 reduced surface expression of CXCR4 in a time- and dose-dependent manner, whereas gp120$_{IIIB}$ (up to 20 nM) did not change CXCR4 level (Figure 2). Incubation with 20 nM CXCL12 for 60 min led to a reduction of CXCR4 staining greater than 50% (Figure 2B).

To determine whether longer treatments were required to observe receptor internalization by gp120$_{IIIB}$, SH-SY5Y cells were incubated with the ligands up to 12 h. In agreement with the previous experiments, gp120$_{IIIB}$ (200 pM) did not affect
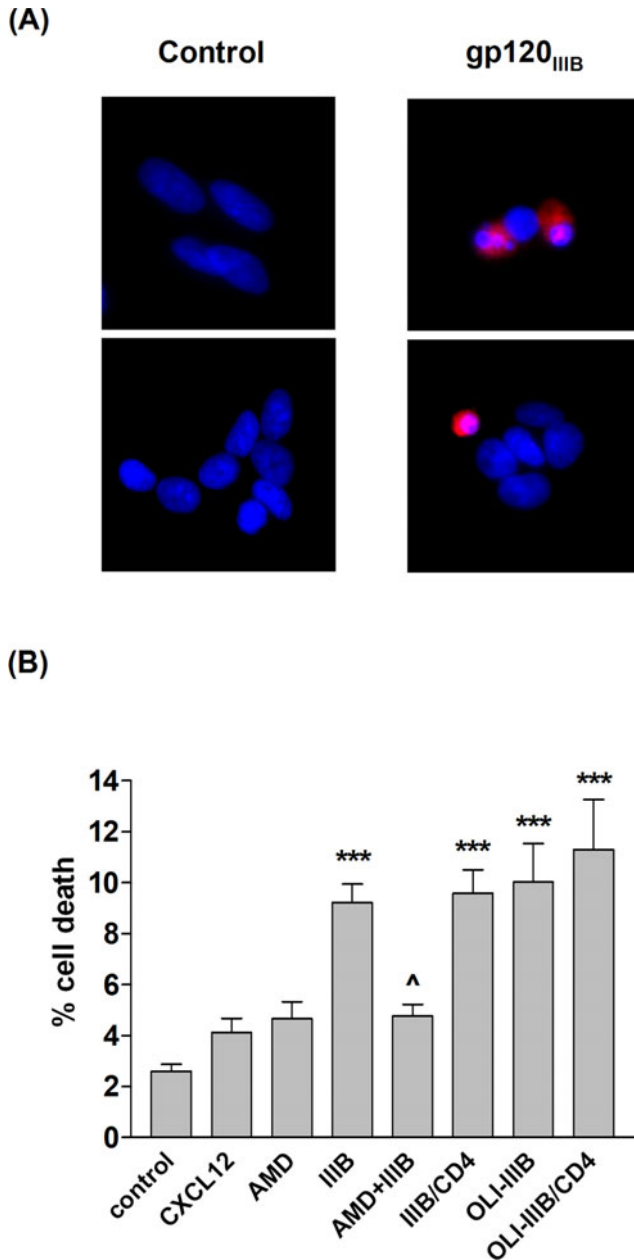
(A)

(B)

Figure 1 HIV gp120 induces cell death in SH-SY5Y cells. SH-SY5Y neuroblastoma cells were serum starved for 48 h and treated with monomeric or oligomeric gp120$_{IIIB}$ (200 pM), monomeric or oligomeric gp120$_{IIIB}$/CD4 complexes (200 pM), or CXCL12 (20 nM) for the following 24 h at 37°C. Two groups of samples were pre-treated for 15 min at 37° C with 100 ng/ml of AMD3100 and subsequently treated with vehicle or monomeric gp120$_{IIIB}$ (200 pM) for 24 h at 37°C. At the end of the treatment, cells were fixed, stained for cleaved caspase 3 (red in **A**), and counted. The graph shows percentage of dead cells (mean ± SEM) from three independent experiments (**B**). Six microscopic fields per coverslip were counted and three coverslips per treatment were used for each experiment. (*** $P < 0.001$ gp120s versus control; ^$P < 0.001$, gp120$_{IIIB}$ versus AMD3100 + gp120$_{IIIB}$).

