

# Apoptotic and Antiapoptotic Effects of CXCR4: Is It a Matter of Intrinsic Efficacy? Implications for HIV Neuropathogenesis

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## ABSTRACT

**CXCR4, the specific receptor for the chemokine SDF-1 $\alpha$  that also binds CXCR4-using HIV gp120s, affects survival of different cell types, including neurons. However, current data show that the outcome of CXCR4 activation on neuronal survival may vary depending on the ligand and/or the cellular conditions. In this study, we have systematically compared the effects of SDF-1 $\alpha$  and gp120<sub>IIIIB</sub> (with or without CD4) on several intracellular pathways involved in cell survival, including MAP kinases and Akt-dependent pathways. Our data show that gp120<sub>IIIIB</sub> and SDF-1 $\alpha$  are both potent activators of MAP kinases in neuronal and nonneuronal cells, though the kinetic of these responses is slightly different. Furthermore, unlike SDF-1 $\alpha$ , and independently of CD4, gp120<sub>IIIIB</sub> is unable to stimulate Akt and some of its antiapoptotic targets (NF- $\kappa$ B and MDM2)—despite its ability to activate other signaling pathways in the same conditions. Finally, the viral protein is more efficient in recruiting some effectors (e.g., JNK) than others in comparison with SDF-1 $\alpha$  (EC<sub>50</sub> = 0.1 vs. 0.6 nM). We conclude that the intrinsic efficacy of the two ligands is significantly different and is pathway dependent. These findings have important implications for our understanding of CXCR4-mediated responses in the CNS, as well as the role of this coreceptor in HIV neuropathogenesis.**

## INTRODUCTION

SINCE THEIR DISCOVERY AS HIV CORECEPTORS,<sup>1</sup> chemokine receptors have been the subject of intense investigation beyond their role in the immune system. In particular, this family of G-protein-coupled receptors (GPCRs) has been implicated in important physiological and pathological processes in the nervous system and in other areas of the body.<sup>2–4</sup> CXCR4, the specific receptor for the chemokine stromal-derived growth factor-1 (SDF-1/CXCL12), regulates various neuronal and glial functions.<sup>5–7</sup> The SDF-1/CXCR4 pair, which is constitutively expressed in the central nervous system (CNS), is also believed to play a role in the neuronal injury associated with HIV-1 infection.<sup>8–12</sup> Abnormal activation of CXCR4 may cause neuronal damage and death via both direct (i.e., mediated by neuronal chemokine receptors) and indirect (i.e., mediated by glial and other nonneuronal receptors) mechanisms.<sup>13,14</sup> For instance, neuronal chemokine receptors affect cell cycle proteins

involved in apoptosis,<sup>15,16</sup> whereas glial chemokine receptors may regulate excitotoxicity.<sup>17</sup>

HIV envelope proteins are potent neurotoxins *in vitro* and *in vivo*.<sup>18–24</sup> The CXCR4-using viral protein gp120<sub>IIIIB</sub>, which binds neuronal chemokine receptors with high affinity,<sup>25</sup> activates many signaling pathways normally stimulated by SDF-1 $\alpha$ .<sup>26,27</sup> Nonetheless, SDF-1 $\alpha$  can promote survival of various cell types, including neurons, and protect them from gp120-induced apoptosis.<sup>16,28,29</sup>

Binding of gp120 to chemokine receptors is influenced by CD4, a membrane glycoprotein that binds to gp120 and induces conformational changes in the viral envelope protein promoting its interaction with chemokine receptors.<sup>30</sup> However, HIV envelope proteins can also act in a CD4-independent manner, and CD4-independent effects of gp120 in neurons and other cells have been reported.<sup>18,25,31,32</sup> In the CNS, CD4 is mainly expressed by microglia/macrophages and other inflammatory/immune cells, major components of HIV neuropathogen-

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esis.<sup>33–35</sup> Thus, we sought to (1) determine whether gp120 signaling is significantly affected by CD4 and (2) evaluate the differences between SDF-1 $\alpha$  and gp120 in the regulation of neuronal survival pathways. We tested the effect of CXCR4-using gp120 on different effectors of survival and differentiation, as well as of gp120 neurotoxicity. Hence, the effects of the HIV protein on intracellular calcium levels ( $[Ca^{2+}]_i$ ), activation of MAP kinases, the Ser/Thr kinase Akt, and some of its downstream targets have been evaluated and compared to the SDF-1 $\alpha$ -induced responses. Our data show that despite its ability to activate mitogen-activated protein kinases (MAPKs) and mobilize intracellular calcium, gp120 was unable to stimulate Akt even in the presence of CD4, which suggests a substantial difference in the intrinsic activities of the two ligands.

## MATERIALS AND METHODS

### Cell cultures

**Human osteosarcoma cells (HOS).** HOS cells transfected with CXCR4 (CXCR4<sup>+</sup>/CD4<sup>-</sup>) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1  $\mu$ g/ml puromycin. Mycophenolic acid (40  $\mu$ g/ml), xanthine (250  $\mu$ g/ml), and hypoxanthine (13.5  $\mu$ g/ml) were included in the medium to culture HOS cells expressing CD4 (alone or with CXCR4). Cells were serum starved before experimental treatments. All HOS cells were obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH (from Dr. Nathaniel Landau).<sup>36,37</sup>

**Human astrocytes.** Cells were purchased from ScienCell Research Laboratories (San Diego, CA) and maintained in a defined culture medium provided by the vendor for three to five passages. Expression of glial fibrillar acidic protein (GFAP) was checked during the entire culture period to verify that all cells were positive to the glial marker. Cells were serum starved before experimental treatments.

