Communication

Activation of the HIV-1 Long Terminal Repeat by Nerve Growth Factor*

(Received for publication, June 3, 1997, and in revised form, July 22, 1997)

Juan A. Recio and Ana Aranda†
From the Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, Arturo Duperier 4, 28029 Madrid, Spain

The brain is an important target for the human immunodeficiency virus type 1 (HIV-1). We show here that nerve growth factor (NGF), which induces neuronal differentiation and survival, causes a strong activation of the HIV-1 long terminal repeat by a Ras/Raf-dependent mechanism in PC12 cells. Mutation of the κB sequences contained within the long terminal repeat reduces NGF-mediated stimulation. NGF does not activate NF-κB in PC12 cells, but rather increases binding of other nuclear factors to the κB sequences. Furthermore, a nuclear receptor response element contributes to the stimulatory effect of NGF. The retinoid receptors have been identified as components of the nuclear binding to the nuclear receptor response element in NGF-treated PC12 cells. These results reveal the importance of neurotrophins and nuclear receptor signaling pathways as specific activators of HIV-1 gene expression in neural cells.

Although lymphocytes and macrophages are the prime target cells for the human immunodeficiency virus type 1 (HIV-1), the central nervous system is also an important target for the virus (1). Viral infection of the brain leads to massive neuronal damage resulting in the AIDS (acquired immunodeficiency syndrome) dementia complex. Studies in transgenic mice have revealed that the promoter of neurotropic HIV-1 strains directs gene expression in neurons throughout the nervous system (2). These results imply that neurons possess a specific transcription machinery capable of HIV-1 activation.

Stimulation of HIV-1 gene expression is mediated through viral regulatory sequences located in the long terminal repeat (LTR). The HIV-1 LTR contains two binding sites for NF-κB (nucleotides −104 to −81) (3, 4) in close proximity to three binding sites for the transcription factor Sp1 (nucleotides −77 to −46) (5), and a cooperative interaction between both is required for activation (6). In primary cultures of neurons it has been described the existence of a constitutive NF-κB activity (7) that appears to be responsible for the strong HIV-1 LTR activity found in transient transfection studies (8). Another transcription factor, BETA, present in neurons can also bind the NF-κB sites (8, 9). Recent data have demonstrated the existence of a nuclear receptor response element (NRRE) at sequences −352 to −320 (10). The orphan nuclear receptor COUP-TF (chicken ovalbumin upstream promoter transcription factor) has been described to activate HIV-1 gene expression in neuroblastoma and oligodendrogliaoma cells (11), and the retinoid receptors RAR and RXR have been described to play a role in stimulating HIV-1 expression in other cell types (12).

PC12 pheochromocytoma cells have been extensively used as a neuronal cell model. Upon treatment with nerve growth factor (NGF), PC12 cells acquire certain characteristics of sympathetic neurons (13). Ligand activation of the NGF receptor tyrosine kinase, the trkA proto-oncogene product, leads to activation of Ras and of the ser/threonine kinase Raf, which acts downstream of Ras in the signaling pathway (14–16). The oncogenic form of Ras has been shown to stimulate the HIV-1 LTR in PC12 cells (17). A low-affinity receptor for NGF, p75NTR, which may also play a role in the signal transduction pathway of this neurotrophin, is also expressed at high levels in PC12 cells (18). It has been recently shown that in the absence of TrkA, NGF binding to p75NTR activates NF-κB in Schwann cells (19).

In this work we show that neuronal differentiation induced by NGF in PC12 cells is accompanied by a significant activation of the HIV-1 promoter. This activation requires Ras and Raf and appears to be mediated both by the κB sites and upstream sequences. However, PC12 cells do not contain constitutively active NF-κB complexes, and NGF treatment does not induce NF-κB. Other nuclear proteins, which bind to the NF-κB sites, are likely responsible for basal expression in PC12 cells. These proteins are induced by NGF. The neurotrophin also increases the amount of nuclear factors interacting with the NRRE, and we have identified RAR and RXR as important components of this in PC12 cells. Our results show the importance of specific cellular transcription factors in the regulation of the HIV-1 promoter in different cell types and suggest that neuronal differentiation could play an important role in the activation of HIV-1 gene expression.

