

The chemokine receptor CXCR4 regulates cell-cycle proteins in neurons

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Neurons express a variety of chemokine receptors that regulate neuronal signaling and survival, including CXCR4 and CCR5, the two major human immunodeficiency virus (HIV) coreceptors. However, the role of chemokine receptors in HIV neuropathology and neuroinflammatory disorders is still unclear. This study aims to determine whether chemokine receptors regulate the activity of cell-cycle proteins in neurons and evaluate the possibility that alterations of these proteins are involved in HIV neuropathogenesis. The authors studied the effect of the chemokine stromal cell-derived factor (SDF)-1 α , the natural CXCR4 ligand, and an X4-using variant of gp120 on the activity of cell-cycle proteins involved in neuronal apoptosis and differentiation, such as Rb and E2F-1. Changes in expression, localization, and phosphorylation/activation of Rb and E2F-1 induced by SDF-1 α (20 nM) gp120_{IIIIB} (200 pM) were analyzed in primary cultures of rat neurons and in a human cell line expressing recombinant CXCR4. The data indicate that changes in the nuclear and cytosolic levels of Rb—which result in the functional loss of this protein—are associated with apoptosis in hippocampal or cerebellar granule neurons and in cell lines. SDF-1 α , which is able to rescue these neurons from apoptosis, induces a time-dependent increase of total Rb expression while decreasing the nuclear content of phosphorylated (Ser780/Ser795) Rb and the transcriptional activity of E2F-1. The HIV envelope protein gp120_{IIIIB} exerts opposite effects at the nuclear level. These data indicate that CXCR4 affects cell-cycle proteins in neurons and raise the possibility that chemokines may contribute to neuronal survival by repressing the activity of E2F-dependent apoptotic genes and maintaining neurons in a highly differentiated and quiescent state. This state may be altered during neuroinflammatory conditions and/or by HIV-derived proteins. *Journal of NeuroVirology* (2003) 9, 300–314.

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Introduction

The chemokine stromal cell-derived factor (SDF)-1 and its receptor CXCR4 are constitutively expressed in the developing and adult brain by both neuronal and non-neuronal cells and are implicated in several physiological processes in the central nervous system (CNS), such as neurogenesis and connectivity (Ma *et al*, 1998; Zou *et al*, 1998; Asensio and Campbell, 1999; Bajetto *et al*, 1999; Hesselgesser and Horuk, 1999; Lu *et al*, 2001, 2002; Zhu *et al*, 2002). The developmental and injury-induced regulation of SDF-1/CXCR4 expression in discrete CNS

areas indicates that this chemokine/receptor pair may play different roles throughout life (Tham *et al*, 2001; Van Der Meer *et al*, 2001; Stumm *et al*, 2002). SDF-1 μ appears to regulate survival of neuronal progenitors and mature neurons (Hesselgesser *et al*, 1998; Meucci *et al*, 1998; Kaul and Lipton, 1999; Klein *et al*, 2001). However, although both neuroprotective and neurotoxic actions of SDF-1 μ have been reported, the role of CXCR4 in the survival of differentiated neurons under physiological conditions is still unclear. Evidence indicates that its inappropriate activation may be involved in neuroinflammatory and neurodegenerative diseases, including human immunodeficiency virus (HIV)-associated dementia (HAD) (Ransohoff *et al*, 1997; Asensio and Campbell, 1999; Miller and Meucci, 1999; Asensio and Campbell, 2001). In addition to its role as HIV coreceptor (Feng *et al*, 1996), CXCR4 may participate in HIV-induced neuropathogenesis through the activation of specific apoptotic pathways in neurons and glia (Hesselgesser *et al*, 1998; Kaul and Lipton, 1999; Zheng and Lee, 1999; Garden *et al*, 2002). These pathways can be activated by the direct interaction of CXCR4 with the HIV-1 envelope protein gp120, as indicated by *in vitro* and *in vivo* studies (Kaul *et al*, 2001).

Alterations of cell-cycle proteins, such as inappropriate activation of the CDK/Rb/E2F-1 pathway—which may result in an abortive attempt to reenter the cell cycle—have often been implicated in neuronal apoptosis (Giovanni *et al*, 2000; O'Hare *et al*, 2000; Osuga *et al*, 2000; Park *et al*, 2000). It is generally believed that the retinoblastoma gene product, Rb, displays neuroprotective functions and that its loss is associated with apoptosis (Macleod, 1999; Harbour and Dean, 2000a; Zheng and Lee, 2001). Although the molecular mechanisms involved are still not clearly defined, many data show that neuronal apoptosis derives from the inability of Rb to control the action of proteins directly involved in the regulation of cell-cycle progression and cell death (Sears and Nevins, 2002). For example, one of the major Rb targets is the transcription factor E2F-1, which activates expression of apoptotic genes (i.e., Apaf-1, p53, and Bax) as well as genes required for entry into S phase, and down-regulates the activation of antiapoptotic signals (i.e., nuclear factor [NF]- κ B) (Phillips *et al*, 1999; Harbour and Dean, 2000b; Moroni *et al*, 2001). E2F-1 is regulated by direct interaction with Rb (Dyson, 1998). Phosphorylation of Rb by cyclin-dependent kinases (CDKs) (i.e., CDK4) abolishes this interaction, leading to loss of repression and promotion of E2F-1 transcriptional activity. Indeed, Rb-null mice show neurological deficits and neuronal apoptosis, which may be reverted by E2F-1 mutation (Jacks *et al*, 1992). Ectopic expression of E2F-1 in proliferating cells as well as neurons induces apoptosis (Phillips *et al*, 1999; O'Hare *et al*, 2000).

Recently, changes in the expression and activity of cell-cycle proteins (Rb, E2F-1, and p53) have been

reported in the brains of HIV-demented patients and monkey with simian immunodeficiency virus (SIV) encephalitis (Jordan-Sciutto *et al*, 2000, 2002), suggesting that alterations of the CDK/Rb/E2F-1 pathway may be implicated in HIV neuropathogenesis. To start investigating this hypothesis, we have asked the question of whether CXCR4 can regulate Rb and E2F-1 activities in cultures of differentiated rat neurons. We have focused on two specific brain areas in which CXCR4 is physiologically relevant, i.e., the cerebellum and the hippocampus (Zou *et al*, 1998; Klein *et al*, 2001; Lu *et al*, 2002). A human cell line overexpressing CXCR4 (HOS cells) has been used to monitor the behavior of the isolated receptor in a different cellular model.

The data presented here indicate that SDF-1 μ modulates the expression and activation state of both Rb and E2F-1, and raise the possibility that the neuroprotective effect of this chemokine may be related to its ability to increase Rb levels, thus inhibiting transcription of apoptotic genes regulated by E2F-1. On the contrary, the apoptotic action of HIV envelope proteins—and possibly of SDF-1 μ in pathological situations—may be related to an opposite effect on this pathway.

Results

SDF-1 μ prevents Rb loss and phosphorylation in cerebellar granule neurons undergoing apoptosis

One of the best-characterized models of neuronal apoptosis in which Rb is implicated is the death of cerebellar granule neurons deprived of depolarizing concentrations of extracellular potassium, i.e., 25 mM KCl (K25) (D'Mello *et al*, 1993; Galli *et al*, 1995). When shifted to a serum-free medium with 5 mM KCl (K5), granule neurons undergo apoptosis, a process that is preceded by several changes of cell-cycle regulators, including increased CDK4 activity, phosphorylation, and subsequent loss of the Rb protein, and up-regulation of E2F-1. Evidence indicates that these alterations are responsible for apoptosis of granule neurons (Boutillier *et al*, 1999; Padmanabhan *et al*, 1999; O'Hare *et al*, 2000; Trinh *et al*, 2001). Thus, we first studied whether SDF-1 μ could affect the changes in Rb and E2F-1 induced by K5 in granule neurons. For these experiments, neurons were cultured in K25 medium with serum for the first 6 to 7 days *in vitro* and then shifted to a K5 serum-free medium according to the experimental design. Control neurons were maintained in K25 and only deprived of serum as previously described (Galli *et al*, 1995). Untouched neurons (i.e., kept in original culture medium with serum and K25) were usually used as an internal control of survival.