CXCR4 surface expression at any time point observed (Figure 2C). Similar results were obtained with a high concentration of gp120$_{IIIB}$ (20nM, up to 4 h) (data not shown). On the contrary, CXCL12
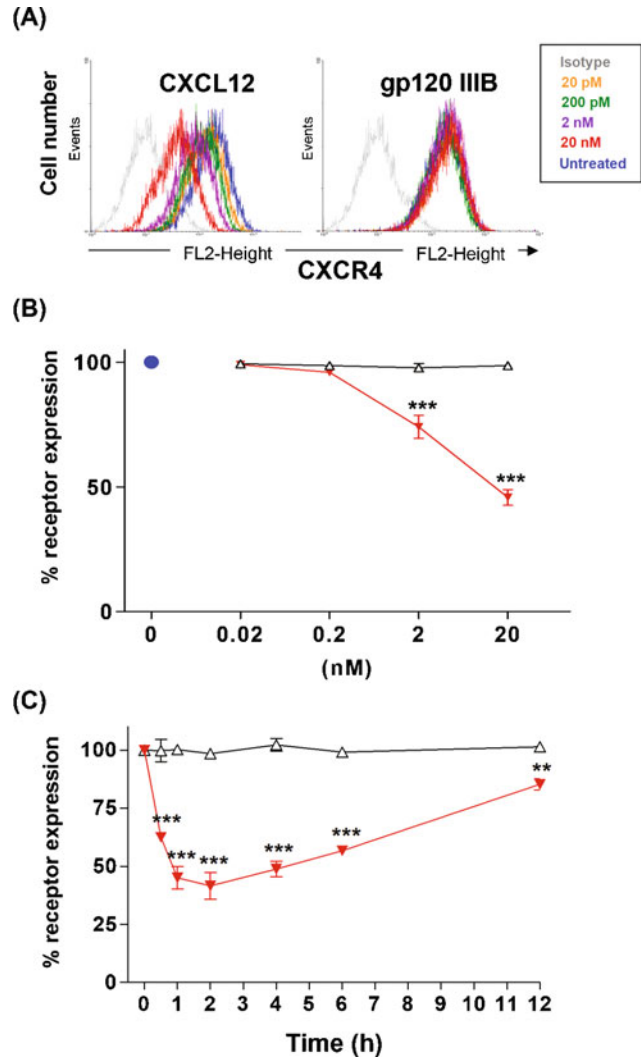
(A)

(B)

(C)

Figure 2 HIV gp120 is unable to induce CXCR4 internalization in SH-SY5Y cells. SH-SY5Y neuroblastoma cells were incubated with different concentrations of CXCL12 and gp120$_{IIIB}$ (20 pM to 20 nM). The relative cell surface expression of CXCR4 was determined by flow cytometry after staining with PE-conjugated monoclonal anti-human CXCR4 antibody. (**A**) Representative traces from experiments in which cells were incubated with the two ligands for 60 min. (**B**) The average of three independent experiments reported as the mean fluorescence intensity of CXCL12-treated (*red filled triangle*) and gp120$_{IIIB}$-treated (*open triangles*) groups, compared to untreated cells (100%, *blue dot*). Some of the error bars of gp120$_{IIIB}$ treated cells are not clearly visible in the graph and thus reported here as well: at 0.02 nM, 99.37% ± 1.26%; at 0.2 nM, 98.63% ± 1.23%; at 2 nM, 97.8% ± 1.72%; at 20 nM, 98.67% ± 1.30%. (**C**) Data from time-course experiments (from 30 min to 12-h) after treatment with 200 pM gp120$_{IIIB}$ (*open triange*) or 20 nM CXCL12 (*filled red triangles*). The graph represents the averages of three independent experiments ± SEM. (treatments versus control, ** $P < 0.01$; *** $P < 0.001$).

(20 nM) internalized the receptor reaching the maximal level within the first 2 h. After this time the receptor expression gradually returned towards basal level reaching about 80% at 12 h (Figure 2C). This surface expression dynamics likely depends on ligand degradation (which reduces ligand-induced

internalization) and recycling of the receptor (which helps restoring the receptor membrane pool) (Neel *et al*, 2005).