**Primary rat neurons.** Hippocampal rat neurons were cultured as previously described.<sup>18,28,38</sup> Neurons were obtained from the brain of 17- to 18-day-old rat embryos and cultured in serum-free medium using a bilaminar cell culture system, i.e., a feeder layer of secondary astrocytes supported the growth and differentiation of the pure neuronal layer. Neurons were separated from glia immediately before the experiments, unless otherwise specified.

### Fura-2 microfluorimetry

Changes of free intracellular calcium concentrations were studied by fura-2-based microfluorimetry and image analysis, as previously reported.<sup>18,28,38</sup> Briefly, cells were loaded for 20 min with 2  $\mu$ M fura-AM at room temperature in a balanced salt solution, washed, further incubated in saline for an additional 30 min, and then mounted on the stage of an inverted microscope (Olympus IX70) connected to a CCD camera (Micromax YS1300, Princeton Instruments) and a computer. The software Metamorph/Metafluor (Universal Imaging Corp.) was used for image acquisition and analysis. Calibration of the fluorescent signals was performed as described previously.<sup>28</sup> Cells were

perfused with either saline alone or saline plus experimental drugs during the whole experiment.

### Western blots

After treatments, cells were washed with ice-cold balanced salt solution and scraped in lysis buffer [25 mM Tris/150 mM NaCl/5 mM NaF/1 mM ethylenediaminetetraacetic acid (EDTA)/1 mM dithiothreitol (DTT)/1% Nonidet P-40/5  $\mu$ g each of aprotinin, leupeptin, and pepstatin/1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride HCE (AEBSF)/1 mM vanadate]. A lower concentration of detergent (0.1%) was used for the extraction of cytosolic proteins when they had to be separated from nuclear proteins, and the pellet was further processed with a hyperosmotic buffer as previously described.<sup>28,38</sup> Histone-1 was used as a nuclear marker and to verify the absence of nuclear proteins in the cytosolic extracts. The protein concentration in cell lysates was determined by bicinchoninic acid protein assay from Pierce.  $\beta$ -Actin expression was assessed to confirm equal sample loading and possible changes in constitutive proteins. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes for immunoblotting. The following primary antibodies were used: anti-Akt, anti-phospho-Akt (Ser-473), and anti-phosphoMDM2 (Ser-166) from Cell Signaling (1:8000, 1:4000, and 1:1000, respectively); anti-MDM2 (SMP14) and anti-NF- $\kappa$ B/p65 from Santa Cruz Biotechnologies (1:2000 and 1:1000, respectively); anti-ERK and phosphoERK from Transduction Laboratories and Cell Signaling (1:10,000 and 1:1000, respectively); anti-JNK and anti-phosphoJNK from Cell Signaling and Promega (1:1000 and 1:3000, respectively); anti-p38 and anti-phosphop38 from Cell Signaling (1:1,000); anti- $\beta$ -actin (polyclonal antibody from Sigma-Aldrich, 1:5000); and anti-histone-1 (polyclonal antibody FL-219 from Santa Cruz Technology, 1:500). An image acquisition and analysis system from Bio-Rad (ChemiDoc System) as well as the U-Scan software were used for detection of chemiluminescent bands and densitometric analysis. Data are reported as mean  $\pm$  SEM with sample size for each experiment. The intensity values from actin bands or total Akt, Erk, Jnk, and p38 band, respectively, were used to normalize phosphoprotein signals and compensate for possible variations in protein loading among samples. Data are expressed as percentage of control after normalization. Paired *t* test has been used to compare differences in the band densities of immunoblots.

### Electromobility shift assay

Nuclear (HOS) or total (neurons) extracts (1.5  $\mu$ g) were incubated with 0.25  $\mu$ g/ $\mu$ l of 3' biotinylated oligonucleotides containing a consensus-binding site for NF- $\kappa$ B (5-agggtt gagg-ggacttcccaggc-3'). After a 20-min incubation at room temperature, the samples were resolved in a 6% nondenaturing PAGE. Streptavidin-horseradish peroxidase conjugate and the chemiluminescent substrates were provided with the LightShift EMSA kit from Pierce.

### Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and incubated with anti-GFAP from Santa Cruz Biotechnologies (1:200) or anti-

CXCR4 (R&D System, 1:100) antibodies. Secondary antibodies conjugated to either AlexaFluor546 (Molecular Probes, 1:1000) or Cy2 (Jackson ImmunoResearch Labs, 1:500) were used. Nuclear counterstaining was obtained with Hoechst 33342 (3  $\mu\text{g}/\text{ml}$ ). The cells were mounted and observed under an epifluorescent microscope connected to a CCD camera (Micro-max), and images were acquired and analyzed using the software Metamorph/Metafluor (Universal Imaging Corp.).<sup>38</sup>

### Materials

Unless otherwise specified tissue culture media are from Life Technology, Rockville, MD and other general reagents are from Sigma, St. Louis, MO. SDF-1 $\alpha$  and CD4 were purchased from R&D System, Minneapolis, MN. Proteins were reconstituted in 0.1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) and aliquots stored at  $-20^{\circ}\text{C}$ . Recombinant HIV-1<sub>IIIB</sub> gp120 (purchased from Intracel Corporation, Issaquah, WA) was prepared and stored as previously described.<sup>28,38</sup> The synthetic peptides V1 and DV1 were prepared as described,<sup>39–41</sup> while AMD3100 is from AnorMED Inc. (Langley, Canada). Custom modified oligonucleotides were obtained from Qiagen.

## RESULTS

As other GPCRs, chemokine receptors are coupled to intracellular calcium ( $[\text{Ca}^{2+}]_i$ ) mobilization in a variety of cell types. We have previously demonstrated that chemokines induce calcium transients in differentiated rat neurons and that CXCR4- and CCR5-using gp120s exerted a similar effect.<sup>28,38</sup> However, it was not clear whether gp120 increases  $[\text{Ca}^{2+}]_i$  through a direct interaction with CXCR4 and what the role of CD4 in this process is. Here we used a CXCR4-transfected human cell line (HOS cells), which expresses CXCR4 with or without hCD4. Besides the advantage of an abundant and homogeneous expression of CXCR4 within the cell population, CXCR4 is also

the only chemokine receptor expressed by these cells. Similar to our previous findings in primary neurons,<sup>28</sup> both SDF-1 $\alpha$  (200 pM–50 nM) and gp120<sub>IIIB</sub> (200 pM) evoked  $[\text{Ca}^{2+}]_i$  rises in HOS cells that expressed CXCR4 in the absence of CD4 (CXCR4<sup>+</sup>/CD4<sup>-</sup>). The  $[\text{Ca}^{2+}]_i$  rises induced by SDF-1 $\alpha$  were blocked by pretreatment with pertussis toxin (PTx, 200 ng/ml for 18–20 hr) or anti-CXCR4 neutralizing antibodies (10  $\mu\text{g}/\text{ml}$ ) in these cells (Table 1). Similarly, responses to gp120<sub>IIIB</sub> were inhibited by PTx and were not observed in wild-type HOS cells (CXCR4<sup>-</sup>/CD4<sup>-</sup>) or in HOS cells transfected only with CD4 (CXCR4<sup>-</sup>/CD4<sup>+</sup>) (Table 1). These results indicate that gp120-induced calcium response is mediated by CXCR4 and is independent of CD4. Indeed, cells preexposed to 50 nM SDF-1 $\alpha$  for 1 hr, which causes CXCR4 receptor desensitization,<sup>42</sup> were unable to respond to gp120<sub>IIIB</sub> or SDF-1 $\alpha$  (not shown). However, further experiments indicate that SDF-1 $\alpha$  and gp120 stimulate CXCR4 in a different fashion. For instance, consecutive and relatively frequent stimulations (i.e., less than 10 min apart) of CXCR4 by SDF-1 $\alpha$  down-regulated calcium responses in HOS cells (CXCR4<sup>+</sup>/CD4<sup>-</sup>) even when low concentrations of SDF-1 $\alpha$  (200 pM) were used (Fig. 1A). Conversely, cells exposed to equimolar concentrations of gp120<sub>IIIB</sub> were still able to respond to a subsequent stimulation with gp120<sub>IIIB</sub> or SDF-1 $\alpha$  (Fig. 1B and C), independently of the washout time. This suggests that receptor down-regulation/desensitization might not occur at the same rate for the two ligands. Thus, activation of CXCR4 by gp120<sub>IIIB</sub> might recruit different intracellular pathways as compared to SDF-1 $\alpha$ . To test this hypothesis, we studied the ability of gp120<sub>IIIB</sub> and SDF-1 $\alpha$  to stimulate MAP kinases and Akt kinase, as these pathways are primarily involved in survival mechanisms. We have systematically compared gp120- and SDF-induced responses using equimolar concentrations of both ligands. In HOS cells we found that both the chemokine and the HIV viral protein induced activation of ERK and JNK (Fig. 2). The response to SDF-1 $\alpha$  was usually faster than that observed for gp120<sub>IIIB</sub> (not shown), but the latter produced significant activation of both kinases, even if used at low concentrations (200 pM) and in the absence of hCD4 (Fig. 2). Maximal responses induced by the two ligands were