As expected, total Rb levels decreased when neurons were deprived of extracellular potassium, an effect observed after a relatively short incubation in K5 (Figure 1). The addition of SDF-1 μ (20 nM) to

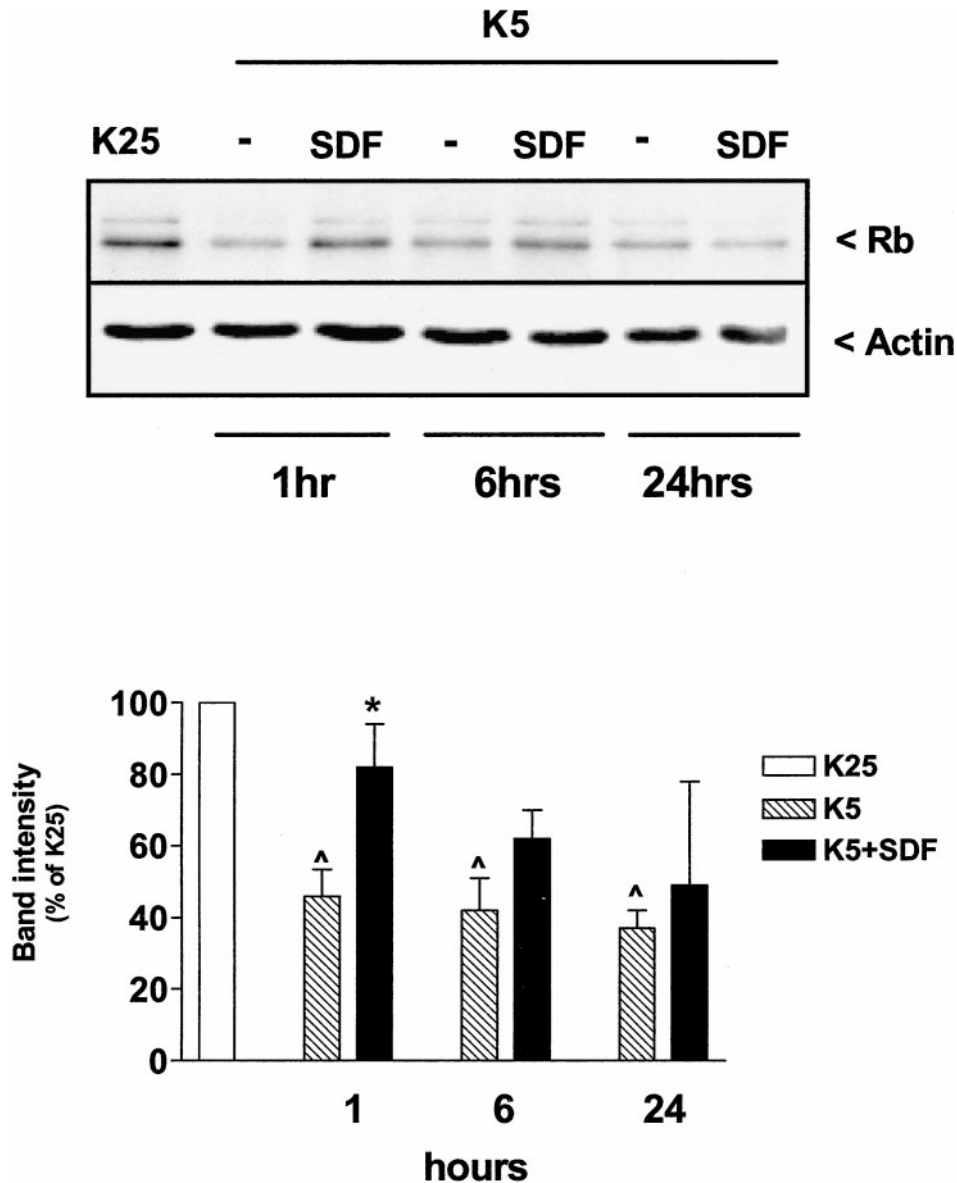


Figure 1 SDF-1 μ affects Rb levels in cerebellar granule neurons. Western blot analysis of Rb expression in control neurons (K25) and neurons maintained in K5 medium in the presence or absence of SDF-1 μ (20 nM) for the indicated time (20 μ g protein/lane). Data reported in the graph (mean \pm SEM) are from three separate experiments. [^]*P* < .05 versus K25 and ^{*}*P* < .05 versus K5 alone.

the K5 medium prevented Rb reduction within the first 6 h (Figure 1). Importantly, this time frame corresponds to the “point of no return” (i.e., commitment to death) for the induction of K5-induced apoptosis (Galli *et al*, 1995). A maximal effect of SDF-1 μ was observed in the very first hour (compared to K25, levels of Rb in K5 + SDF and K5 alone were around 80% and 45%, respectively). Similar results were observed with higher concentrations of SDF-1 μ (50 and 100 nM, not shown). A modest increase of Rb levels (20% to 30%) was also observed when neurons were exposed to SDF-1 μ (20 nM) in K25 medium, whereas SDF-1 μ was unable to affect Rb when added to neurons that had been deprived of potassium 5 h earlier

(data not shown). This does not seem to be due to a reduced expression of CXCR4 in neurons cultured in low potassium, as we found no significant differences in the expression of CXCR4 at the single-cell level between control neurons (K25) and neurons cultured in K5 for several hours (up to 24 h; Figure 2). These data indicate that the chemokine interferes with the earliest steps of the death signal cascade triggered by K5.

The ability of Rb to inhibit E2F-1 depends on its phosphorylation state, which also affects Rb localization and degradation (Dyson, 1998). Although several different sites of phosphorylation have been identified on Rb, four residues C-terminal to the pocket domain (Ser975/807/811/780) are critical for