Similar results were obtained with the oligomeric-gp120$_{IIIB}$, which was unable to induce internalization of the chemokine receptor on SH-SY5Y neuroblastoma cells (Figure 3A). Also, binding of gp120$_{IIIB}$ to equimolar concentrations of human soluble CD4 did not change CXCR4 expression on the surface of treated cells (Figure 3A). We then tested a gp120 from a different HIV strain, i.e., the gp120$_{SF2}$. Treatment with 20 nM gp120$_{SF2}$ at 37°C for 60 min (Figure 3A) or 4 h (data not shown) also did not alter CXCR4 expression on the surface of SH-SY5Y cells. Finally, to determine whether this phenomenon was limited to neuroblastoma cells, we used a mouse pre-B-cell line stably transfected with human CXCR4 (300-19/CXCR4) (Figure 3B). These cells were treated with increasing concentration of gp120$_{IIIB}$ or CXCL12 for 60 min (Figure 3B). Experiments with the oligomeric gp120$_{IIIB}$ (20 nM) were also performed (Figure 3C). Altogether, these studies showed that, though CXCL12 induces a dose-dependent internalization of CXCR4, reaching almost 50% of receptor internalization at a concentration of 2 nM, gp120$_{IIIB}$ was unable to induce CXCR4 internalization in the mouse pre-B cells. This is shown by the complete overlay of the traces of untreated cells (blue) and cells treated with either monomeric-gp120$_{IIIB}$ (orange) or oligomeric-gp120$_{IIIB}$ (green) (Figure 3C).

The results of the flow cytometry studies could raise concerns about the ability of gp120 to signal through the chemokine receptor on SH-SY5Y cells. Thus, we tested the ability of the viral protein to activate CXCR4-dependent pathways, such of ERK1/2 phosphorylation, caspase activation or other apoptotic pathways (Garden *et al*, 2002; Khan *et al*, 2005; Meucci *et al*, 1998). As previously reported in different cells (Khan *et al*, 2004; Lazarini *et al*, 2000; Meucci *et al*, 1998), CXCL12 and gp120$_{IIIB}$ are both able to induce phosphorylation of ERK1/2 in SH-SY5Y cells (Figure 4A), with peak responses generally observed at 5 min for both ligands (data not shown). This effect was completely blocked by pretreatment of cells with the CXCR4 specific inhibitor AMD3100 (100 ng/ml) (Figure 4). The HIV protein also stimulated, in an AMD3100-dependent manner, caspase 3 cleavage (Figure 1A) and E2F1 activity (Shimizu *et al*, submitted). These results demonstrate the presence of a functional chemokine receptor on the surface of SH-SY5Y neuroblastoma cells and show that gp120$_{IIIB}$-induced cell death and signaling do not require CXCR4 internalization in the neuroblastoma cells.

Discussion

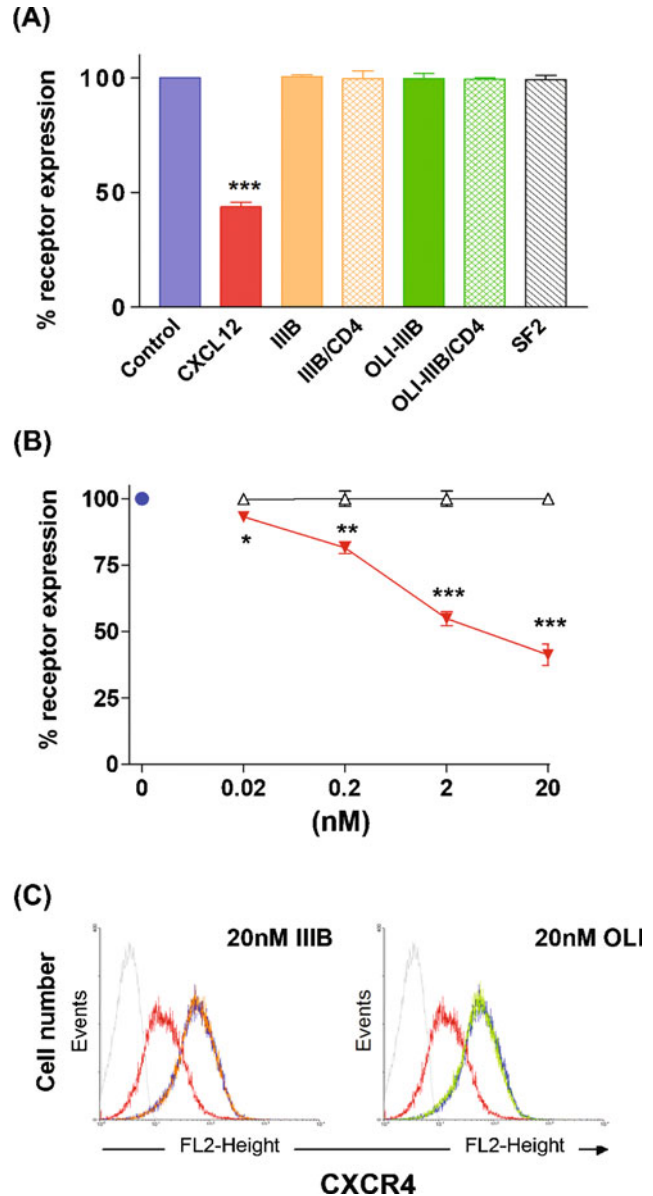Chemokine receptors expressed on the cell surface undergo a basal level of internalization and