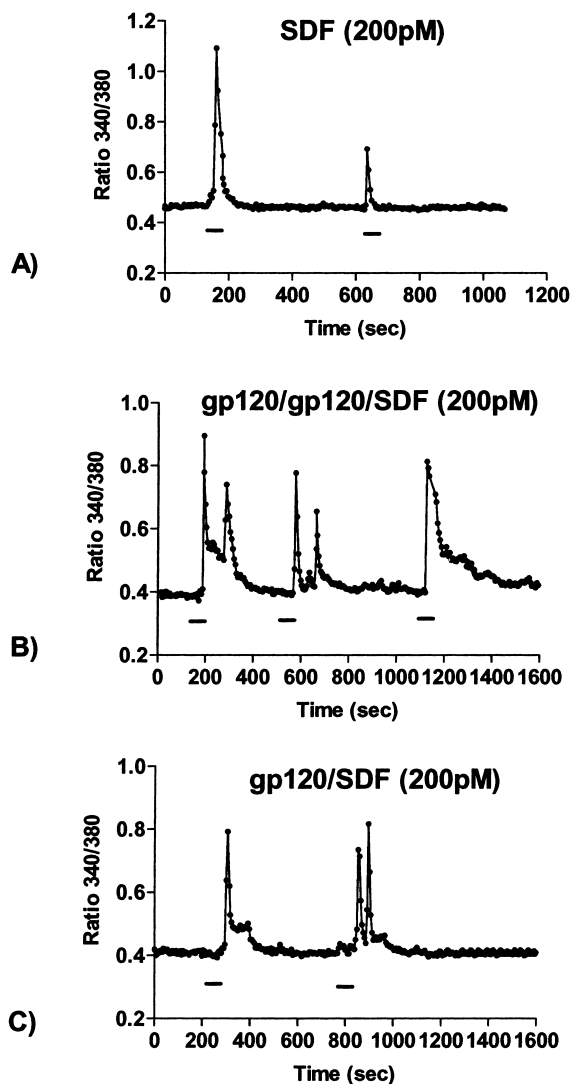
TABLE 1. CALCIUM RESPONSES IN HOS CELLS

Treatment	Total number of cells	Responsive cells	Peak ratio range
CXCR4 <sup>+</sup> cells			
SDF 200 pM	176	30%	0.6–0.9
SDF 2 nM	59	35%	0.6–0.9
SDF 20 nM	207	67%	0.6–1.3
SDF 50 nM	135	100%	0.8–1.6
SDF 20 nM (PTx)	34	5%	—
SDF 20 nM (anti-X4)	40	10%	—
gp120 200 pM	131	49%	0.6–0.9
gp120 200 pM (PTx)	74	1%	—
CXCR4 <sup>-</sup> cells			
gp120 200 pM (CD4 <sup>-</sup> )	30	0	—
gp120 200 pM (CD4 <sup>+</sup> )	23	0	—

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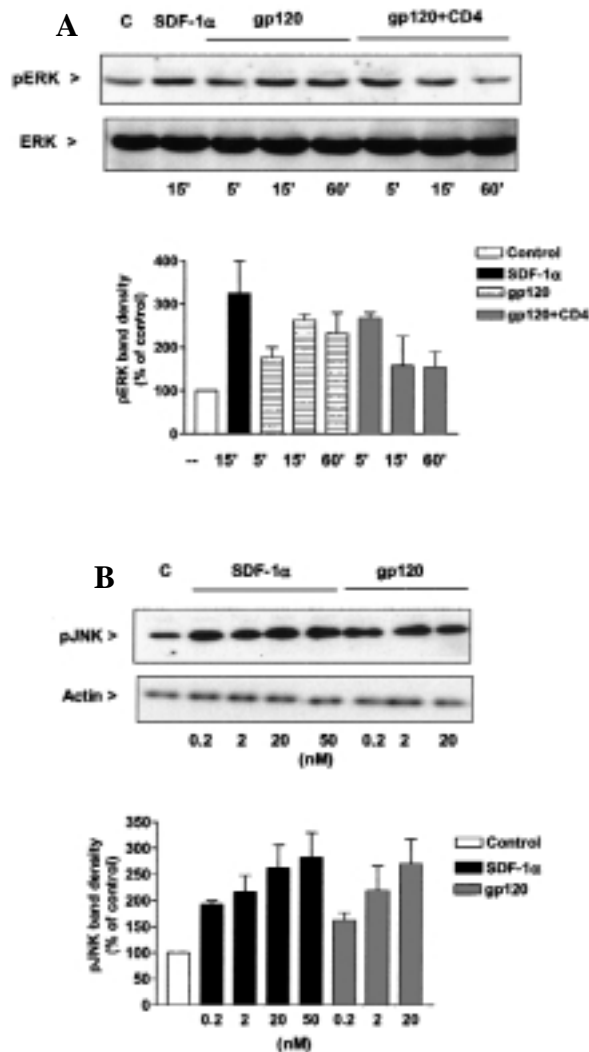
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**FIG. 1.** Effect of SDF-1 $\alpha$  and gp120<sub>III</sub>B on intracellular calcium. SDF-1 $\alpha$  and gp120<sub>III</sub>B increase intracellular calcium in CXCR4<sup>+</sup>/CD4<sup>-</sup> HOS cells. Quick and consecutive stimulation of cells with SDF-1 $\alpha$  down-regulates calcium responses (A) whereas when stimulated with gp120<sub>III</sub>B, the cells generally respond to subsequent stimulations with gp120 or SDF-1 $\alpha$  irrespective of washout time (B, C).

comparable. Coincubation with soluble hCD4 (2 nM) slightly accelerated the gp120<sub>III</sub>B- (200 pM) induced increase in ERK phosphorylation (Fig. 2A). Equimolar concentrations of the two agonists activated JNK to a comparable extent (Fig. 2B).