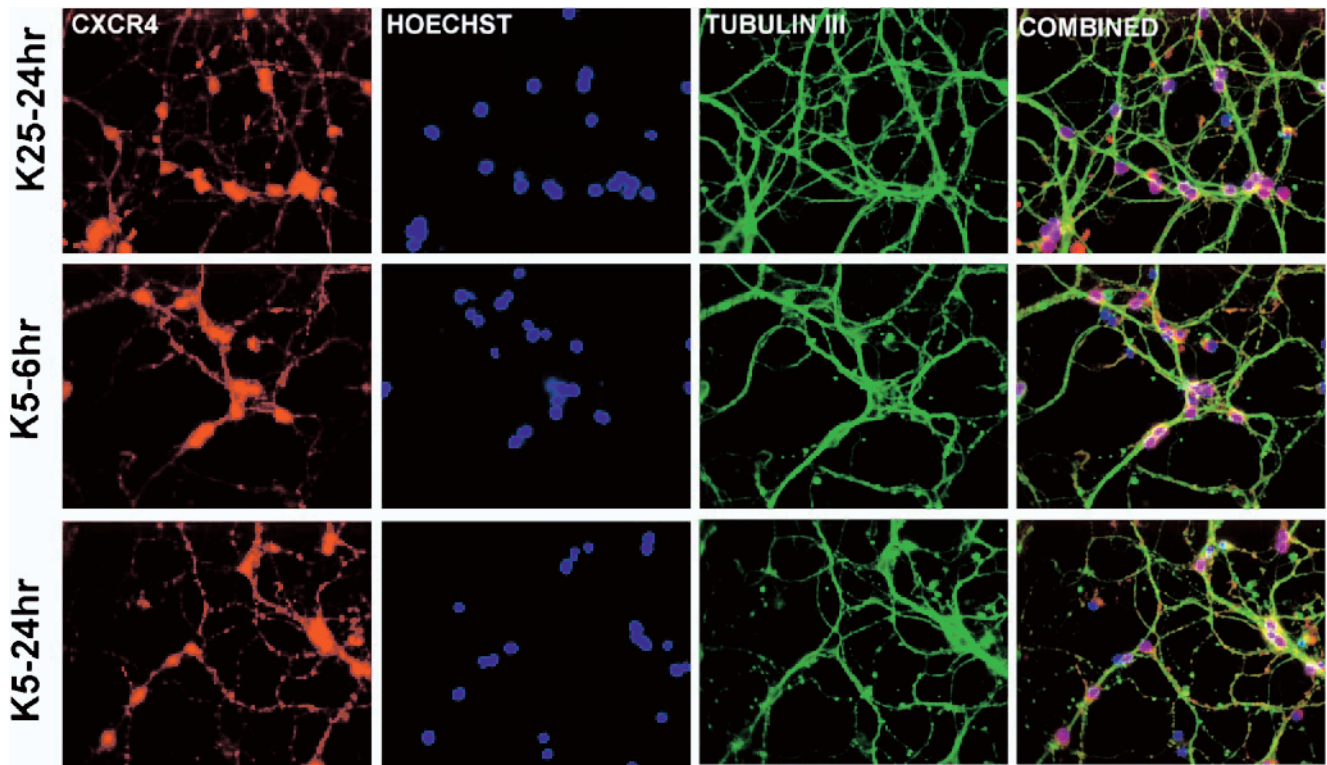


Figure 2 Expression of CXCR4 in cerebellar granule neurons cultured in high or low potassium. Control neurons (K25) and neurons maintained in K5 for 6 to 24 h were immunostained with a polyclonal antibody against CXCR4 (amino acids 176 to 293) and a monoclonal antibody for the specific neuronal marker μ -tubulin III (green). Nuclei were stained with Hoechst 33342 (blue).

its interaction with E2F proteins and are targets of CDK4/6 (Taya, 1997). Thus, to evaluate whether the effect of SDF-1 μ on Rb may affect E2F-1 function, we probed the neuronal extracts with antibodies against Rb phosphorylated at Ser795 or Ser780. According to previous reports on cerebellar granule neurons, Rb phosphorylation on these residues by CDK4 is one of the earliest events in the K5-induced apoptotic cascade (Boutillier *et al*, 1999; Padmanabhan *et al*, 1999). As in the case of total Rb levels, we found that SDF-1 μ treatment prevented the phosphorylation of Rb caused by potassium deprivation (Figure 3), which is generally associated to the subsequent degradation of the protein and activation of apoptotic genes by E2F-1.

SDF-1 μ regulates Rb expression and localization in HOS cells

To investigate the mechanism(s) involved in the action of SDF-1 μ at the total Rb level, we tested the effect of SDF-1 μ on a human cell line (HOS) that stably overexpresses CXCR4. The goal of these experiments was to gather information about potential changes in the cellular localization of Rb induced by SDF-1 μ . Despite its major role as a nuclear protein, Rb is also expressed in the cytosol and it seems to translocate to the nucleus in association with other cellular factors (such as E2F-1) (Zacksenhaus *et al*, 1996, 1999).

The subcellular localization of these proteins may, in fact, contribute to their regulation.

In case of HOS cells, cytosolic levels of Rb are greatly reduced when cells are incubated for 2 to 4 h in a saline solution (Figure 4A), even though its nuclear level remains quite stable during that period, indicating that the lack of trophic factors is responsible for Rb degradation and loss. When we examined the effect of serum starvation on Rb expression over a longer period of time (from 6 to 48 h), we also noticed that Rb accumulates in the nucleus in conjunction to its cytosolic decrement (Figure 4B). This effect could be related to the suppressive role of Rb in cell-cycle progression. Interestingly, both the decrease in cytosolic Rb and its accumulation into the nucleus were inhibited by SDF-1 μ (Figure 4B) for the first 24 h. After 48 h of starvation, the differences between SDF-treated and untreated cells are less dramatic (and even disappear at the nuclear level), possibly due to chemokine degradation and/or to prolonged serum starvation. Overall, these experiments indicate that the chemokine may affect Rb synthesis and subcellular localization in these cells. Indeed, total Rb content increased in cells maintained in regular culture medium and treated with SDF-1 μ for 15 to 180 min, as demonstrated by immunocytochemistry and Western blot analysis (Figure 5A, B). To determine whether this effect was related to new protein

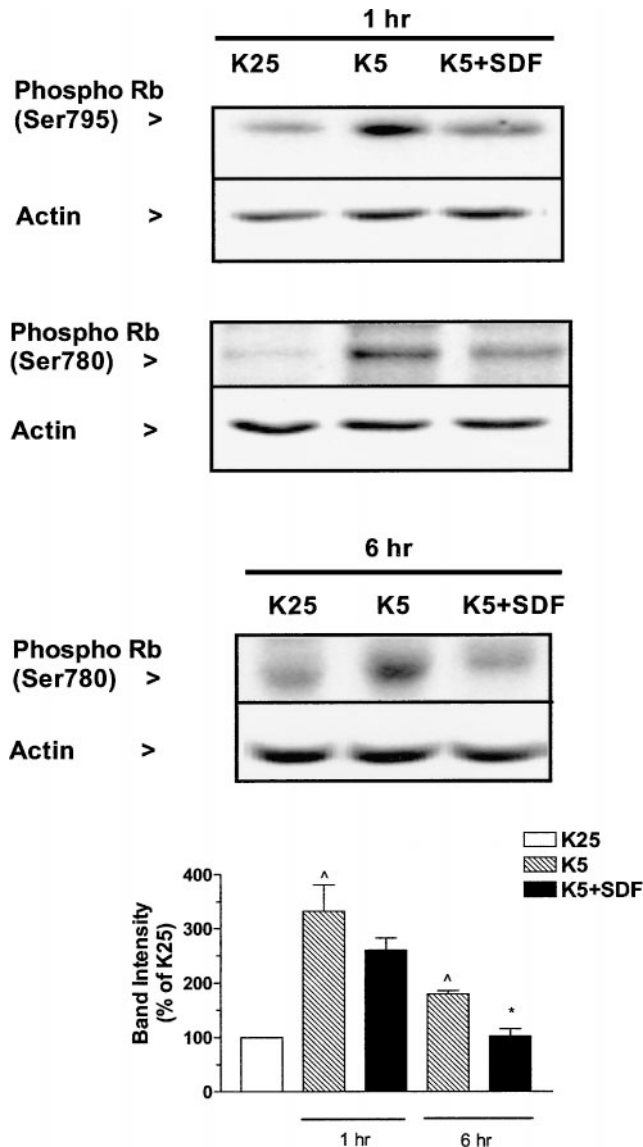


Figure 3 SDF-1 μ prevents Rb hyperphosphorylation induced by low potassium. Control neurons (K25) and neurons maintained in K5 medium in the presence or absence of SDF-1 μ (20 nM) for 1 or 6 h were used to determine variations in the expression of phosphoRb in cerebellar granule neurons by using antibodies for either Ser795pRb or Ser780pRb. Twenty micrograms of protein were loaded in each lane. Data reported in the graph (mean \pm SEM) are from three separate experiments (Ser780pRb). [^]*P* < .05 versus K25 and ^{*}*P* < .01 versus K5.

synthesis, HOS cells were pretreated for 4 to 5 h with cycloheximide (CHX, 1 μ M) before being exposed to SDF-1 μ plus CHX (1 h). As shown in Figure 5B, SDF-1 μ did not affect Rb level in CHX-treated cells. Thus, it is likely that in this experimental condition, the chemokine stimulates Rb expression. Finally, SDF-1 μ is also able to regulate the phosphorylation state of Rb in HOS cells, as indicated by Western blot analysis and *in vitro* kinase assays (Figure 5C). A reduction in the phosphorylated (Ser780) band of Rb was found in cells treated with SDF-1 μ and lower levels

of phosphorylation were observed in *in vitro* kinase assays performed with protein extracts from SDF-treated cells. For these experiments, a glutathione *S*-transferase (GST)-Rb fusion protein containing the C-terminus region of Rb was used as a substrate for phosphorylation.

