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Tat Protein from HIV-1 Activates MAP Kinase in Granular Neurons and Glial Cells from Rat Cerebellum

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We have investigated the effect of extracellularly applied Tat protein of the human immunodeficiency virus type 1 (HIV-1) on tyrosine phosphorylation processes, which represent a major signal transduction pathway of cells of the central nervous system. Primary cultures of rat cerebellar astrocytes or granule cells were incubated with synthetic Tat (10 ng/ml) for various periods of time and analyzed for their phosphotyrosine content by Western blotting. In both types of cultures Tat was able to induce the phosphorylation of mitogen-activated protein kinase (MAP kinase) on tyrosine residues, although with different kinetics and isoform specificity. In addition, in neuronal cells, but not in astrocytes, Tat increased the phosphotyrosine content of Shc, a protein involved in signal transduction downstream of receptor tyrosine kinase activation. This study shows that Tat applied extracellularly is able to induce the generation of intracellular signals in neuronal as well as glial cells. © 1997 Academic Press

The AIDS/dementia complex is a late complication of HIV infection whose importance is increasing with the development of therapeutic strategies that allow long-term survival of AIDS patients. The pathogenesis of the AIDS/dementia complex has not been clarified yet. In the absence of a detectable infection of neuronal cells [1], the neurotoxic and neurodegenerative consequences of HIV infection have been hypothesized to be brought about via indirect mechanisms by multiple factors, including excessive release of neurotransmitters and/or excitotoxic metabolites, alterations in the glial trophic support, release of inflammatory cytokines and viral toxins [2].

The regulatory HIV-1 transactivator protein, known as Tat, beside being able to transactivate the viral genome, has the unusual property of being released by infected cells and taken up by neighboring cells [3,4]. In non-neuronal cells in culture, extracellularly applied Tat has been shown to influence cell proliferation and metabolism [see, e.g., 4-6]. We have now investigated whether this protein is able to modulate signal transduction in cells of the central nervous system.

EXPERIMENTAL PROCEDURES

Materials. Full-length chemically synthetized Tat was a kind gift of Dr. G. Fassina (Tecnogen, Italy). The activity of the synthetic protein has been described elsewhere [7]. The following reagents were purchased from the indicated sources: anti phosphotyrosine antibody, UBI (Lake Placid NY); anti-MAP kinase and anti-Shc antibodies, Santa Cruz (Santa Cruz CA); peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies, Bio-Rad (Hercules CA); anti-glial fibrillar acidic protein (GFAP) antibody and chemiluminescence detection system, Amersham (Buckinghamshire, England); BCA Protein Assay Reagent, Pierce (Rockford IL); anti-neurofilament antibody, Sigma (St. Louis MO); goat anti-mouse FITC-conjugated and goat anti-rabbit Texas Red-conjugated antibodies, Jackson (West Grove PA); Sprague Dawley rats, Charles River (Calco, Italy).

Cell cultures and Tat stimulation. Cerebella of 5-day-old Sprague Dawley rats were dissected in Hanks' solution with no Ca^{2+} and Mg^{2+} (Biowhittaker, Belgium), manually cut into 1 mm-thick slices and incubated for 10 min at 37° C in the same solution supplemented with 5 mg/ml trypsin (Sigma) and 0.5 mg/ml DNase (Calbiochem, La Jolla CA). Trypsin was then blocked by incubation for 10 min at 37° C in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker) plus 10% fetal calf serum (Hyclone, Logan UT). After two washes in Hanks' solution the slices were mechanically disrupted with a 10 ml pipette in Hanks' solution with no Ca^{2+} and 5 mM Mg^{2+}. Cell suspensions were then centrifuged at 68 × g for 10 min and the pellets resuspended in the final culture medium. In order to obtain pure neuronal cultures, cells were plated on poly-L-ornithine (10 mg/ml)-treated coverslips at high density (1.5×10^6/ml) and cultured in DMEM plus 10% fetal calf serum in the presence of 7.5 μM cytosine-β-D-arabinofuranoside (SIGMA) to prevent glial proliferation and of 20 mM KCl to favour cerebellar granule cell survival. To obtain pure glial cultures, cells were plated on poly-L-ornithine (10 mg/ml)-treated coverslips at low density (1.5×10^5/ml) and cultured in DMEM plus 10% fetal calf serum.

