Extracellular Human Immunodeficiency Virus Type-1 Tat Protein Activates Phosphatidylinositol 3-Kinase in PC12 Neuronal Cells*

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We have here investigated the effect of the regulatory Tat protein of the human immunodeficiency virus type 1 (HIV-1) on the PI 3-kinase catalytic activity in PC12 rat pheochromocytoma cells. After as early as 1 min from the beginning of the treatment with recombinant HIV-1 Tat protein, a significant increase in the tyrosine phosphorylation levels of the p85 regulatory subunit of PI 3-kinase was noticed in 48 h serum-starved PC12 cells. Moreover, the addition of Tat to PC12 cells induced a great increase in PI 3-kinase immunoprecipitated with an anti-phosphotyrosine antibody with a peak of activation (19-fold increase with respect to the basal levels) after a 15-min treatment. This increase in PI 3-kinase activity was significantly higher in PC12 cell cultures supplemented with Tat protein than in cultures stimulated by 100 ng/ml nerve growth factor (NGF; 8-fold increase with respect to the basal levels). Further experiments showed that Tat protein was able to specifically activate PI 3-kinase at picomolar concentrations. In fact: (i) maximal activation of PI 3-kinase was observed at concentrations as low as 1 ng/ml and was specifically blocked by anti-Tat neutralizing antibody; (ii) a Tat-dependent activation was also observed in experiments in which PI 3-kinase activity was evaluated in either anti-Tyr(P) or anti-phosphotyrosine immunoprecipitates; (iii) 100 nM wortmannin completely blocked the Tat-mediated increase in PI 3-kinase activity both in vitro and in vivo. Our data strongly support the concept that extracellular Tat acts as a cell stimulator, inducing intracellular signal transduction in uninfected cells.

The regulatory HIV-1 Tat protein is a small (86–104 amino acid) protein encoded by two exons. Tat can be divided into five distinct domains called N-terminal, cysteine-rich, core, basic, and C-terminal sequences. While the cysteine-rich region is responsible for the formation of intramolecular disulfide bonds, the basic region contains nuclear localization signals and the binding site for the transactivation response element RNA, located at the 5' end of all viral mRNAs (1).

The best characterized biological effect of Tat is the transactivation of HIV-1 genome, which takes place after specific interactions of Tat protein with the stem-loop transactivation response element sequence of the long terminal repeat viral RNA at the nuclear level. In addition to acting intracellularly, Tat protein shows the unique property, for a viral protein, to be actively released in culture by HIV-1-infected and tat-transfected cells (2) and displays pleiotropic activities on the survival, growth, and function of various cell types by acting as a viral growth factor (2–6). In particular, we have previously demonstrated that picomolar concentrations of recombinant Tat (0.1–10 ng/ml) promote the survival of PC12 rat pheochromocytoma cells under serum-free culture conditions (3). Additionally, PC12 cells stably transfected with tat cDNA show an increased resistance to apoptotic death (4). However, the addition in culture of anti-Tat neutralizing antibodies to these tat-transfected cells completely blocked their increased resistance to apoptosis (7), indicating that Tat released extracellularly by tat-transfected cells is required to promote cell survival.

Although experimental evidence for the existence of high affinity receptor for Tat is still lacking, our data suggested that recombinant Tat might act by inducing intracellular signal transduction. Since it has been recently shown that NGF protects PC12 cells from apoptosis by activating PI 3-kinase (8), we here investigated whether extracellular Tat was also able to activate PI 3-kinase in PC12 cells.