Taken together, these data suggest that SDF-1 μ may be involved in the regulation and trafficking of Rb being able to affect its synthesis and possibly degradation, nuclear translocation, as well as phosphorylation.

Effects of SDF-1 μ on E2F-1

Overexpression of E2F-1 can induce apoptosis of cerebellar granule neurons as well as other cells and increase in the level and activity of this transcription factor have been implicated in K5-induced apoptosis (O'Hare *et al*, 2000). In line with these data, we found that the DNA-binding activity of E2F-1 was higher than normal in preapoptotic neurons (Figure 6A). This effect was inhibited by SDF-1 μ , suggesting that the chemokine may interfere with the apoptotic action of E2F-1. In support of this hypothesis, we found that in HOS cells, SDF-1 μ not only reduced E2F-1-binding activity (Figure 6A), but also led to accumulation of E2F-1 in the cytosol (Figure 6B). Thus, the chemokine may reduce E2F-1 proapoptotic activity by "retaining" the protein into the cytosol—a mechanism that regulates the activity of other members of the E2F family—and favoring its degradation. This last possibility is also suggested by the reduced levels of total E2F-1 expression in cerebellar granule neurons treated with SDF-1 μ (Figure 6C). Further studies are in progress to confirm this hypothesis and determine the effect of SDF-1 μ on E2F-1 transcription activity. Interestingly, gp120_{IIIIB} did not reduce E2F-1 expression in these conditions (Figure 6C), in line with its inability to rescue neurons from K5-induced apoptosis (Figure 7A).

The neuroprotective effect of SDF-1 μ correlates with the changes in cell-cycle proteins

The effects of SDF-1 μ on Rb and E2F-1 expression suggest that SDF-1 μ may affect neuronal apoptosis induced by K5. For instance, SDF-1 μ may contribute to the survival of differentiated neurons by inhibiting cell-cycle progression and/or activation of apoptotic genes. Thus, we tested the ability of the chemokine to block K5-induced neuronal death. In agreement with our findings on Rb levels and phosphorylation, cell-survival experiments showed that SDF-1 μ was able to reduce neuronal cell death induced by potassium deprivation. The neuronal death induced by K5 after 20 to 22 h was reduced by almost 50% by SDF-1 μ (Figure 7A)—an effect abolished by the specific CXCR4 antagonist AMD3100 (Figure 7B). A similar degree of protection was observed by incubating neurons with the specific CDK4/6 inhibitor PD0183812 (Fry *et al*, 2001) (Figure 7A), suggesting that the neuroprotective action of SDF-1 μ might be

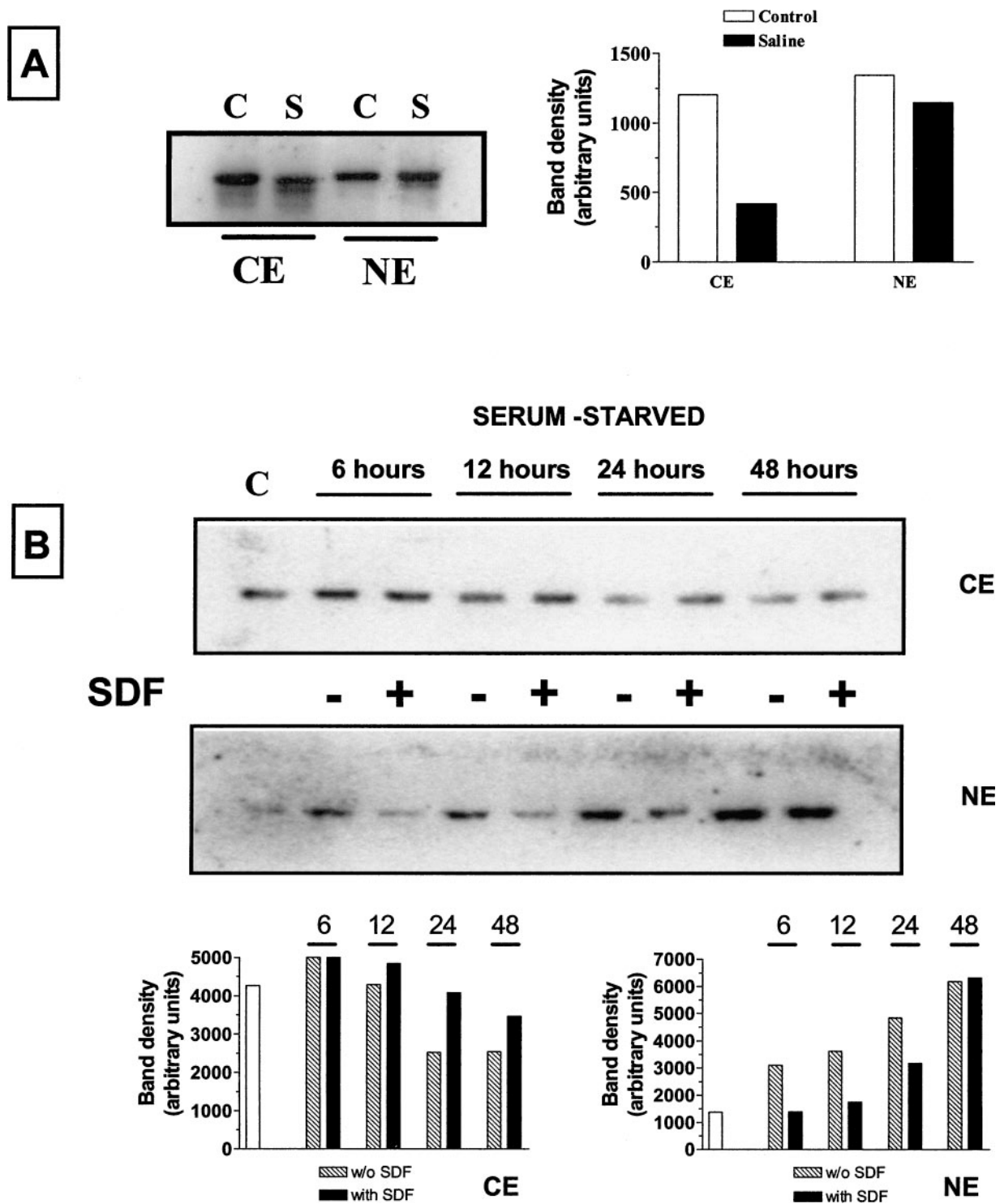


Figure 4 Effect of SDF-1 μ on Rb cellular localization in HOS-CXCR4 cells. (A) Cytosolic and nuclear extracts (CE and NE, respectively) were obtained from control cells (C) and cells incubated for 4 h in saline solution (S). Immunoblots for Rb show that trophic factors deprivation caused a reduction in the Rb content mainly at the cytosolic level. (B) SDF-1 μ prevents changes in Rb expression and subcellular localization induced by long-term serum starvation. Similar data were obtained from two additional experiments. (CE = 25 μ g protein/lane; NE = 5 μ g protein/lane.)