All treatments were performed at 37° C on cultures after 12 days *in vitro* (DIV). A 30 min preincubation in KRH buffer (130 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 1.1

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mg/ml glucose and 25 mM HEPES-Na pH 7.4) was applied before treatments in order to avoid serum starvation artefacts. Synthetic Tat (10 ng/ml) was applied to the buffer in the absence or presence of 1 mM sodium orthovanadate, as indicated.

Immunoblot analysis. At various time points after the addition of Tat, cerebellar neurons or glial cells were solubilized by scraping in 1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 10 mM HEPES-Na (pH 7.4) and immediately frozen in liquid nitrogen. After thawing, the lysates were boiled for two min and sonicated. Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as previously described [8].

For anti-phosphotyrosine immunoblotting, filters were blocked for 2 h at room temperature (RT) with 5% bovine serum albumin (BSA) in Tris-buffered saline, incubated overnight at 4° C in the same solution supplemented with an anti-phosphotyrosine monoclonal antibody (1:5,000), washed 5 times for 5 min with 5% BSA in Tris-buffered saline/ 0.5% Tween 20, incubated for 2 h with an anti-mouse peroxidase-conjugated secondary antibody (1:10,000), washed five times for 5 min and finally developed using a chemiluminescence detection system.

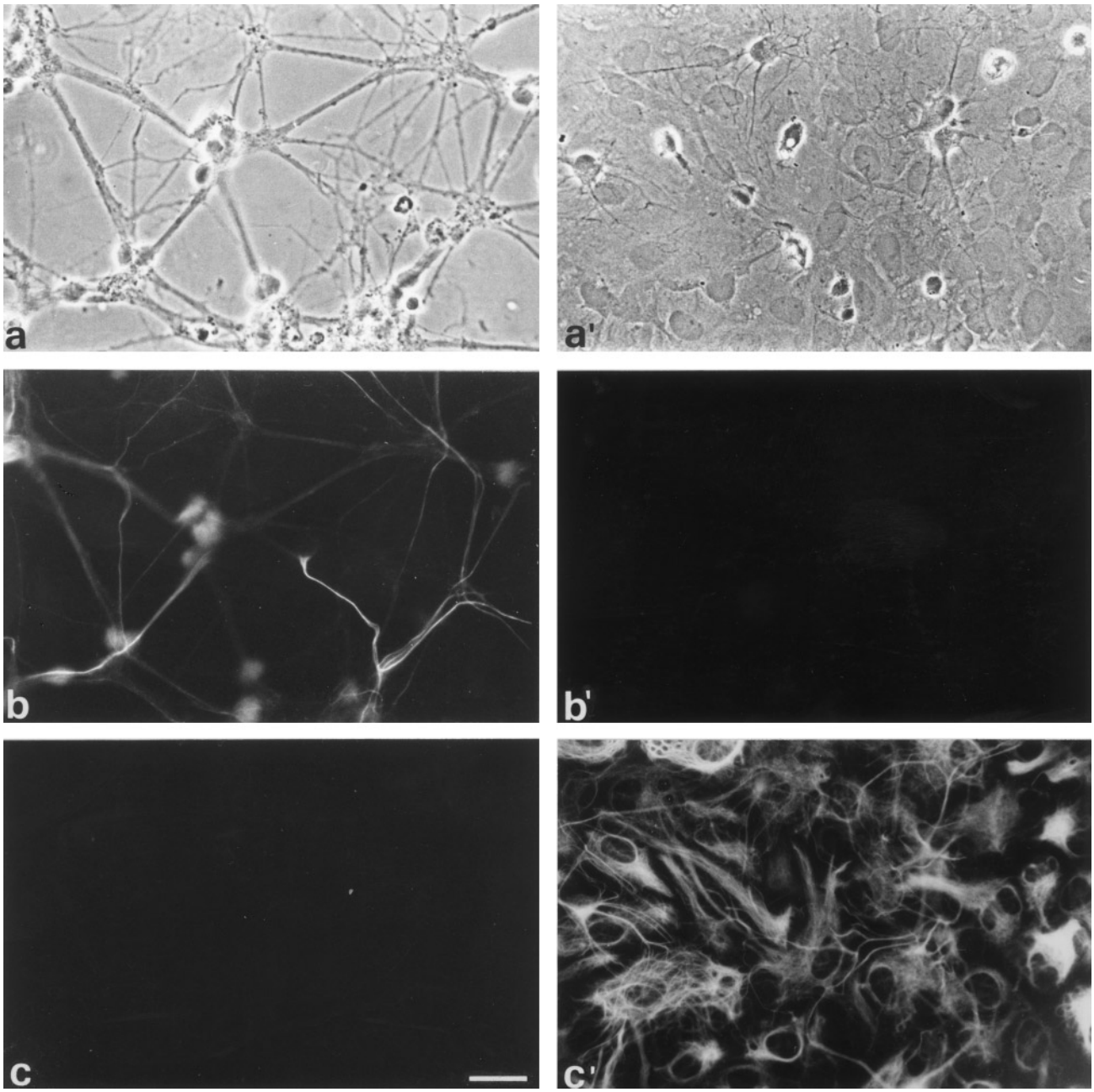

FIG. 1. Characterization of neuronal and glial cerebellar cultures. a–c: cultures enriched in granular neurons, viewed at 12 DIV under phase-contrast (a) and double-stained for neurofilament protein 200 kDa (b) and for GFAP (c). a′–c′: corresponding images of cultures enriched in glial cells at 12 DIV. Note the virtual absence of staining for the glial marker in the neuronal culture and for the neuronal marker in the glial culture. Bar, 100 μm.