EXPERIMENTAL PROCEDURES

Cells—PC12 cells and the PC12 subline M-M17-26 (20), that expresses the dominant negative mutant Ha-Ras<sup>Val12</sup> were cultured in RPMI medium containing 10% donor horse serum and 5% fetal calf serum (21).

Plasmids—A plasmid containing HIV-1 LTR sequences from −453 to −80 fused to luciferase (−453HIV-Luc) has been described previously (17). Additional deletion mutants of the HIV-1 LTR extending to −453, −104, −76, and −28 of the HIV-LTR were constructed by polymerase chain reaction with primers which provide a 5′ XhoI site and a 3′ HindIII site. In the plasmid −453HIVmut-Luc, the GGC motif of both NF-κB binding sites located at −104/−76 has been mutated to TCT (17). Constitutive expression vectors for oncogenic Ha-ras<sup>Val12</sup> or raf, the dominant inhibitory Ha-ras<sup>mut</sup> mutant or a dominant negative raf lacking the catalytic domain under control of the Rous sarcoma virus (RSV) promoter were also used (21). Expression vectors for the truncated receptors RAR419 and RXR445 have been described previously (22).
DNA Transfection—The cells were transiently transfected by the calcium phosphate method with 4 \( \mu \)g of the reporter constructs (21). After overnight incubation with the calcium phosphate precipitate, the cells were incubated with 7 S NGF or TNF-\( \alpha \) and luciferase activity determined. When the cells were cotransfected with the reporter plasmid and expression vectors, the amount of DNA was kept constant by addition of the same amount of an “empty” noncoding vector (RSV-0). Each treatment was performed in triplicate cultures that normally exhibited less than 10–15% variation, and each experiment was repeated at least three times with similar differences in regulated expression.

Gel Mobility Shift Assays—Electrophoretic mobility shift assays (EMSA) were performed using 5 \( \mu \)g of nuclear extracts in a buffer containing approximately 30,000 cpm of \( ^{32}P \)-labeled oligonucleotide, 0.1 \( \mu \)g of poly(dI-dC), 40 mM Hepes, pH = 7, 140 mM NaCl, 4 mM dithiothreitol, 0.01% Nonidet P-40, 100 \( \mu \)g/ml bovine serum albumin, 4% Ficoll. After incubation, the samples were loaded onto a 4 or 6% non-denaturing polyacrylamide gel. The \( \kappa \)-B oligonucleotides used were: 5'-CAAGGGACTTTCCGCTGGGACTTTCCAGG-3' with sequences –104 to –76 of HIV-1 LTR containing the \( \kappa \)-B-binding sites, 5'-CAATCT-TACTTTCCGTCTACTTTCCAGG-3' in which both binding sites are mutated, and 5'-TCGACAGGGGACTTTCCGAGGAAGG-3' containing the NF-\( \kappa \)-B site from the \( \kappa \) light chain enhancer. The B1 sequence, a \( \kappa \)B sequence that binds BETA, but not NF-\( \kappa \)-B, complexes was 5'-GCCGGGAGGCCTGGACTTTCCGAGGAAGG-3'. For supershift assays, antibodies against \( \kappa \)B, \( \kappa \)P, and NGFI-B purchased from Santa Cruz Biotechnology, as well as specific antibodies against RAR\( \beta \) and RXR kindly provided by P. Chambon, were mixed with the nuclear proteins for 15 min at 4°C before addition of the probe. Oligonucleotides containing nuclear receptor binding sites were: 5'-CACGGGGCTAGATATCCACTGACCTTTGGG-3', encompassing the NF-\( \kappa \)-B-related element in the HIV-1 LTR and the palindromic element 5'-AGCTTCAGTCTAGTTACGTACCTGA-3', a strong response element that binds retinoic acid receptors (23).

SouthWestern Assays and Detection of Proteins Bound to the HIV-1 \( \kappa \)-B Sequences—For SouthWestern analysis, 30 \( \mu \)g of nuclear extracts were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After renaturation with guanidine hydrochloride (from 6 to 0.35M), the membrane was blocked with 5% nonfat milk and hybridized with 3.5\( \times \)10\(^{6} \)cpm of labeled probe. The retarded band was excised and the same DNA was subjected to EMSA with 800,000 cpm of labeled probe. The retarded band was excised and the protein bands were detected by silver staining.