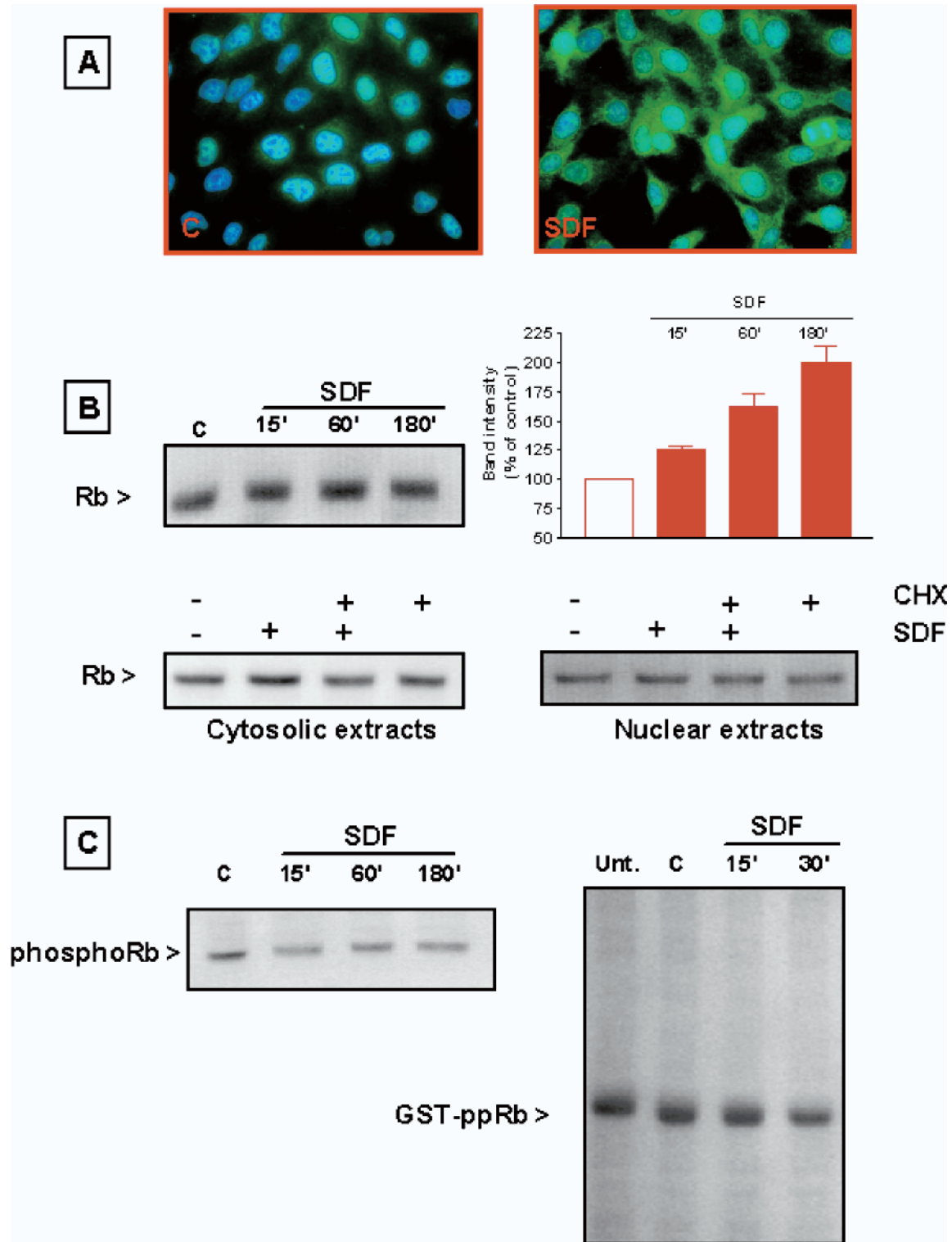


Figure 5 SDF-1 μ regulates Rb expression and phosphorylation in HOS-CXCR4 cells. (A) Immunostaining for Rb in control cells (*left panel*) and SDF-treated cells (SDF-1 μ 20 nM, 3-h treatment in regular medium) showing higher levels of Rb (*green*) in treated cells. (B) The same antibody was used for immunoblots (SDF-1 μ 20 nM): total cell lysates (*top*) or cytosolic and nuclear extracts (20 μ g protein/lane). Treatment with CHX prevents the effect of SDF-1 μ on Rb (*bottom*). (C) The left panel shows immunoblots for pRb (Ser795) with the same total cell lysates used in B. *In vitro* kinase assay showing a reduction in GST-Rb phosphorylation following SDF-1 μ (20 nM) treatment.

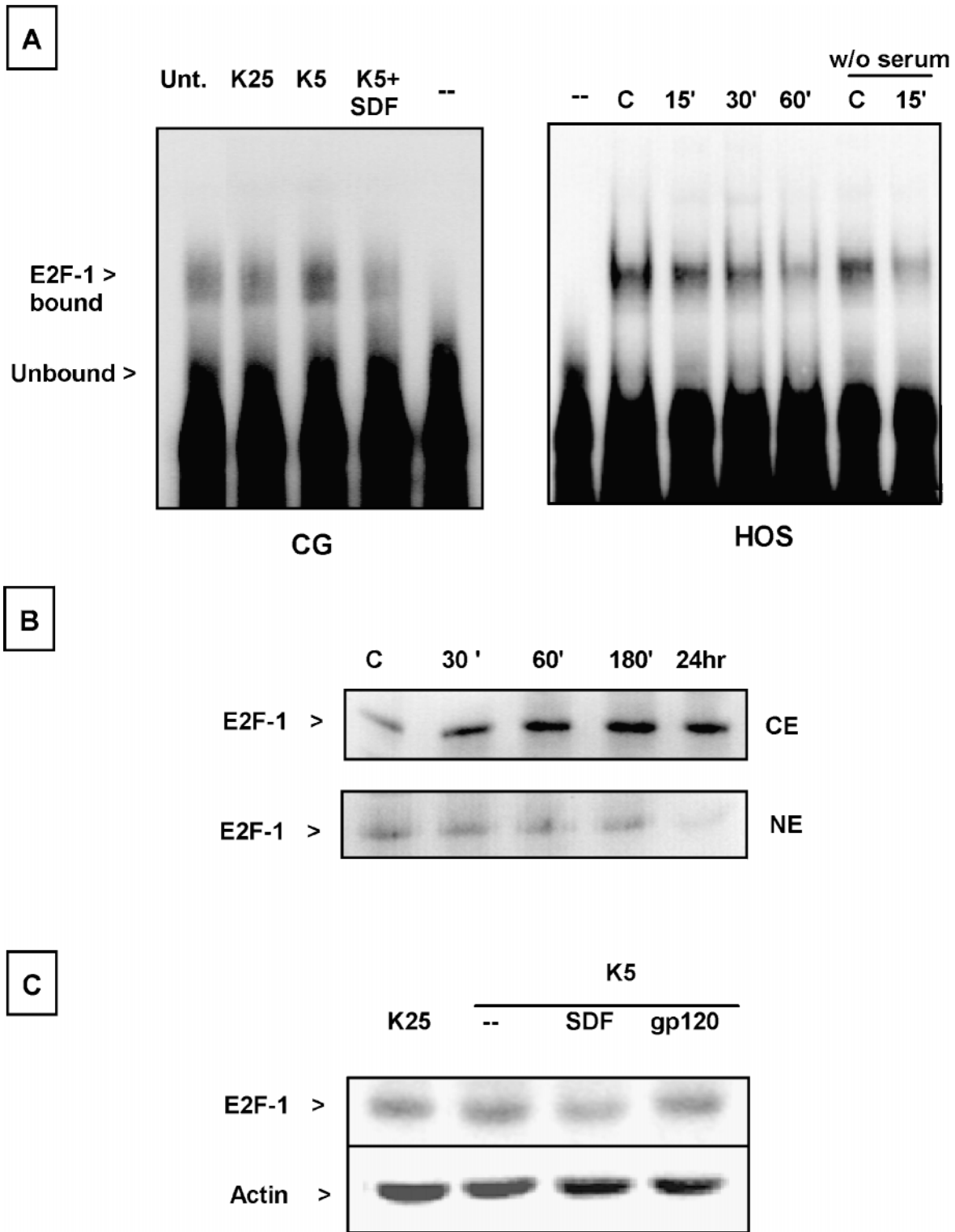


Figure 6 Effect of SDF-1 μ on E2F-1 in granule neurons and HOS-CXCR4 cells. (A) SDF-1 μ prevents the increase in E2F-1 DNA-binding activity induced by K5 (6 h treatment) in cerebellar granule neurons (*left panel*). The chemokine also inhibits E2F-1 activity in HOS cells (*right panel*) in the presence or absence of serum (last two lanes). Lanes marked with (o) do not contain nuclear extracts. (B) Western blot analysis in HOS cells: SDF-1 μ (20 nM) increases the cytosolic content of E2F-1 and slightly decreases its nuclear level (CE = 25 μ g protein/lane; NE = 5 μ g protein/lane). (C) Neuronal extracts (20 μ g protein/lane) from control and treated cells (6 h) were immunoblotted with E2F-1 antibody: SDF-1 μ but not gp120 reduces E2F-1 expression in granule neurons.