After exposure to X-ray films, in order to remove the bound antibodies the filters were incubated for 30 min at 50° C with 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.7. The filters were then washed twice for 10 min with Tris-buffered saline/ 0.1%Triton X-100 at RT, re-blocked for 1 h at RT with 5% non fat dry milk in Tris-buffered saline and incubated for 2 h with either an anti-MAP kinase polyclonal antibody (1:5,000) or an anti-Shc monoclonal antibody (1:2,000), washed 5 times for 5 min with Tris-buffered saline/0.1%Triton X-100, incubated for 1 h with either an anti-rabbit or an anti-mouse peroxidase-conjugated secondary antibody (1:10,000) and finally developed by chemiluminescence.

Immunofluorescence analysis. Cerebellar neurons or glial cells were fixed for 30 min at 37° C in 4% formaldehyde (freshly prepared from paraformaldehyde) dissolved in 120 mM sodium phosphate buffer (pH 7.4) and 4% sucrose. After washing in phosphate-buffered saline (PBS), cells were processed for double immunofluorescence with a monoclonal anti-GFAP antibody (1:1,000) and with a polyclonal anti-200 KDa neurofilament antibody (1:200), followed by goat anti-mouse FITC-conjugated and goat anti-rabbit Texas Red-conjugated (1:100). Incubation and washing conditions were those described [8].

RESULTS

The effects of extracellularly applied synthetic tat on tyrosine phosphorylation processes were investigated in primary cultures from rat cerebellum. In order to be able to separate the effects of the protein on neuronal and glial cells, we employed culture conditions which lead to selective enrichment in either neuronal or astrocytic cells. Phase-contrast microscopy and double-immunofluorescence labeling with antibodies to neuronal and astrocytic markers (i.e. neurofilaments and GFAP, respectively) confirmed the effectiveness of the method of selective enrichment (Fig. 1).