**EXPERIMENTAL PROCEDURES**

Viral Proteins, Growth Factors, Antibodies, and Chemicals—Full-length recombinant Tat and p24 proteins of human immunodeficiency virus type 1 (HIV-1), produced in *Escherichia coli* (American Bio-Technologies, Inc., Cambridge, MA), were stored at −70 °C with 0.1 mM dithiothreitol to prevent oxidation and resuspended in PBS containing 0.1% bovine serum albumin (Sigma) before use. Murine NGF was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). 0.5PiATP (specific activity, 3000 Ci/mmol) was purchased from DuPont NEN. Anti-Tyr(P) antibody, used in both Western blot and immunoprecipitation experiments, was a murine monoclonal antibody (mAb) generated using a phosphotyramine as an immunogen and was a generous gift from Dr. Thomas Roberts (Dana Farber Hospital, Boston, MA). Antibody to the 85-kDa subunit of PI 3-kinase (anti-p85) was raised in rabbits by Dr. Brian Schaffhausen (Tufts University, Boston, MA) and is commercially available from Upstate Biotechnology Inc. Anti-Tat and anti-p24 neutralizing monoclonal antibodies were purchased from American Bio-Technologies, Inc. Wortmannin, purchased from Sigma, was dissolved in dimethyl sulfoxide at 10 mM, stored at −20 °C in the dark, and diluted with distilled water immediately before use.

Cell Cultures and Growth Factor Stimulation—Rat pheochromocytoma PC12 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal calf serum (all from Seromed, Biochrom KG, Berlin, Germany). Before stimulation...
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with Tat, p24, or NGF, the medium was replaced, and PC12 cells were cultured for 48 h with Eagle’s modified Eagle’s medium alone in the absence of serum. The cells were then stimulated by adding 100 ng/ml NGF or various concentrations of HIV-1 Tat or p24 proteins to the culture medium.

Immuno precipitation and Western Blotting Experiments—PC12 cells were lysed in lysis buffer (137 mM NaCl, 20 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol (v/v), 1% Nonidet P-40, 0.1 mM vanadate, and 1% PMSF, and debris were removed by centrifugation. PI 3-kinase was immunoprecipitated with either 1 µl of anti-p85 or 3 µl of anti-Tyr(P) (3.5 mg/ml) for 3 h at 4 °C. The immunoprecipitates were collected with protein A-Sepharose, washed three times in PBS, 1% Nonidet P-40; twice in 0.1 M Tris, 0.5 M LiCl; and twice in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5). All wash solutions contained 100 mM vanadate. The samples were then boiled for 5 min and electrophoresed on 7.5% polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane. After incubation with TBS containing 3% bovine serum albumin at 37 °C for 1 h, the membranes were incubated with a nitrocellulose membrane. After incubation with TBS containing 3%

Assay of PI 3-Kinase Activity—HIV-1 Tat or p24 proteins or NGF were added to intact cells for the indicated times at 37 °C. Cells were washed twice with ice-cold buffer A (137 mM NaCl, 20 mM Tris, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM vanadate, pH 7.5), and lysed in lysis buffer (buffer A plus 10% glycerol (v/v), 1% Nonidet P-40, and 1% PMSF). The lysates were vortexed and centrifuged at 14,000 rpm. The cleared supernatant was transferred to a fresh microcentrifuge tube, and incubated with anti-Tyr(P) (3.5 mg/ml lysate) or anti-p85 (2 µg/ml lysate) antibody for 2 h at 4 °C. Protein A-Sepharose (4 mg/ml lysate) was then added for 1 h at 4 °C to the lysates. The immunoprecipitates were washed three times in PBS, 1% Nonidet P-40; twice in 0.1 M Tris, 0.5 M LiCl; and twice in TNE. All wash solutions contained 100 µM vanadate.

The PI 3-kinase assay on the immunoprecipitate was performed by adding sonicated PI after 10 min of PI-HEPES, 1 mM EDTA, pH 7.5; 0.5 mg/ml final concentration) and [γ-32P]ATP (5–10 nCi/ml) to the immunoprecipitates for 10 min at room temperature. The reaction was stopped by the addition of 80 µl of HCl (2 M) and 160 µM of methanol:chloroform (1:1 mixture). The lipid-containing organic phase was resolved on a precoated thin-layer chromatography plates (Silica Gel 60, Merck) developed in chloroform:methanol:water:ammonium hydroxide (60:40:3:2). Radiolabeled spots, identified by autoradiography on Kodak X-Omat S films, were excised and quantified by scintillation counting.