RESULTS AND DISCUSSION

As shown in Fig. 1A, incubation of PC12 cells with NGF caused a dose-dependent activation of a luciferase construct containing the HIV-1 LTR (–453HIV-Luc). A half-maximal luciferase induction was obtained with approximately 20 ng/ml NGF, and this response was almost maximal at 50 ng/ml. The effect was maximal after 16 h of incubation with NGF and was maintained for at least 48 h (data not shown). However, UV light and TNF-\( \alpha \), which are potent inducers of the HIV-1 LTR in other systems, had a weak effect (less than 2-fold induction) in PC12 cells, although both increased significantly the response to NGF (Fig. 1B). These results indicate that NGF functions as a potent HIV-1 transcriptional activator in PC12 cells.

A series of 5'-deletion constructs derived from –453HIV-Luc, as well as a construct (–453mutHIV-Luc) in which both \( \kappa \)B sites were mutated, were used to define the elements responsible for basal and NGF-stimulated HIV-1 expression in PC12 cells. It was shown that mutation of the \( \kappa \)B sites very significantly decreased basal luciferase levels, showing that these elements are important for HIV-1 LTR activity in PC12 cells. However, a significant response to NGF was still observed with the mutated LTR. When expressed as fold induction over the corresponding basal levels, the response to NGF decreased from 7-fold in the wild type promoter to approximately 3-fold in the \( \kappa \)B mutant. These results suggest that additional elements to the \( \kappa \)B sites contribute to HIV-1 LTR activation by NGF. Deletion of the LTR from –453 to –104 did not alter basal luciferase activity. However, the response to the neurotrophin decreased significantly, showing that this promoter fragment is also involved in the regulation by NGF. This fragment is known to contain and Ets-1 binding site (24) and a NRRE (10). A 2-fold increase was obtained after 16 h of treatment with increasing concentrations of NGF. B, the cells were exposed to 40 J/m² of ultraviolet light (U.V.) 2 h after transfection or were incubated with 10 ng/ml TNF-\( \alpha \) in the presence or absence of 30 ng/ml NGF for 16 h. C, elements of the HIV-1 LTR-mediating regulation by NGF. The positions of the arrows indicate the deletion mutants of the HIV-1 LTR which were used. D, constructs extending to –453, –104, –76, and –28 of the HIV-1 LTR were transfected into PC12 cells. In the plasmid –453HIVmut-Luc, the GGG motif of both NF-\( \kappa \)-B binding sites located at –104/–76 has been mutated to TTC. This mutation abolishes the binding of NF-\( \kappa \)-B (5). Luciferase activity was determined in untreated cells and in cells treated with 30 ng/ml NGF for 16 h. All luciferase data presented are mean ± S.D.

Ras and Raf proteins are involved in the responses to NGF in PC12 cells (14–16). Expression of either oncogenic ras or raf dramatically enhanced the activity of the –453HIV-Luc construct in PC12 cells (Fig. 2A). In the presence of the activated oncogenes the signaling pathway was maximally stimulated and NGF did not induce a further increase. It has been reported in NIH-3T3 cells that the ras and raf oncogenes activate HIV-1 LTR expression through the \( \kappa \)B binding sites (25). However, the mutated promoter was still significantly activated by Ras and Raf in PC12 cells, showing that, as it occurs for NGF, additional sequences must be responsible for HIV-1 activation in this cell type.

As shown in Fig. 2B, the HIV-1 LTR was not stimulated by NGF in M-M17-26 cells, a PC12 subclone that constitutively expresses the dominant inhibitory ras\( ^{\text{AVain}} \) mutant (20), showing that endogenous Ras is required for the effect of NGF.
These results were confirmed in parental PC12 cells transiently transfected with ras<sup>dom1</sup>. Fig. 2C shows that transfection with dominant inhibitory ras blocked NGF induction of luciferase activity. Expression of a negative inhibitory raf mutant also inhibited the stimulation of luciferase activity, showing that endogenous Raf proteins are also required for the regulation of the HIV-1 LTR by NGF.