When analyzed by antiphosphotyrosine immunoblotting, neuronal cells appeared to contain several proteins phosphorylated on tyrosine residues. The application of synthetic Tat (10 ng/ml) for 30 min in the absence of sodium orthovanadate did not significantly alter the overall pattern of tyrosine phosphorylation, with the exception of a selective increase in the phosphotyrosine content of two bands of 42 and 44 kDa and of a poorly resolved band of approximately 52 kDa (Fig. 2, left panel). The 42 and 44 kDa bands were found to correspond to the two isoforms of MAP kinase, p42 and p44, by immunoblotting (Fig.2, right panel) as well as by a mobility shift assay (data not shown).

The identity of the 52 kDa band could be better studied in high resolution 12-18% gradient gels. This band appeared to exactly co-migrate with the high mobility isoform of the adaptor protein Shc (Fig. 3). Immunoblotting revealed that in our neuronal preparation three isoforms of Shc were present. However, the increase in phosphotyrosine content upon treatment with Tat appeared to be selective for the isoform with the highest mobility, which was the least represented in this preparation.

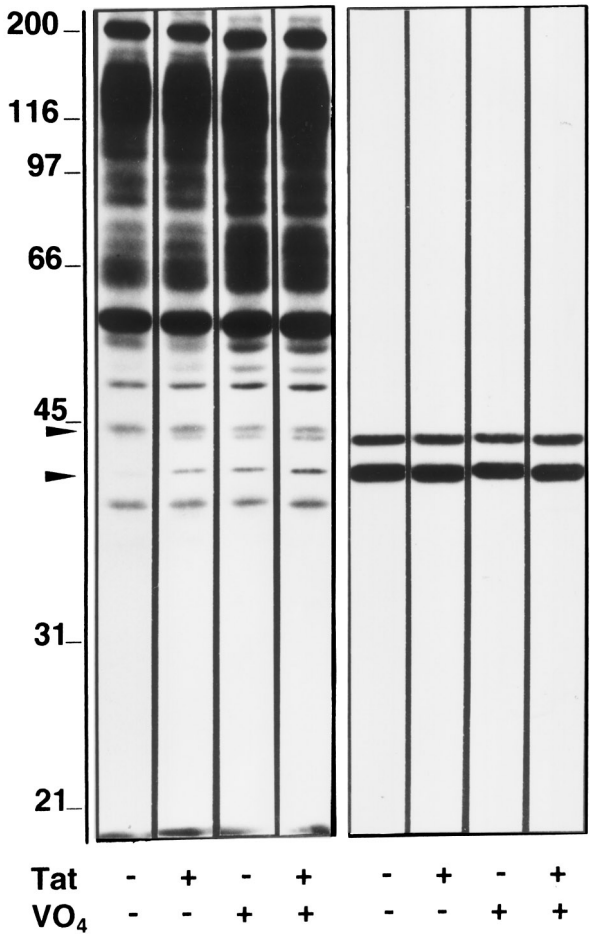

FIG. 2. Tat increases the tyrosine phosphorylation of MAP kinase in neuronal cultures. Neuronal cultures at 12 DIV were incubated in KRH with synthetic Tat (10 ng/ml) for 30 min in the presence or absence of sodium orthovanadate (as indicated) and processed for immunoblotting with anti-phosphotyrosine antibody (left panel) and with antibodies to MAP kinase (right panel). The arrowheads point to two bands of 42 and 44 kDa, respectively, whose mobilities are identical to those of the two isoforms of MAP kinase.

When cells were solubilized after a 30 min incubation with sodium orthovanadate (a tyrosine phosphatase inhibitor [9]), several protein bands, including p42 and p44 MAP kinase, appeared to have an increased phosphotyrosine content. However, under these conditions no difference in MAP kinase or Shc tyrosine phosphorylation could be detected between control neurons and neurons treated with Tat (Figs. 2 and 3).