HPLC Analysis—Separation of deacylated phosphoinositides was carried out on a Paratissphere SAX, 120 × 4.6-mm column, equipped with a Six precolumn insert following a previous described procedure (9). The chromatographic apparatus used was composed of a 9012 ternary gradient pump and a 2550 UV-visible detector, both from Varian (Palo Alto, CA). To verify that the 32P labels was present on the 3-OH instead of the 4-OH, radiolabeled phosphoinositides were scraped off the TLC plate, the silica was incubated at 53 °C for 90 min with 0.6 ml of 40% methylene, cooled, transferred to a new tube, and dried by vacuum. The samples were transferred into 0.6 ml of water and washed twice with 1 ml of n-butanol/petroleum ether/ethyl formate (20:4:1 by volume), after which the aqueous layer was dried under vacuum and stored at −20 °C. To achieve glycerophosphoinositol separation, the sample was reconstituted in water, filtered on a 0.2-µm filter, and injected into the HPLC apparatus previously equilibrated in water. After 1 min, a gradient between 0 and 15% of 1 x (NH₄)₂HPO₄ was developed over a time of 50 min.

RESULTS

Stimulation of PI 3-Kinase Activity by HIV-1 Tat in PC12 Cells—Since previous studies (10) demonstrated that the exposure of PC12 cells to NGF induces an increase in anti-Tyr(P)-immunoprecipitable PI 3-kinase activity, we first compared the ability of Tat protein and NGF to stimulate PI 3-kinase activity in PC12 cells.

Fig. 1A shows PI 3-kinase activity immunoprecipitated using anti-Tyr(P) antibody at different time points after addition of Tat or NGF. Both Tat protein (1 ng/ml) and NGF (100 ng/ml) potently stimulated PI 3-kinase activity. Both proteins showed significant stimulation within 1 min, but Tat gave rise to a more prolonged stimulation, peaking at 15 min. On the other hand, HIV-1 p24 protein at concentrations of 1–100 ng/ml was unable to stimulate PI 3-kinase activity over the base line (data not shown). Activation of PI 3-kinase was further investigated using an antibody against the 85-kDa subunit of the p85/p110 type PI 3-kinase. As shown in Fig. 1B and C, Tat (1 ng/ml) stimulated the anti-p85 precipitable PI 3-kinase activity, and maximal stimulation was observed at 15 min after addition of Tat. The product of the lipid kinase activity was analyzed by deacylation and HPLC separation and shown to be phosphatidylinositol 3-phosphate (data not shown).

Since it has been observed that Tat displays different and sometimes opposite effects on cell survival depending on the concentrations used in various studies (2, 4–6), in the next group of experiments various concentrations of Tat protein in
PC12 rat pheochromocytoma cells. PI 3-kinase phosphorylates HIV-1 Tat protein promotes the activation of PI 3-kinase in which also suppressed the basal catalytic activity still present inng/ml Tat protein. The Tat-mediated elevation of PI3-kinase was observed in various cell types when used at nanomolar concentrations (11). Metabolite Wt has been shown to inhibit PI3-kinase activity in the range 0.1–1000 ng/ml were added in culture to 48-h serum-starved PC12 cells for 10 min before performing the PI3-kinase assay on anti-p85 immunoprecipitates. A concentration-dependent activation was observed, with maximum stimulation occurring at 1 ng/ml Tat. Higher concentrations of Tat were less effective (data not shown).