Following the same experimental approach, we studied the signaling of the two CXCR4 ligands in secondary cultures of human astrocytes as these cells constitutively express functional chemokine receptors *in vitro*, including CXCR4.<sup>43</sup> SDF-1 $\alpha$  and gp120 stimulated MAP kinases in astrocytes (Fig. 3). The effects of the two ligands are blocked by various CXCR4 antagonists, such as the bicyclam AMD3100 (Fig. 3) and the synthetic peptides V1 and DV1 (not shown), confirming the



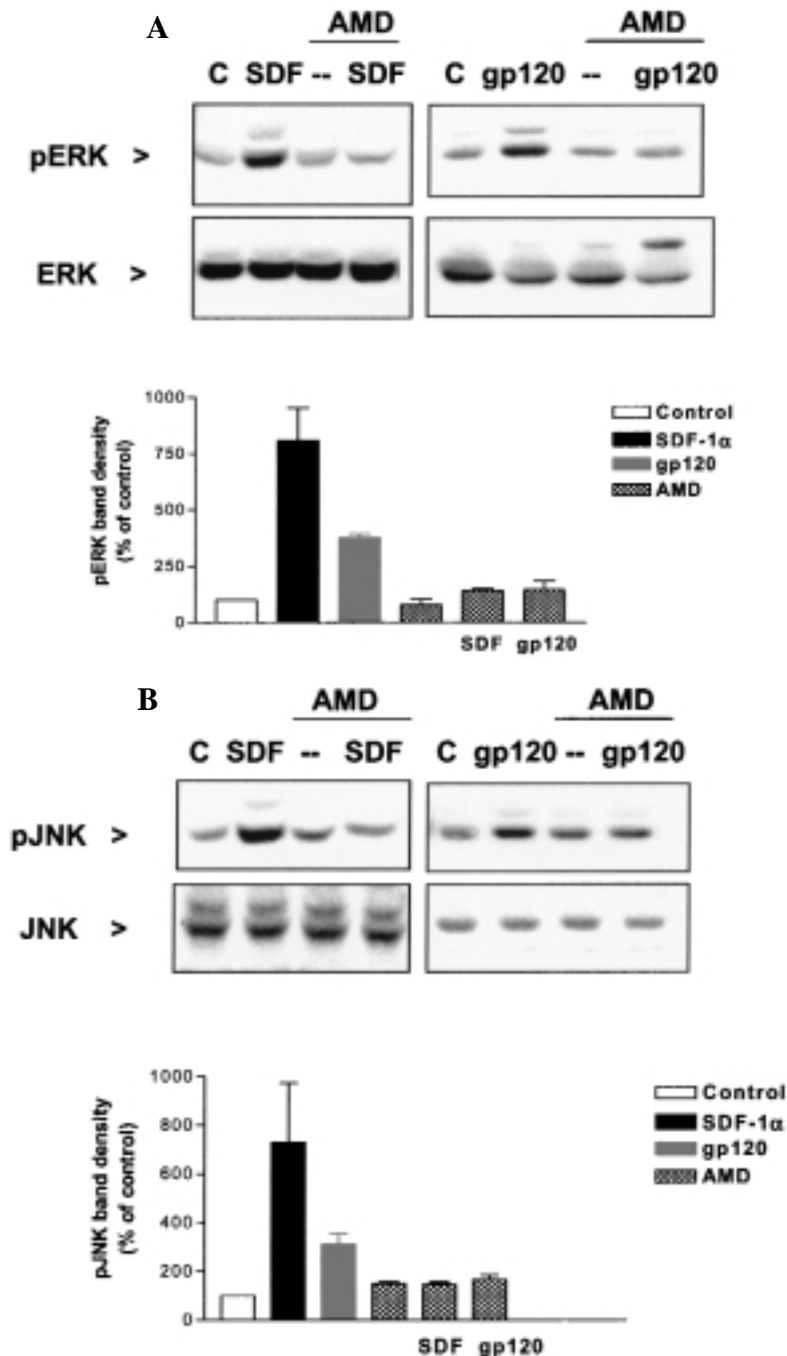
**FIG. 2.** Effect of SDF-1 $\alpha$  and gp120<sub>III</sub>B on ERK and JNK. (A) SDF-1 $\alpha$  (20 nM) and gp120<sub>III</sub>B (200 pM) stimulate ERK phosphorylation in CXCR4<sup>+</sup>/CD4<sup>-</sup> HOS cells. Cells treated with gp120 in the presence of soluble hCD4 show a more rapid and transient phosphorylation of ERK as compared to gp120 alone. The graph indicates the densitometric analysis from three independent experiments. As in these cells the p42 ERK band is much more evident than the p44 band; it has always been used for densitometric analysis. (B) SDF-1 $\alpha$  and gp120<sub>III</sub>B stimulate JNK phosphorylation in CXCR4<sup>+</sup>/CD4<sup>-</sup> HOS cells in a dose- and time-dependent manner. The graph shows the analysis of three independent experiments (15 min treatments).

involvement of this receptor in both gp120<sub>III</sub>B and SDF-1 $\alpha$  action. However, gp120<sub>III</sub>B appears more potent than SDF-1 $\alpha$  in activating MAP kinases, particularly JNK and p38 (EC<sub>50</sub>: 0.1 vs. 0.6 nM, for gp120<sub>III</sub>B and SDF-1 $\alpha$ , respectively).