Similarly to what observed in neuronal cells, also in pure glial cultures Tat did not alter the overall pattern of tyrosine phosphorylation; rather, it selectively increased the phosphotyrosine content of p42 and p44 MAP kinase (Fig. 4). However, in this case the effect

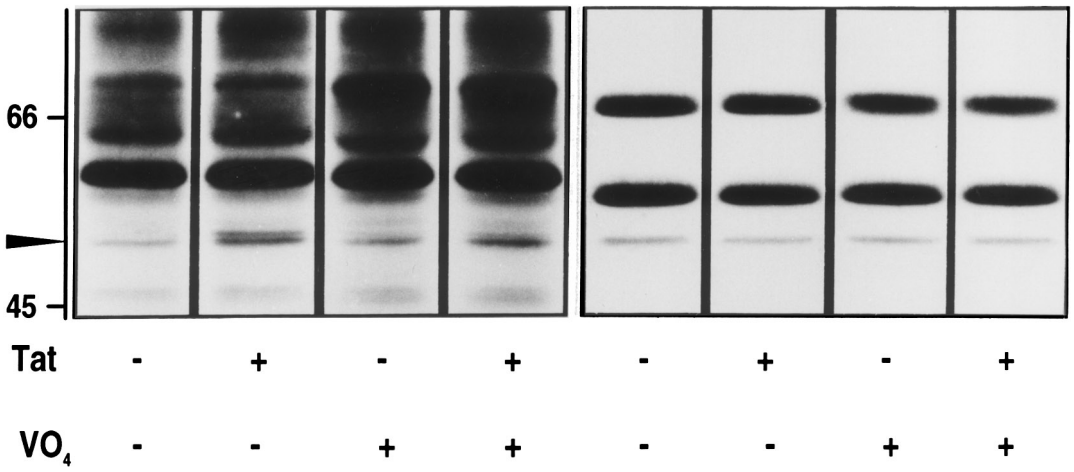

FIG. 3. Tat increases the tyrosine phosphorylation of Shc in neuronal cultures. Solubilized proteins from neuronal cultures at 12 DIV were separated on SDS 12–18% polyacrylamide gradient gels, transferred to nitrocellulose and sequentially probed with anti-phosphotyrosine (left panel) and anti-Shc (right panel) antibodies. The arrowhead points to a 52 kDa band whose mobility is identical to that of the highest mobility isoform of Shc.

was apparent also in cultures treated with Tat in the presence of sodium orthovanadate. In addition, in these cultures no effect on the tyrosine phosphorylation of any of the Shc isoforms could be observed (Fig. 4), although in glial cultures the highest mobility isoform of the protein was more abundant than in granule neurons (data not shown).

The kinetics of MAP kinase activation appeared to be faster in glial than in neuronal cells. Thus, in glial cells phosphorylation of the kinase was already evident after 5 min of treatment with Tat. In addition, p42 and p44 appeared to be independently regulated. p42 was already maximally phosphorylated after 5 min and successively decreased in intensity, whereas p44 phosphorylation reached a peak after 15 min of treatment. In contrast, in neuronal cells an increase in tyrosine phosphorylation of MAP kinase was detectable only after 30-45 min of treatment, with no significant difference between the two isoforms (Figs. 2 and 5).

DISCUSSION

Our results show that HIV-1 Tat protein, when applied extracellularly, is able to induce the generation of intracellular signals in neuronal as well as glial cells cultured *in vitro*. The protein is active at picomolar concentrations, which are likely to be