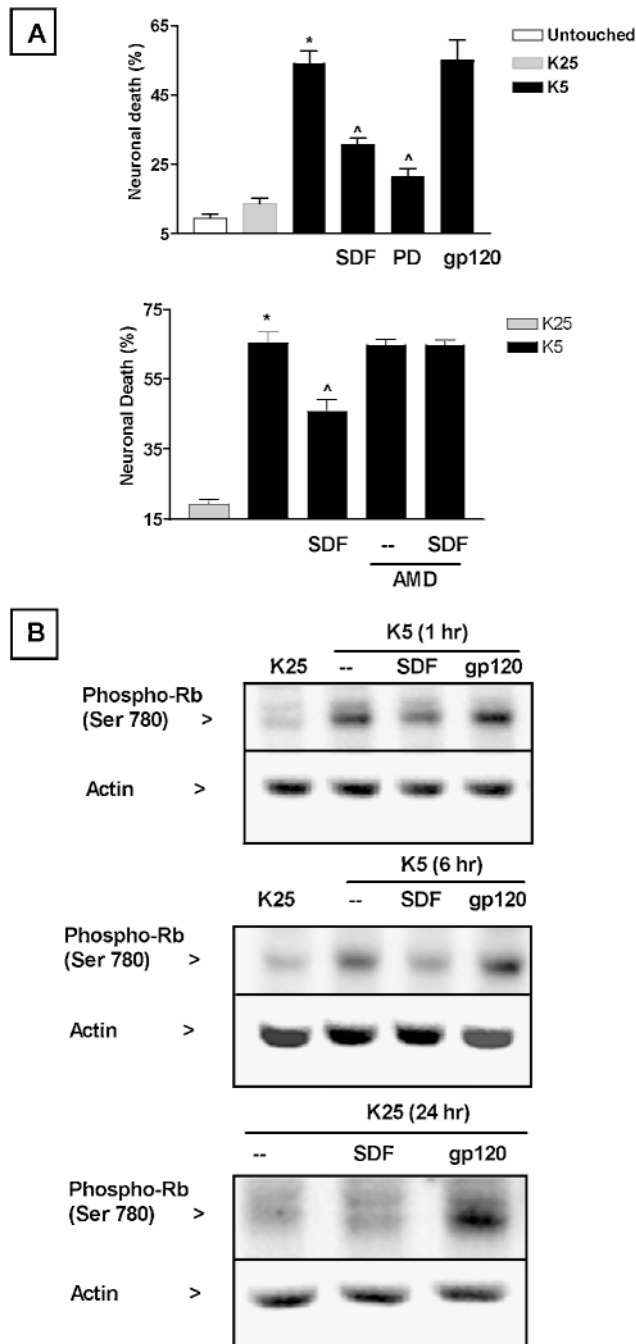


Figure 7 Effect of SDF-1 μ , PD0183812, and gp120 on granule neurons survival and Rb phosphorylation. (A) Neuronal death induced by potassium deprivation is inhibited by SDF-1 μ (20 nM) and by PD0183812 (0.5 μ M), a specific inhibitor of CDK4/6. The CXCR4 antagonist AMD3100 (100 ng/ml) blocked the effect of SDF-1 μ . Data from three different experiments are reported in the graph as mean \pm SE; * P < .05 versus K25 and ^ P < .05 versus K5. (B) gp120_{IIIIB} (200 pM) does not inhibit the increase in Rb phosphorylation induced by potassium deprivation (*top two panels*), whereas increases the level of phosphoRb when neurons are maintained in high potassium (K25, *bottom panel*). Twenty micrograms of proteins were loaded in each lane.

related to the inhibition of Rb phosphorylation and its subsequent degradation. As expected, PD0183812 was also able to reduce phosphorylation and loss of Rb in these neurons (data not shown). Furthermore, preliminary experiments indicate that both SDF-1 μ and PD0183812 reduce the overexpression of cyclin D1 caused by potassium deprivation. In contrast, the HIV envelope protein gp120_{IIIIB} did not inhibit K5-induced apoptosis (Figure 7A) and Rb phosphorylation (Figure 7B)—but rather increased Rb phosphorylation in normal conditions, i.e., K25 mM (Figure 7B, *bottom*), in agreement with previous evidence reporting gp120 neurotoxicity in these cultures (Savio and Levi, 1993). The viral protein seems also able to reduce nuclear expression of Rb in the HOS cells (data not shown).

To further evaluate the effect of gp120 on cell-cycle proteins, we performed similar experiments using primary cultures of hippocampal neurons. We have previously used this *in vitro* model of neuronal cultures to study the intracellular mechanisms implicated in gp120 neurotoxicity as well as the effect of chemokines on major survival pathways (Meucci and Miller, 1996; Meucci *et al*, 1998, 2000). Chemokines, including SDF-1 μ , are able to rescue hippocampal neurons from the apoptosis induced by gp120_{IIIIB} or by withdrawal of glial trophic factors and activate several antiapoptotic pathways, such as inositol triphosphate kinase (PI3-K)/Akt and NF- κ B in these neurons (Meucci and Miller, 1996; Meucci *et al*, 1998, 2000). Our data so far indicate that low levels of phosphorylated Rb are normally found in the nucleus of hippocampal neurons under control conditions (i.e., in the presence of trophic factors), and that SDF-1 μ regulates Rb and E2F-1 in a similar manner to granule neurons (Figure 8). SDF-1 μ also reduced the increase of Rb phosphorylation induced by trophic factors withdrawal, whereas gp120_{IIIIB} was ineffective in these conditions and increased Rb phosphorylation when neurons were treated in their glia-conditioned medium (data not shown). In addition, we found that gp120_{IIIIB} also increased the DNA-binding activity of E2F-1 in hippocampal neurons (Figure 8B), suggesting a novel potential role of cell-cycle proteins in HIV neuropathogenesis. However, whether activation of E2F-1 is required for gp120 neurotoxicity still remains to be determined.