Next, we compared the effects of SDF-1 $\alpha$  and gp120 on Akt, a pro-survival factor directly involved in the neuroprotective effect of chemokines.<sup>38</sup> As reported in Figure 4, SDF-1 $\alpha$  stimulates Akt phosphorylation in HOS-CXCR4<sup>+</sup> cells and glia. Activation of the kinase was time and dose dependent, and max-

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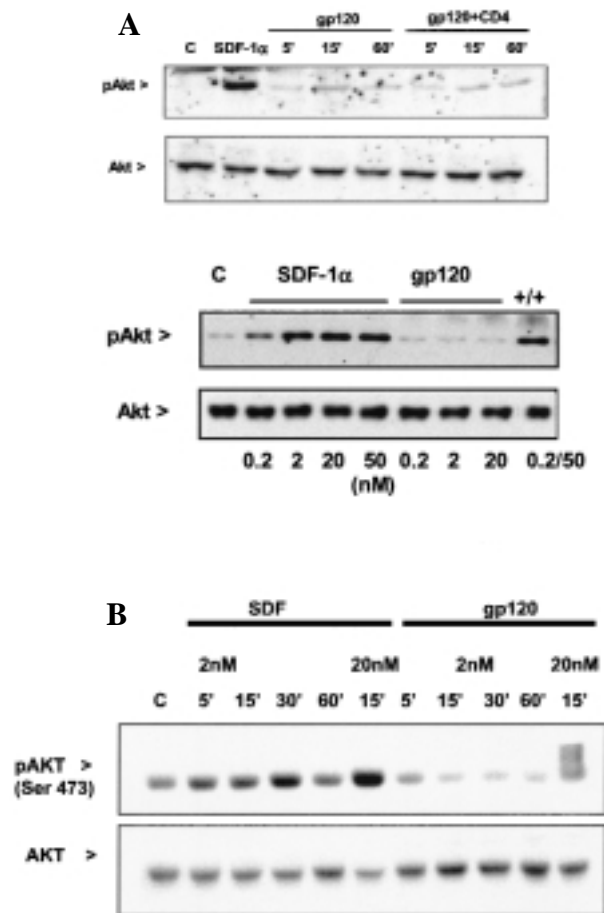


**FIG. 3.** Phosphorylation of MAP kinases induced by SDF-1 $\alpha$  or gp120<sub>IIIIB</sub> is mediated by CXCR4 in human astrocytes. The CXCR4 antagonist, AMD3100 (200 ng/ml), is able to block the ERK (**A**) and JNK (**B**) activation by SDF-1 $\alpha$  (2 nM) or gp120<sub>IIIIB</sub> (200 pM). The graphs show the analysis of three independent experiments normalized with total ERK or JNK, respectively.

imal responses were observed within 15 min of treatment with maximal concentrations of SDF-1 $\alpha$ , as in case of MAPKs (not shown). In contrast, gp120<sub>IIIIB</sub> did not affect Akt, even after prolonged treatments (up to 60 min), at high concentrations (up to 20 nM) and/or in the presence of soluble hCD4 (Fig. 4) or in cells expressing both CXCR4 and hCD4 (not shown). SDF-1 $\alpha$  stimulation of Akt was slightly inhibited by coincubation with

gp120<sub>IIIIB</sub> (percentage inhibition of SDF-1 $\alpha$  stimulation was  $28 \pm 9$ ,  $n = 4$ ) and completely blocked by treatment with PTx (not shown), as expected.

Parallel experiments in neurons (in the absence of soluble hCD4) also demonstrated an analogous pattern of CXCR4 activation: whereas both SDF-1 $\alpha$  and gp120<sub>IIIIB</sub> stimulated Ca fluxes and MAP kinases,<sup>28,38</sup> SDF-1 $\alpha$  but not gp120<sub>IIIIB</sub> was



**FIG. 4.** Effect of SDF-1 $\alpha$  and gp120<sub>IIIIB</sub> on Akt. SDF-1 $\alpha$  is able to induce the phosphorylation of Akt in CXCR4<sup>+</sup>/CD4<sup>-</sup> HOS cells, whereas gp120<sub>IIIIB</sub> (200 pM) is unable to do so in the presence or absence of soluble CD4 (A) as well as at high concentrations (B). Similar results were obtained in human astrocytes (C).

substrate of Akt,<sup>44,45</sup> as determined by immunoblots with a phospho-specific antibody against MDM2 (not shown).