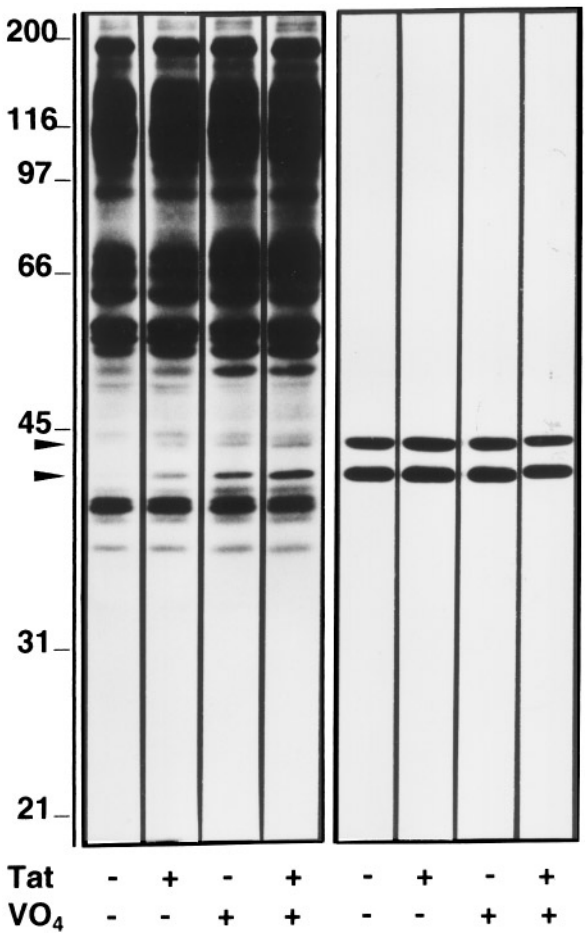

FIG. 4. Effects of Tat treatment on the protein tyrosine phosphorylation pattern in glial cultures. Glial cultures at 12 DIV were incubated with synthetic Tat under the experimental conditions described in the legend to Fig. 2. Left panel: total pattern of phosphotyrosine labeling of samples separated on an SDS 7.5% polyacrylamide gel. The arrowheads point to two bands of 42 and 44 kDa, respectively, whose phosphotyrosine content is increased upon treatment with Tat. Right panel: immunoblotting of the same filter with antibodies to p42 and p44 MAP kinase.