Discussion

Several studies have recently suggested that, beyond their classical involvement in neuroinflammatory and infectious processes, chemokines may play additional roles in the CNS, such as regulation of neuronal survival, differentiation, synaptic transmission, and plasticity (Meucci *et al*, 1998; Asensio and Campbell, 1999; Limatola *et al*, 2000; Bezzi *et al*, 2001; Lu *et al*,

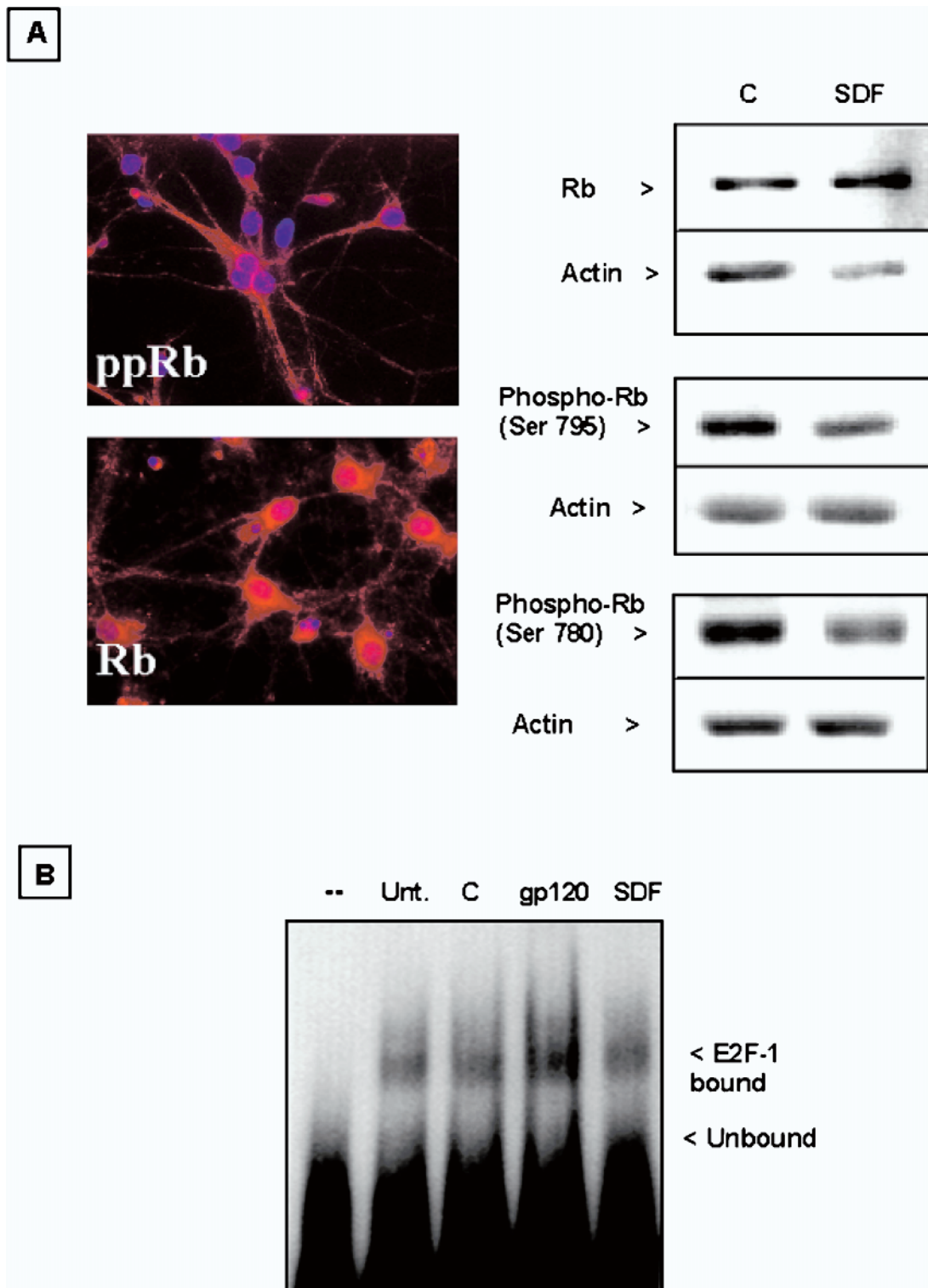


Figure 8 Effect of SDF-1 μ and gp120^{III}B on E2F-1 activity in hippocampal neurons. (A) *Left:* Immunostaining of phosphoRb (Ser795) and Rb (both in red) in control hippocampal neurons (7DIV with glia). Nuclei are counterstained with Hoechst 33342 (*blue*). *Right:* Immunoblots using total cell lysates of hippocampal neurons showed that treatment with SDF-1 μ (20 nM, 1 h, without glia) increased Rb expression and reduces phosphoRb expression. (B) gp120^{III}B increases the DNA-binding activity of E2F-1 as assessed by gel retardation assay. The first lane only contains labeled oligonucleotide. Neurons were treated with SDF-1 μ (20 nM) or gp120 (200 pM) in the absence of the glia feeder layer but in glia-conditioned medium (i.e., original culture medium) for 15 min before extraction of nuclear proteins.

2002; Stumm *et al*, 2002). In analogy to what is observed in hematopoietic cells (Broxmeyer *et al*, 2001), emerging data indicate that SDF-1 μ can regulate proliferation and maturation of developing neurons (Zou *et al*, 1998; Klein *et al*, 2001; Lu *et al*, 2002). For instance, SDF-1 μ contributes to the maintenance of the external granule cell layer (EGL) in the cerebellum by localizing granule cells to the EGL and enhancing the activity of Sonic hedgehog (Klein *et al*, 2001; Zhu *et al*, 2002). One of the effects of SDF-1 μ in primitive hematopoietic cells is the regulation of cell-cycle progression. As recently reported by different groups, SDF-1 μ selectively and reversibly blocks S-phase entry of quiescent erythroid and granulopoietic colony forming cells, and promotes survival of CD34⁺ cells via a PI3-K/Akt-dependent pathway (Vicente-Manzanares *et al*, 1999; Cashman *et al*, 2002; Lataillade *et al*, 2002).

We had previously shown that chemokines—including SDF-1 μ —regulate a number of intracellular pathways in neurons, leading to activation of Akt and other prosurvival factors, such as NF- μ B and CREB (Meucci *et al*, 1998, 2000). Akt also regulates the activity of cell-cycle proteins and transcription factors (i.e., Mdm2, p53) involved in cell-cycle progression (Yamaguchi *et al*, 2001; Gottlieb *et al*, 2002; Sears and Nevins, 2002). Thus, we hypothesized that chemokine receptors may contribute to neuronal survival and differentiation by maintaining neurons in a quiescent state. To test this hypothesis, we have studied the effect of SDF-1 μ on Rb and E2F-1, two major regulators of cell-cycle progression, differentiation, and apoptosis.

The data presented here show that activation of CXCR4 by SDF-1 μ up-regulates Rb expression, as indicated by experiments with rat primary neuronal cultures and human cell lines. SDF-1 μ also affects the phosphorylation state and subcellular localization of Rb in a way that promotes the ability of this protein to block the transcriptional activity of E2F-1. This is supported by the diminished DNA-binding activity of E2F-1 in cells or neurons treated with SDF-1 μ and by the neuroprotective action of SDF-1 μ in cerebellar granule cultures deprived of depolarizing concentrations of extracellular K⁺. In fact, overactivation of E2F-1, resulting from the functional loss of Rb, is primarily responsible for the neuronal death in these experimental conditions (Padmanabhan *et al*, 1999; O'Hare *et al*, 2000). As deregulation of cell-cycle proteins has been implicated in different *in vitro* and *in vivo* models of neurodegeneration, we believe this may represent a novel general mechanism whereby chemokines affect neuronal survival and differentiation, which could be altered in neuropathological conditions. Of note in this regard is the recent discovery that alterations of the expression and localization of cell-cycle proteins (Rb, E2F-1, and p53) have been found in the brain of HIV-demented patients (Jordan-Sciutto *et al*, 2002), suggesting their involvement in HIV neuropathogenesis. Although this

is still a matter of investigation, our preliminary data with the HIV envelope protein gp120_{IIIB} would support this conclusion; in contrast to SDF-1 μ , gp120_{IIIB} does not prevent the Rb loss and phosphorylation induced by K⁺ deprivation in cerebellar granule neurons, whereas it increases E2F-1-binding activity under normal conditions. Interestingly, gp120_{IIIB} does not enhance the neurotoxicity or Rb phosphorylation induced by K5. Thus, the inappropriate activation of the CDK/Rb/E2F-1 pathway could be implicated in HIV-induced neuronal damage. Whether this turns to be the case or not, the ability of SDF—and possibly other chemokines—to promote the neuronal “resting” state provides an interesting setting for studying the multiple (sometimes contradictory) roles of these proteins in the CNS, and could account for a variety of effects exerted by chemokines on developing and mature neurons. However, further investigation is needed to identify the mechanisms of action involved and, more importantly, to verify the relevance of these effects *in vivo*. Our data so far, show that de novo protein synthesis is required for the effect of SDF-1 μ on total Rb, and suggest that SDF-1 μ might reduce the level of phosphoRb either by inhibiting upstream kinase(s) (i.e., CDK4) or by promoting the activity of protein phosphatases. In addition, activation of CXCR4 might also be coupled to mechanisms that regulate Rb translocation to and from the nucleus, as suggested by the experiments with HOS deprived of serum in the presence and in the absence of SDF-1 μ .