To examine whether activation of NF-κB was involved in the stimulation of the HIV-1 LTR by NGF in PC12 cells, nuclear proteins from untreated cells and from cells treated with NGF were subjected to EMSA. Fig. 3A shows that NGF increased the abundance of proteins bound to sequences −104/−76 of the HIV-1 LTR. This effect was already observed after 4 h of NGF treatment and was maintained for at least 24 h (lanes 1–4). However, supershift experiments using specific antibodies against p65 and p50 did not show the presence of these proteins in the complexes (A, lanes 7–9). This absence was confirmed using the consensus NF-κB binding site of the murine κ-light chain enhancer. As shown in A, lane 10, nuclear extracts from PC12 cells produced the appearance of several retarded bands with this element. The amount of complexes with the slowest mobility was increased after NGF treatment, with a maximal effect again found at 4 h of incubation (lanes 11–13). As a control, the effect of NGF was compared with that caused by TNFα. Lane 14 shows that incubation for 30 min with TNFα activates NF-κB in PC12 cells. The identity of these complexes was confirmed by supershift analysis using antibodies against p50 (lane 19) and p65 (lane 20). It can be observed that the mobility of the p50 dimers and p50/p65 heterodimers is different from that of the complex whose intensity is augmented by NGF. These results show that PC12 cells do not contain significant amounts of constitutive NF-κB in the nucleus and that, in contrast to the results obtained in Schwann cells (19), NGF binding to p75<sup>NeT</sup> does not activate NF-κB in PC12 cells. BETAT, a brain-specific zinc finger transcription factor, can also bind to κB sites in neuronal cells (8, 9). An oligonucleotide (B1) that recognizes BETAT but does not bind neuronal NF-κB was also used. Lane 21–23 show that nucleic from PC12 cells contain factors that bind strongly to B1 and that treatment with NGF weakly increases this binding. Although our data do not demonstrate whether the retarded band contains BETAT or other still unidentified factors, these results confirm that factors different from NF-κB are constitutively present in PC12 cell nuclei.

As shown in B, SDS-polyacrylamide gel electrophoresis of the factors bound to the HIV-1 sequences −104/−76, demonstrated the presence of different proteins with varying sizes (from less than 40 to 115 kDa). C shows that a major band running approximately with the 63-kDa protein standard is detected by Western analysis with the HIV-1 probe. This species, whose expression is increased in NGF-treated PC12 cells, corresponded in size with the most prominent bands detected in B, which had an apparent molecular mass between 58 and 72 kDa.

Taken together the results shown in Fig. 3 suggest that several κB-binding proteins could be involved in basal activity of the HIV-1 LTR in PC12 cells and that the level of these factors appear to increase after NGF treatment.

The data shown in Fig. 1D demonstrated that sequences upstream of the κB sites also significantly contribute to the response to NGF. These sequences contain Ets sites that appear to be important for activation of the HIV-1 enhancer in T cells (24). However, Ets proteins do not appear to be involved in HIV-1 stimulation in PC12 cells (data not shown).

Other potentially important sequence for HIV-1 expression is the NRRE at −356/−320. This element binds multiple members of the nuclear receptor superfamily (10–12), including orphan receptors and retinoid receptors (RAR and RXR). Recently, retinoids have been shown to activate several viruses, including HIV-1 (12). To analyze a possible role of the retinoid receptors on the transactivation of the HIV-1 LTR by NGF, PC12 cells were transfected with expression vectors for tran-
These results show that the retinoid receptors are involved in the regulation of HIV-1 expression in PC12 cells, demonstrating a cross-coupling of the signal transduction of NGF with nuclear receptor pathways. Orphan receptors also appear to play a role in HIV-1 activation in neuronal and glial cells (12) reinforcing the importance of this family of transcription factors as modulators of virus expression in brain and immune cells.

In conclusion, our data suggest that the state of differentiation and the availability of neurotrophic factors may dictate the efficiency of the HIV-1 LTR function in neuronal cells. Because the LTR represents the main regulatory region that determines HIV-1 transcription and replication, these results suggest that the transduction of the NGF signal and its cross-coupling with nuclear receptor pathways may play a role in the infectious process of brain cells. The essential role of neurotrophins in the differentiation, survival, and function of neural cells is well established, and our data show that HIV-1 appears to opportunistically use an essential stimulator of these cells to favor its own expression. Interestingly, it has been very recently suggested that NGF may play an important role in the expression of the neuropathology caused in rat brain by administration of the HIV-1 viral protein gp120 (27). At this point, further experiments are required to demonstrate that NGF can indeed play a role in virus replication and in the development of neurological damage in AIDS patients.