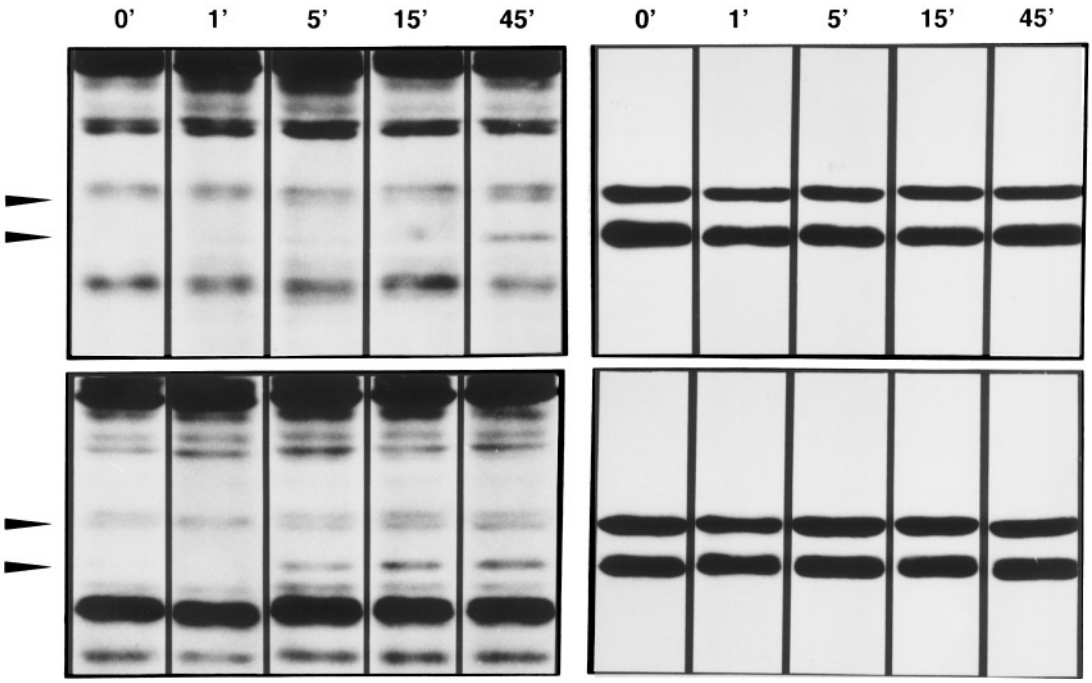

FIG. 5. Time-course of the effects of Tat on tyrosine phosphorylation in neuronal and glial cultures. Neuronal (upper panel) or glial (lower panel) cultures at 12 DIV were incubated in KRH with synthetic Tat (10 ng/ml) in the absence of sodium orthovanadate for various periods of time, as indicated. Left panels: patterns of phosphotyrosine labeling of samples separated on an SDS 7.5% polyacrylamide gel. Right panels: Immunoblotting of the same filters with antibodies to p42 and p44 MAP kinase.

reached in the cerebrospinal fluid of AIDS patients. Indeed, these concentrations of Tat are similar to those detected in the sera of HIV-1 positive individuals [10] and encephalitic AIDS patients have been shown to express Tat in cells of the central nervous system [11].

The intracellular signals generated by Tat that we detected consisted in an activation of the tyrosine phosphorylation of selected substrates, the main of which was MAP kinase. Although this effect was present in both neuronal as well as glial cells, major differences were observed in terms of kinetics, regulation and pattern of activation. These differences might be related to differences in the physiology of the two kinds of cells (e.g. a differential regulation in the turnover of MAP kinase phosphotyrosine), rather than to a distinct mechanism of action of Tat.

The activation of MAP kinase elicited by Tat might have important consequences in redirecting cell physiology. Indeed, MAP kinase plays a critical role in cell activation, phosphorylating transcription factors and linking the activation of cell surface receptors to the regulation of gene expression [12].

In neurons, but not in glial cells, tyrosine phosphorylation of one isoform of Shc was also stimulated by Tat. Shc is an adaptor protein linking activation of growth factor receptors as well as of seven-transmembrane receptors to Ras signaling and MAP kinase activation by forming a complex with Grb2/Sos1. Thus, Shc phosphorylation is also a key event in cell activation, elicited by various stimuli [13]. The reasons for the selectivity of Tat effects on one isoform of Shc are at present unclear. However, they might be due to differential regulation and/or subcellular localization of the three isoforms of Shc.

Tat might induce cell activation by acting either at the plasma membrane or at the nuclear level. Thus, the protein might be taken up by the target cells and transactivate growth-related genes or it might act as a growth factor, binding to a cell surface receptor and leading to the generation of intracellular signals. Extracellularly applied exogenous Tat has been shown to rapidly enter into cells and accumulate in the nucleus [3,14]. However, it has also been recently reported that Tat can activate endothelial cells by binding to the Flk-1/KDR receptor for vascular endothelial growth factor-A, promoting tyrosine phosphorylation of the receptor and its association with downstream effectors [15]. Since the Flk-1/KDR receptor is specific for endothelial cells [16], it is unlikely to be responsible for the effects observed in our preparations. However, it is possible that Tat is able to mimic other growth factors and interact with growth factor tyrosine kinase receptors present in cells of the central nervous system.

Although its precise mechanism of action still remains to be fully elucidated, to our knowledge this is the first demonstration that picomolar concentrations of Tat can activate signal transduction pathways in cells of the nervous system. In fact, previous reports, suggesting that Tat activates NMDA receptors and is toxic to neurons [17,18], were based on the application of micromolar concentrations of the protein, which are unlikely to be reached *in vivo*. Being exerted on key molecules in the signal transduction pathways, the observed neuronal and astrocytic responses to Tat may lead to cell disfunction and/or affect cell survival. In non-neuronal cells, exogenous Tat has been shown to influence cell proliferation and survival in opposing directions, based on the cell type [4,6,10]. Studies aimed at identifying the long-term effects of Tat exposure as well as the complete sequence of events elicited by the protein on neuronal and glial cells are currently under way.

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