Figure 3 -HIV gp120$_{IIIB}$/CD4 complex does not affect CXCR4 expression. (**A**) CXCR4 cell surface expression on SH-SY5Y cells after incubation for 60 min at 37°C without (control) or with 20 nM of the indicated ligands. Bars represent the averages of two to four independent experiments ± SEM. (**B**) 300-19/CXCR4 cells were incubated for 60 min at 37°C with increasing concentrations of gp120$_{IIIB}$ (*open triangles*) or CXCL12 (*red filled triangles*). The percentage of receptor expression from two independent experiments as compared to control cells is shown (mean ± SEM) (treatments versus control, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (**C**) Representative traces from a FACS analysis experiment in 300-19/CXCR4 cells (60 min at 37°C). A total of three independent experiments with both monomeric and oligomeric gp120$_{IIIB}$ as well as the CXCL12 control were performed; untreated cells (*blue trace*), 20 nM monomeric-gp120$_{IIIB}$ (*orange trace*), 20 nM oligomeric-gp120$_{IIIB}$ (*green trace*), 20 nM CXCL12 (*red trace*). The gray trace represents the antibody isotype control.

degradation in absence of specific ligand (Signoret *et al*, 2000). A certain amount of receptors on the cell membrane is maintained by new synthesis and recycling mechanisms. Internalization of chemokine
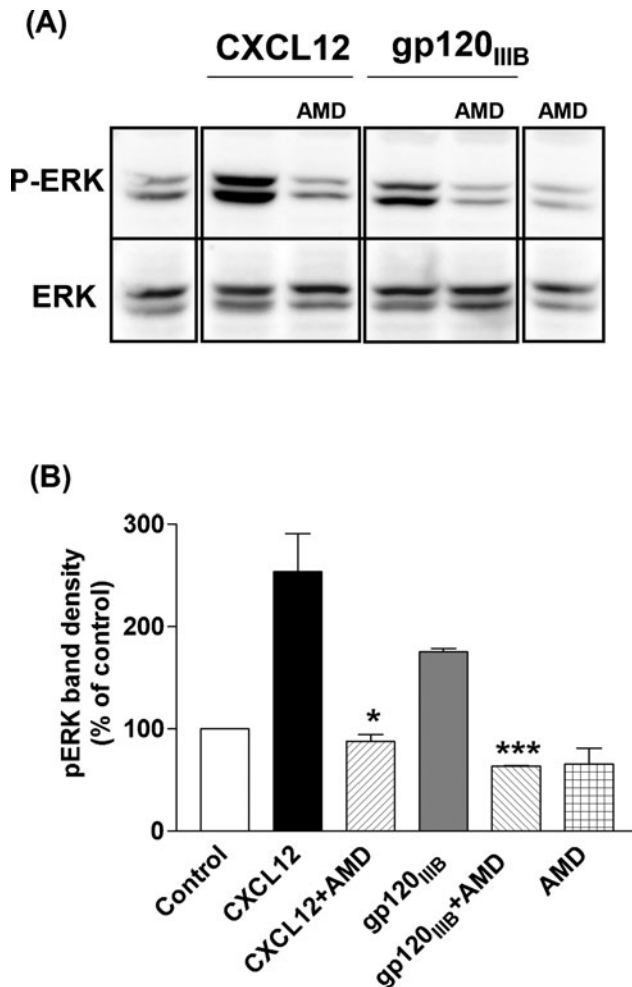
Figure 4 HIV gp120$_{IIIB}$ signals through CXCR4 on SH-SY5Y cells. Pretreatment of SH-SY5Y cells with the specific CXCR4 antagonist AMD3100 (100 ng/ml) abolishes CXCL12- (2 nM, 5 min) and gp120$_{IIIB}$- (200 pM, 5 min) induced phosphorylation of ERK (**A**). (**B**) The densitometry analysis of the bands (mean ± SEM) of three independent experiments (CXCL12 + AMD versus CXCL12, $P^* < 0.05$; gp120$_{IIIB}$ + AMD versus gp120$_{IIIB}$, $^{***}P < 0.001$).