In Vivo and in Vitro Suppression of the Tat-mediated Activation of PI 3-Kinase by WT and Anti-Tat mAb—The fungal metabolite WT has been shown to inhibit PI3-kinase activity in various cell types when used at nanomolar concentrations (11). 48 h serum-starved PC12 cells were pretreated for 20 min with 100 nM WT and then supplemented for additional 10 min with 1 ng/ml Tat protein. The Tat-mediated elevation of PI3-kinase activity was completely blocked by a brief exposure to WT, which also suppressed the basal catalytic activity still present in serum-starved PC12 cells left untreated. Pretreatment of 1 ng/ml Tat with 1 μg/ml anti-Tat but not anti-p24 mAb for 30 min also completely abolished the Tat-induced increase in PI3-kinase activity. In addition, the in vitro treatment with WT completely blocked PI3-kinase activity and Nonidet P-40, an in vitro inhibitor of PI3-kinase but not of PI4-kinase or other lipid kinases (12), suppressed the catalytic activity associated with p85 immunoprecipitates (data not shown).

Tat Stimulates Phosphorylation of Proteins on Tyr—Since an anti-Tyr(P) antibody precipitated PI3-kinase activity from Tat-stimulated cells, we investigated the effect of Tat on protein-Tyr phosphorylation in PC12 cells. Increased Tyr phosphorylation was observed on at least five proteins of molecular masses between 200 and 66 kDa (Fig. 2, left panel). In next experiments, p85 immunoprecipitates were blotted with anti-Tyr(P) to determine whether Tat stimulates direct phosphorylation of this protein. A small increase in a doublet band at 85 kDa was observed in anti-Tyr(P) blotting (Fig. 2, right panel), but this increase peaked at 1 min, while PI3-kinase activity peaked much later (Fig. 1).

**FIG. 2.** Tat protein induces the tyrosine phosphorylation of p85. Left panel, 48-h serum-starved PC12 cells were either left untreated (lane 1) or treated with 1 ng/ml recombinant Tat for 1 (lane 2), 10 (lane 3), or 30 (lane 4) min. Whole cell homogenates were then subjected to Western blot analysis with an anti-Tyr(P) mAb. Right panel, 48-h serum-starved PC12 cells were treated with 10% fetal calf serum (serum +, positive control), left untreated (serum −, negative control), or treated for 1–30 min with Tat protein (1 ng/ml). Cell lysates were immunoprecipitated (IP) with anti-p85 polyclonal antibody, and then the same filter was first revealed in Western blotting (WB) with anti-Tyr(P) (A), then stripped and reprobed with anti-p85 (B) antibody. A representative of five separate experiments is shown.

The D-3 position of the inositol ring of phosphoinositols and produces D-3 phosphoinositides (phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate). Increasing experimental evidence indicates that D-3 phosphorylated inositides represent a class of second messenger molecules, which can activate specific protein kinase C isomers and Akt-Ser/Thr kinase, interact with SH2 domains and induce cytoskeletal rearrangement (13–15). PI3-kinase is often found in cellular complexes with ligand-activated growth factor receptors and oncogene protein-tyrosine kinases.

Activation of the mammalian PI3-kinase complex can play a critical role in transducing growth factor responses. The lipid kinase complex has been implicated in a variety of cell functions, including mitogenesis, cell transformation, etc. (16). Moreover, a role for PI3-kinase in the promotion of neuronal cell survival has been recently demonstrated by Yao and Cooper (8). These authors have shown that NGF counteracts the apoptotic cell death program induced by serum withdrawal in PC12 cells by activating PI3-kinase. In this respect, it is particularly noteworthy that we have previously demonstrated that recombinant Tat is also able to protect PC12 cells from apoptosis induced by serum withdrawal (4).

We have shown here that 1 ng/ml Tat potently stimulates PI3-kinase, reaching values of activation (approximately 20-fold increase with respect to the basal level) significantly higher than those observed in PC12 cell cultures supplemented with 100 ng/ml NGF (8-fold increase with respect to the basal level). Therefore, PI3-kinase may represent a common intracellular target for both NGF and Tat protein.

**DISCUSSION**

In this report, we have demonstrated that extracellular HIV-1 Tat protein promotes the activation of PI3-kinase in PC12 rat pheochromocytoma cells. PI3-kinase phosphorylates...
possibility that Tat must be internalized in order to activate PI 3-kinase.