## DISCUSSION

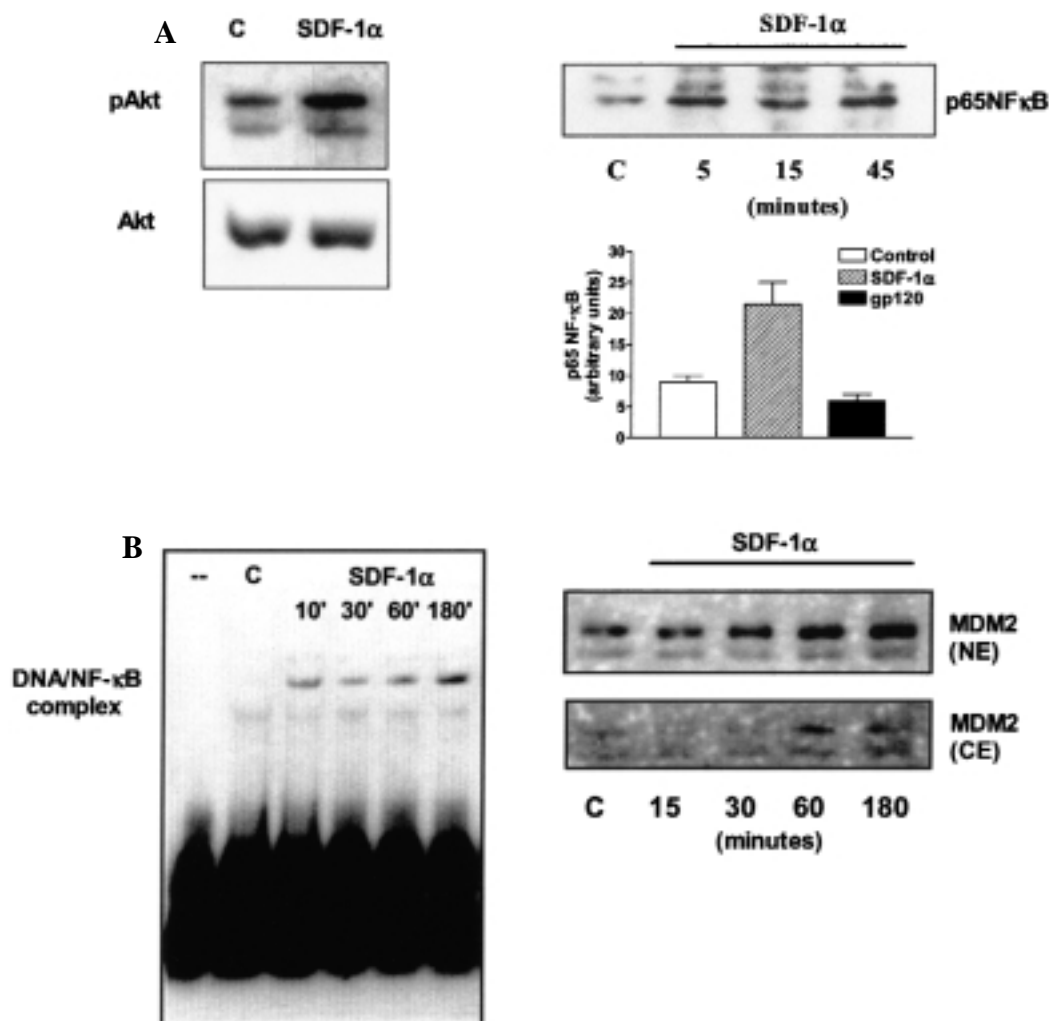
The data presented here show that although gp120 is a potent stimulator of MAP kinases, it is unable to activate pro-survival pathways such as Akt, either in the presence or in the absence of hCD4. This implies that the opposing effects of gp120 and SDF-1 $\alpha$  on neuronal survival cannot be ascribed to the absence of hCD4, but rather to differences in the “intrinsic efficacy” of the two CXCR4 ligands to affect pro-survival pathways. Indeed, the chemokine and the viral protein differ in their ability to induce receptor internalization and desensitization in neuronal cells, as indicated by the results of our calcium imaging experiments and by studies with YFP-tagged CXCR4 showing that stimulation with SDF-1 $\alpha$  resulted in greater endocytosis compared to gp120.<sup>31</sup> That receptor internalization/degradation depends on the activation of specific intracellular pathways (i.e.,  $\beta$ -arrestins, GRKs),<sup>46–48</sup> which, in turn, are linked to other downstream effects<sup>47–49</sup> (e.g., src activation), strengthens our conclusion that different transduction mechanisms are activated by gp120 and SDF-1 $\alpha$  upon CXCR4 stimulation. These results are in agreement with the recent observation that gp120-induced apoptosis of dorsal root ganglia neurons is mediated by CXCR4 independently of hCD4,<sup>31</sup> and with our previous findings regarding the involvement of Akt in the neuroprotective action of chemokines.<sup>28,38</sup>

The possibility that binding of different ligands to CXCR4 may result in pro-survival or apoptotic events is further corroborated by a very recent study showing that while full-length SDF-1 $\alpha$  functions as a survival factor, the cleaved form of this chemokine (SDF-1 $\alpha$  aa 5–67) is highly neurotoxic *in vivo* and *in vitro*.<sup>50</sup> Though the N-terminal truncated molecule has a much reduced affinity for CXCR4 and is unable to induce chemotaxis of CD34<sup>+</sup> cells, it still activates ERK (better than the full length SDF-1 $\alpha$ ) and its neurotoxicity is blocked by PTx<sup>50</sup>—suggesting that it still functions via a GPCR.