receptors can be enhanced by ligand binding leading to a clathrin-mediated or lipid raft/caveolae-dependent endocytosis (Neel *et al*, 2005). This agonist-mediated receptor internalization represents an important physiological feedback mechanism that protects the cell against acute and chronic over-stimulation. The signaling through CXCR4 in brain-derived cells can result in opposite biological events, such as survival or apoptosis (Chalasani *et al*, 2003; Kaul and Lipton, 1999; Khan *et al*, 2004; Meucci *et al*, 1998). Activation of these pathways may depend on the nature of the ligand and the subsequent intracellular events (Khan *et al*, 2005; Zhang *et al*, 2003a). The present study suggests that CXCR4 internalization may also contribute to the different effects of CXCR4 on cell survival. The fact that gp120 does not induce CXCR4 internalization emphasizes a potential role of CXCR4 internalization

in the activation of prosurvival pathways—although this still remains to be proven. However, previous studies showing that CXCL12, but not gp120, activates the PI3K/AKT pathway (Khan *et al*, 2004) support this hypothesis. In addition, studies from other investigators indicate that beta-arrestins (which mediate receptor internalization) are instrumental in the coupling of GPCRs and tyrosine kinases receptors to antiapoptotic pathways, including AKT (Goel and Baldassare, 2002; Povsic *et al*, 2003; Revankar *et al*, 2004). Thus, pathways regulated by AKT (such as the Rb/E2F pathway) could be altered as well (Khan *et al*, 2003).

The inability of gp120 to induce CXCR4 internalization was also reported in a CXCR4-transfected cell line (Wang *et al*, 1998). These authors found that a specific anti-CD4 antibody was required for the internalization of the gp120/CXCR4/CD4 complex, and suggest that CXCR4 endocytosis is secondary to CD4-dependent signaling. Indeed, our results in SH-SY5Y cells and those from other investigators in hematopoietic CD34$^+$ cells demonstrate that soluble CD4 is not sufficient for gp120 to trigger CXCR4 internalization, whereas gp120 causes CXCR4 internalization in CD4+ lymphocytes (Aiuti *et al*, 1999; Fernandis *et al*, 2002). Altogether, these data suggest that gp120-induced CXCR4 endocytosis is strictly dependent on the cellular expression of CD4, at least in certain cells. As neurons do not generally express the CD4 molecule, it is possible that gp120 does not cause CXCR4 internalization in neurons *in vivo*. This hypothesis is also supported by our previous studies (Khan *et al*, 2004) showing that pretreatment of CD4$^-$/CXCR4$^+$ cells with gp120$_{IIIB}$ did not abolish calcium responses stimulated by the physiological CXCR4 agonist (CXCL12), suggesting that the viral protein is unable to induce proper CXCR4 down-regulation despite that it activates several intracellular responses (Meucci *et al*, 1998, and this study). However, a recent confocal microscopy study reported a time-dependent internalization of recombinant gp120 in cerebellar granule neurons (Bachis *et al*, 2003). The authors find that internalization of biotin-conjugated gp120 is blocked by brain-derived neurotrophic factor, which also prevents gp120 neurotoxicity and reduces CXCR4 expression, i.e., CXCR4 total protein levels. The effect of gp120 on CXCR4 internalization/surface expression, however, is not reported in their study. Moreover, gp120 internalization was not observed in glial cells (Bachis *et al*, 2003), which also express CXCR4, thus questioning whether this is a CXCR4-dependent event (Lazarini *et al*, 2003). Thus, the apparent discrepancy with our findings could be due to various scenarios, including differences in the cell types and experimental approaches, the reported binding of gp120 to other molecules expressed on the neuronal surface, such as glycosphingolipids (Cook *et al*, 1994; Long *et al*, 1994), and/or the expression of unknown CD4-like molecules in

granule neurons. Alternatively, the HIV envelope protein could enter the cell via ligand-independent chemokine receptor internalization (Neel *et al*, 2005; Signoret *et al*, 2000), which would be consistent with the kinetics of gp120 accumulation in cerebellar granule neurons (Bachis *et al*, 2003). On the other hand, the dynamics of CXCR4 expression on the membrane of SH-SY5Y cells treated with CXCL12 (Figure 2) is a typical example of ligand-induced receptor endocytosis, a common and reversible mechanism of desensitization of chemokine receptor and GPCRs in general (Ferguson, 2001; Neel *et al*, 2005).