In conclusion, our findings indicate that SDF-1 μ is able to affect the expression and activity of Rb and E2F-1 in differentiated neurons, suggesting that the action of the chemokine on neuronal survival may be related to its ability of interfering with the transcription of genes regulated by E2F-1. Alternatively, SDF-1 μ may affect the interaction of Rb and/or E2F-1 with other proteins implicated in cell survival and apoptosis (i.e., Mdm2, p53), an issue we are currently investigating. Thus, the inappropriate activation of CXCR4 by viral-derived proteins—or by SDF-1 μ under pathological conditions—may contribute to neuronal dysfunction in patients with acquired immunodeficiency syndrome (AIDS) and other neuroinflammatory diseases.

Materials and methods

Cell cultures

Neurons: Primary cultures of cerebellar or hippocampal rat neurons were cultured as previously described (Galli *et al*, 1995; Meucci *et al*, 1998, 2000) with minor modifications. Briefly, (a) *cerebellar granule neurons* were obtained from the cerebella of 7- to 8-day-old rats and rapidly collected in a culture dish containing ice-cold dissection medium. After removing the meninges, tissue was minced, incubated with 0.25% trypsin for 15 to 20 min and

dissociated with a flame-narrowed pipette. Cells were plated onto polylysine-coated (100 $\mu\text{g/ml}$) dishes (4 \times 10⁶ cells/60-mm dish) or coverslips (5 \times 10⁵ cells/15-mm coverslip) in basal Eagle medium supplemented with KCl (final concentration 25 mM), 10% fetal calf serum, 2 mM glutamine, and 100 $\mu\text{g/ml}$ gentamycin. Cytosine- μ -D-arabinofuranoside (10 μM) was added to the culture 18 to 20 h after plating to halt proliferation of non-neuronal cells. (b) *Hippocampal neurons* were obtained from the brain of 17- to 18-day-old rat embryos and cultured in serum-free medium using the bilaminar cell culture system, as previously described (Meucci and Miller, 1996; Meucci *et al*, 1998, 2000). 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.

HOS cells: HOS.CXCR4 cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum and 1 $\mu\text{g/ml}$ puromycin. These cells were obtained through the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (from Dr. Nathaniel Landau) and are CD4 negative (Landau and Littman, 1992; Deng *et al*, 1996).

Western blots

After treatment, 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 EDTA, 1 mM DTT, 1% Nonidet P-40, 5 μg each of aprotinin, leupeptin, and pepstatin, 1 mM 4-(2-aminoethyl)benzylsulfonamide 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 (Meucci *et al*, 1998, 2000). 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. μ -Actin expression was assessed to confirm equal sample loading and possible changes in constitutive proteins during apoptosis. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes for immunoblotting. The following primary antibodies were used: anti-Rb (monoclonal G3-245 at 1:4000; Pharmingen), ppRb (Ser795 or Ser780 at 1:2000; Cell Signalling Technology), E2F-1 (either a monoclonal, KH95, or the polyclonal antibody C20 at 1:2000 and 1:500, respectively; Santa Cruz Technology), μ -Actin (polyclonal antibody at 1:5000; Sigma-Aldrich), histone-1 (polyclonal antibody FL-219 at 1:500; Santa Cruz Technology). An image acquisition

and analysis system from BioRad (ChemiDoc System) as well as the U-Scan software were used for detection of chemiluminescent bands and densitometric analysis.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde. The anti-Rb (1:500; Pharmingen) and anti-phosphoRb (Ser795, 1:100; Cell Signalling Technology) were followed by a biotinylated secondary antibody and streptavidin conjugated with Cy2 or Cy3. The antibody against CXCR4 (sc-9046; Santa Cruz Biotechnology) was used at a dilution of 1:50. As this antibody recognizes an internal region of the receptor, cells were previously permeabilized with 0.1% Triton \times 100. Cells were also stained for the neuronal specific marker μ -tubulin III (1:500; Covance, Berkeley, CA). Secondary antibodies conjugated to either AlexaFluor546 (1:1000; Molecular Probes) or Cy2 (1:500; Jackson Immunoresearch Labs) were used for CXCR4 and μ -tubulin III staining, respectively. Nuclear counterstaining was obtained with Hoechst 33342 (3 $\mu\text{g/ml}$). The cells were mounted and observed under an epifluorescent microscope (Olympus IX70) connected to a CCD (Micromax), and images were acquired using the software Metamorph (Universal Imaging) (Meucci *et al*, 2000).

In vitro kinase assay

A GST-Rb fusion protein (amino acids 769 to 921 of mouse Rb; Santa Cruz Biotechnology) was used as substrate for the *in vitro* reactions after binding to glutathione beads. The reaction buffer contained 50 mM Tris, pH 7.4, 10 mM MgCl₂, and 5 mM DTT. GST-Rb (0.5 $\mu\text{g/sample}$) was incubated with cytosolic extracts (10 μg) for 30 min at 30°C in the presence of 5 μCi [μ -³²P]-ATP and 10 μM ATP. Incubations were stopped by placing tubes in ice. Samples were immediately centrifuged (at 4°C) to pellet Rb/beads. Pellets were washed, resuspended in sample buffer, boiled, and separated by electrophoresis. Then they were transferred to a nitrocellulose membrane and visualized by autoradiography.

Electromobility shift assay (EMSA)

Nuclear (HOS) or total (neurons) extracts (1.5 μg) were incubated with 0.25 $\mu\text{g}/\mu\text{l}$ of 3' biotinylated oligonucleotides containing a consensus-binding site for E2F-1 (5'-ATTTAAGTTTCGCGCCCTTCTCAA-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 (Pierce).

Survival assay

Neuronal survival studies were performed as previously described using different fluorescent dyes (fluorescein diacetate, propidium iodide, and Hoechst

33342) (Meucci and Miller, 1996; Meucci *et al*, 1998, 2000). Cerebellar granule neurons were cultured in the presence of serum and high potassium (25 mM) for the first week and then switched to low-potassium (5 mM) medium to induce apoptosis, in the presence or absence of SDF-1 μ and/or other drugs. Neuronal viability was assessed 18 to 24 h later.

Statistical analysis

Data are reported as mean \pm SEM with sample size for each experiment. One-way analysis of variance (ANOVA), followed by Newman-Keuls multiple-comparison procedure, was used for analysis of survival experiments. Paired *t* test was used to compare differences in the band densities of immunoblots.

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- Analysis of immunostaining was performed using the software Metamorph.
- #### Materials
- Unless otherwise specified, tissue culture media are from Life Technology and other general reagents are from Sigma. SDF-1 μ was purchased from R&D System. The lyophilized protein was reconstituted (100 μ g/ml) in 0.1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) and aliquots stored at \pm 20°C. Recombinant HIV-1_{IIIIB} gp120 from Intracel Corporation was prepared and stored as previously described (Meucci *et al*, 1998, 2000). Custom modified oligonucleotides were obtained from Qiagen.
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