Acknowledgments—We thank Drs. M. Karin, P. Chambon, and H. Stunnenberg for plasmids and antibodies used in this study.

REFERENCES

1. Dubois-Dalq, M., Altmeier, R., Chiron, M. & Wilt, S. (1995) Curr. Opin. Neurobiol. 5, 647–655
2. Corboy, J. R., Buzy, J. M., Zink, M. C. & Clements, J. E. (1992) Science 258, 1804–1808
3. Kawakami, K., Scheidereit, C. & Roeder, R. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4700–4704
4. Nabel, G. & Baltimore, D. (1987) Nature 326, 711–713
5. Jones, K. A., Kadonaga, T. J., Luciw, P. A. & Tjian, R. (1986) Science 223, 755–759
6. Perkins, N. D., Edwards, N. L., Duckett, C. S., Agranoff, A. B., Schmidt, R. M. & Nabel, G. J. (1990) EMBO J. 9, 3551–3558
7. Kaltschmidt, C., Kaltschmidt, B., Neumann, H., Wekerle, H. & Baeuerle, P. A. (1994) Mol. Cell. Biol. 14, 3981–3992
8. Rattner, A., Konter, M., Walker, M. D. & Citri, J. (1993) EMBO J. 12, 2461–2467
9. Korner, M., Rattner, A., Mauxion, F., Sen, R. & Citri, Y. (1989) Neuron 3, 563–572
10. Ladas, J. A. (1994) J. Biol. Chem. 269, 5944–5951
11. Sawaya, B. E., Rohr, O., Aunis, D. & Schaeffer, E. (1996) J. Biol. Chem. 271, 23572–23576
12. Lee, M.-O., Hobo, P. D., Zhang, X.-k., Dawson, M. I. & Pfahl, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5632–5636
13. Greene, L. A. & Tischler, A. (1982) Adv. Cell. Neurobiol. 5, 737–743
14. Wood, W. & Sarnecki, C., Roberts, T. M. & Blenis, J. (1992) Cell 68, 1031–1050
15. D’Arcangelo, G. & Hagedoorn, S. (1993) Mol. Cell. Biol. 13, 3146–3155
16. Jaiswal, R. K., Moodie, S. A., Wolfman, A. & Tischler, A. (1992) Mol. Biol. Rep. 14, 6941–6953
17. Devery, V., Rossete, C. DiDonato, J. A. & Karin, M. (1993) Science 261, 1442–1445
18. Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A. & Shooter, E. M. (1987) Nature 325, 593–597
19. Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Böhm, Matthaei, R., Rau, S. & Barde, Y.-A. (1996) Science 272, 342–345
20. Szeberényi, J., Cai, H. & Cooper, G. M. (1996) Mol. Cell. Biol. 10, 5324–5332
21. Cosgaya, J. M. & Arnada, A. (1996) Oncogene 12, 2651–2660
22. Valarezo, R., Holt, H., García Jiménez, C., Barettoni, D. & Stunnenberg, H. G. (1994) Genes & Dev. 8, 3968–3979
23. Uneson, K., Murakami, K. K., Thompson, C. C. & Evans, R. M. (1991) Cell 65, 1255–1266
24. Sheridan, P. L., Sheline, C. T., Cannon, K., Pazin, M. J., Kadonaga, J. T. & Jones, C. A. (1995) Genes & Dev. 9, 2090–2104
25. Bruder, J. T., Geidecker, G., Tan, T.-H., Weske, J. C., Derse, D. & Rapoport, U. R. (1993) Nucleic Acids Res. 21, 3129–3133
26. Napol, S., Saunders, M., Kastner, P., Durand, B., Nakashita, H. & Chambon, P. (1993) EMBO J. 12, 2349–2356
27. Bagetta, G., Corasaniti, M. T., Aloe, L., Berlischchi, L., Costa, N., Finazzi-Agro, A. & Nesti, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 926–933