In conclusion, our findings show that gp120-induced signaling and toxicity is independent of CXCR4 internalization. Thus, gp120 and CXCL12 differ in their interaction with CXCR4 at multiple levels, which may also include receptor trafficking. However, it remains to be established whether this phenomenon is responsible for the altered coupling of CXCR4 to survival pathways upon stimulation by the HIV envelope protein.

Materials and methods

Cells cultures

SH-SY5Y human neuroblastoma cells were obtained from ATCC and cultured in 45% minimum essential medium, 45% Ham's F-12 medium, 10% fetal bovine serum (FBS) containing $50 \mu g/ml$ gentamycin. Medium was replaced every 3 days. Mouse pre-B 300-19 cells stably transfected with human CXCR4 were a kind gift from Dr. Bernhard Moser (Institute of Cell Biology, University of Bern, Switzerland). These cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% nonessential amino acids, 1mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM glutamine, and $1.5 \mu g/mL$ puromycin.

Flow cytometry

SH-SY5Y or 300-19/CXCR4 cells were treated with CXCL12 or gp120s as indicated in the appropriate figures for different times at 37°C. After treatments, cells were washed and resuspended in cold Ca^{2+}-free phosphate-buffered saline (PBS), and then incubated in PBS supplemented with 10% horse serum (30 min on ice). Cells were then resuspended in FACS buffer (1% bovine serum albumin (BSA)/PBS) and incubated (30 min on ice in the dark) with $12.5 \mu g/ml$ ($5 \mu g/ml$ for 300-19/CXCR4 cells) phycoerythrin (PE)-conjugated antibodies (FAB173P; R&D System). We used a mouse monoclonal anti-human CXCR4-PE and a mouse IgG2B-PE as the isotype control. After incubation, cells were washed and fixed with 1% paraformaldehyde in FACS buffer. Samples acquisition was performed with a FACS Calibur (BD).

Survival

Cell death was evaluated 24 h after treatment. Hoechst 33342 ($2 \mu g/ml$) was used to evaluate differences between normal and apoptotic nuclei (Meucci *et al*, 1998). Additionally, cleaved caspase 3 staining with an antibody that specifically recognizes cleaved/active caspase 3 (80 ng/ml; Cell Signaling) was also used to identify apoptotic cells as described previously (Khan *et al*, 2005). Six microscopic fields per coverslip were counted and three coverslips/treatment were used for each experiment. Analysis of the staining has been performed using Metamorph (Universal Imaging) software.

Western Blots

Following drug treatment, cells were washed with ice-cold PBS, scraped with lysis buffer (150 mM NaCl, 50 mM Tris, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mM $Na_4P_2O_7$, 5 mM EDTA, 1% Triton X, $5 \mu g$ each of aprotinin, leupeptin, and pepstatin/1 mM 4-(2 aminoethyl)benzeresulfonyl fluoride HCE (AEBSF)/1 mM vanadate], and incubated for 30 min on a rotor at 4°C. After centrifugation at 20,800 × g for 10 min, the protein concentration of the supernatants was determined by the bicinchoninic acid protein assay (PIERCE). Equal amout of protein ($40 \mu g$) were loaded in each lane. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes for immunoblotting. After blotting for pERK (11.25 ng/ml), membranes were stripped and reprobed for total ERK (1.25 ng/ml). Both antibodies are from Cell Signaling Technology. Horseradish peroxidase (HRP)-conjugated Protein A (1:20000; Sigma) was used to resolve the bands. An image acquisition and analysis system, from Alpha Innotech (FluorChem 8900) was used for detection of chemiluminescent bands and densitometry analysis. Intensity levels from total ERK bands were used to normalize phosphoprotein signals and compensate for possible variations in protein loading. Data are reported as mean ± SEM.

Reagents

Unless otherwise specified, tissue culture media are from Gibco-Invitrogen and other general reagents are from Sigma. CXCL12 was purchased from PeproTech (NJ). Recombinant HIV-1 gp120$_{IIIB}$ (both monomeric and oligomeric) was from ImmunoDiagnostics. Stock solutions of gp120s and CXCL12 were prepared and stored as previously described (Meucci *et al*, 1998). AMD3100 was obtained from Sigma and dissolved in sterile water before further dilution in culture medium. gp120-sCD4 binding was achieved after 30 min incubation at 37°C of equimolar amount of the two reagents as previously described by (Bodner *et al*, 2003). Recombinant soluble CD4 was from Intracel Corporation.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test was used for CXCR4 internalization and survival experiments. Paired *t* test was used to compare differences in the band densities of immunoblots.

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