In conclusion, this is the first report demonstrating that HIV-1 Tat protein, which is actively released by infected cells, is able to generate intracellular signals activating PI 3-kinase.

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REFERENCES
1. Jones, K. A., and Peterlin, M. B. (1994) Annu. Rev. Biochem. 63, 717–720
2. Ensoli, B., Barillari, G., Zaki Salahuddin, S., Gallo, R. C., and Wong-Staal, F. (1990) Nature 345, 84–86
3. Milani, D., Zauli, G., Neri, L. M., Marchisio, N., Previati, M., and Capitani, S. (1993) J. Gen. Virol. 74, 2587–2594
4. Zauli, G., Gibellini, D., Milani, D., Mazzoni, M., Borgatti, P., La Placa, M., and Capitani, S. (1993) Cancer Res. 53, 4481–4485
5. Vescidi, R. P., Mayor, K., Lederman, H. M., and Frankel, J. D. (1989) Science 246, 1606–1608
6. Li, C. J., Friedman, D. J., Wang, C., Metelev, V., and Pardee, A. B. (1995a) Science 268, 229–2318
7. Zauli, G., La Placa, M., Vignoli, M., Re, M. C., Gibellini, D., Furlini, G., Milani, D., Marchisio, M., Mazzoni, M., and Capitani, S. (1995) J. AIDS 10, 306–316
8. Yao, R., and Cooper, M. G. (1995) Science 267, 2003–2006
9. Auger, K. R., Serunian, L. A., Soltsof, S. P., Libby, P., and Cantley, L. C. (1989) Cell 57, 167–175
10. Soltsof, S. P., Rabin, S. L., Cantley, L. C., and Kaplan, D. R. (1992) J. Biol. Chem. 267, 17472–17477
11. Okada, T., Sakuma, I., Fukui, Y., Hazeki, O., and Ui, M. (1994) J. Biol. Chem. 269, 3563–3567
12. Fukui, Y., and Hanafusa, H. (1989) Mol. Cell. Biol. 9, 1651–1658
13. Nakashima, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
14. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Cell 81, 727–736
15. Kapeller, R., Chakrabarti, R., Cantley, L. C., Fay, F., and Corvera, S. (1993) Mol. Cell. Biol. 13, 6052–6063
16. Kapeller, R., and Cantley, L. C. (1994) BioEssays 16, 565–576
17. Westendorp, M. O., Frank, R., Ochsenbauer, C., Stricker, K., Dhein, J., Walczak, H., Debatin, K. M., and Krammer, P. H. (1995) Nature 375, 497–500
18. Magnuson, D. S., Knudsen, B. E., Geiger, J. D., Brownstone, R. M., and Nath, A. (1995) Ann. Neural. 37, 373–380
19. Zauli, G., Davis, B. R., Re, M. C., Visani, G., Furlini, G., and La Placa, M. (1992) Blood 80, 3036–3043
20. Buonaguro, L., Barillari, G., Chang, H. K., Bohan, C. A., Kao, V., Morgan, R., Gallo, R. C., and Ensoli, B. (1992) J. Virol. 66, 7159–7167
21. Frankel, A. D., and Pabo, C. O. (1988) Cell 55, 1189–1193
22. Mann, D. A., and Frankel, A. D. (1991) EMBO J. 10, 1733–1739
23. Brake, D. A., Debouck, C., and Biesecker, G. (1990) J. Cell. Biol. 111, 1275–1281
24. Barillari, G., Gendelman, R., Gallo, R. C., and Ensoli, B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7941–7945
25. Vogel, B. E., Lee, S. J., Hildebrand, A., Craig, W., Pierschbacher, M. D., Wong-Staal, F., and Ruoslahti, E. (1993) J. Cell. Biol. 121, 461–468
26. Weeks, B. S., Desai, K., Loewenstein, P. M., Klotman, M. E., Klotman, P., Green, M., and Kleinman, H. K. (1995) J. Biol. Chem. 268, 5279–5284
27. Zachary, I., and Rozengurt, E. (1992) Cell 71, 891–894
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