Intrinsic efficacy (i.e., the ability of a ligand to activate or inactivate a receptor) is an elusive, yet fundamental concept in molecular pharmacology, which is currently under reevaluation.<sup>51,52</sup> Recent evidence suggests that intrinsic efficacy might not be a single, ligand-dependent parameter but that agonists might have multiple intrinsic efficacies and that each agonist efficacy varies depending on which response (receptor behavior) is measured.<sup>51,53</sup> Thus, intrinsic efficacy is becoming a pathway dependent attribute and different ligands can have a range of efficacies for different receptor behaviors. Several groups have now shown that the potency and efficacy order of different drugs that act on the same receptor can, in fact, differ depending on the pathway analyzed. These concepts have been applied to a wide variety of GPCRs, including chemokine receptors.<sup>53</sup> The idea that agonists produce only varying degrees of receptor activation is therefore becoming obsolete as it does not reconcile with the situations in which ligands induce differing patterns of responses.<sup>54</sup> All these activities rely on the same molecular mechanism, namely the selective affinity (mi-

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able to activate Akt (Fig. 5).<sup>38</sup> Finally, we tested the ability of the two ligands to modulate Akt targets that are implicated in cell fate, the transcription factor NF- $\kappa$ B, and the oncoprotein MDM2. Increased nuclear translocation of the p65NF- $\kappa$ B subunit was observed upon treatment of neurons with SDF-1 $\alpha$  (Fig. 5). We did not find similar effects with gp120<sub>IIIIB</sub> (Fig. 5). In addition, DNA retardation assays showed that SDF-1 $\alpha$  increases the DNA-binding activity of p65NF- $\kappa$ B in HOS cells (Fig. 5). Another recently discovered target of Akt is the protein MDM2, which regulates the stability/activity of cell cycle proteins involved in apoptotic processes (i.e., p53, E2F-1 and Rb).<sup>44</sup> MDM2 is a predominantly nuclear protein. Phosphorylation of MDM2 by Akt is critical for its nuclear localization and activation.<sup>44,45</sup> The nuclear levels of MDM2 were significantly increased by SDF-1 $\alpha$  in HOS (CXCR4<sup>+</sup>/CD4<sup>-</sup>) cells (Fig. 5). This effect was observed in cells treated with SDF-1 $\alpha$  for up to 3 hr. In HOS cells, SDF-1 $\alpha$  (but not gp120<sub>IIIIB</sub>) also increased phosphorylation of MDM2 on the Ser-166, a known



**FIG. 5.** Effects of SDF-1 $\alpha$  on Akt-mediated pathways. SDF-1 $\alpha$  (50 nM) stimulates Akt phosphorylation in hippocampal neurons (A); the chemokine also increases p65NF $\kappa$ B levels in the nuclear fraction of these neurons, unlike gp120<sub>IIIb</sub> (200 pM 45 min) (B); SDF-1 $\alpha$  (20 nM) also induces a time-dependent increase in the DNA-binding activity of p65 NF $\kappa$ B in CXCR4<sup>+</sup>/CD4<sup>-</sup> HOS cells. (C). In HOS cells, SDF-1 $\alpha$  (20 nM) also induces up-regulation of MDM2 levels in the nucleus (D).

croaffinity) of a given ligand for a particular conformational state of the receptor.<sup>52</sup>

Therefore the neurotoxicity of gp120 (and perhaps cleaved SDF-1 $\alpha$ ) may derive from a strong affinity of the ligand for CXCR4 in a conformational state coupled to apoptotic pathways (p38, JNK) with a concomitant negligible affinity for those CXCR4 conformations that are coupled to the activation of pro-survival pathways (Akt). The idea of multiple CXCR4 conformations is also supported by previous structure-activity analyses with CXCR4 peptide antagonists, which show the remarkable flexibility of the CXCR4-ligand interface.<sup>39</sup> The balance among the signaling cascades triggered by the various receptor conformations would ultimately determine cell survival. Akt is a major controller of these mechanisms. This kinase is not only responsible for generating pro-survival signals, but also counteracts several effectors of apoptosis, such as caspases, JNK, and the transcription factor E2F1.<sup>55-57</sup> This is underscored

by the finding that the neuroprotective activity of chemokines is lost when Akt is inhibited,<sup>38</sup> and by the different effects of SDF-1 $\alpha$  and gp120 on E2F1.<sup>16</sup> In addition, a recently discovered function of Akt is the control of synaptic strength and plasticity.<sup>58</sup> Chemokines regulate synaptic activity in neurons.<sup>28,59</sup> Therefore, the inability of gp120 to affect Akt might have negative consequences at various levels, even those preceding apoptosis, providing a possible explanation for the discrepancy between serious neurological deficits and low degree of cell death observed in some neuroAIDS patients.

In conclusion, we have shown that SDF-1 $\alpha$  and gp120 differ in their ability to activate intracellular pathways downstream of the activation of CXCR4 in neuronal and nonneuronal cells, and that gp120 may possess a different intrinsic efficacy for CXCR4 as compared to that of the natural CXCR4 ligand. A better understanding of the regulation of CXCR4-dependent pathways in normal and pathological conditions is essential, not

only to improve our knowledge of HIV neuropathogenesis and foster the development of new pharmacological tools, but also to elucidate the role of chemokines in the CNS.

### ACKNOWLEDGMENTS

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