

HIV-1 gp120 Receptor on CD4-Negative Brain Cells Activates a Tyrosine Kinase

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Human immunodeficiency virus (HIV-1) infection in the human brain leads to characteristic neuropathological changes, which may result indirectly from interactions of the envelope glycoprotein gp120 with neurons and/or glial cells. We therefore investigated the binding of recombinant gp120 (rgp120) to human neural cells and its effect on intracellular signalling. Here we present evidence that rgp120, besides binding to galactocerebroside or galactosyl-sulfatide, specifically binds to a protein receptor of a relative molecular mass of approximately 180,000 Da (180 kDa) present on the CD4-negative glioma cells D-54, but not on Molt4 T lymphocytes. Binding of rgp120 to this receptor rapidly induced a tyrosine-specific protein kinase activity leading to tyrosine phosphorylation of 130- and 115-kDa proteins. The concentration of intracellular calcium was not affected by rgp120 in these cells. Our data suggest a novel signal transducing HIV-1 gp120 receptor on CD4-negative glial cells, which may contribute to the neuropathological changes observed in HIV-1-infected brains. © 1992 Academic Press, Inc.

INTRODUCTION

Neuropathological changes in the central nervous system (CNS) of AIDS patients, which lead to the so-called AIDS dementia complex (ADC), are of great clinical importance. Manifestations of ADC are progressive cognitive, motor, and behavioral dysfunctions that have been linked to the persistence of HIV-1 infections in the CNS and are mainly found in CD4-positive (CD4⁺) microglial cells and circulating macrophages (Budka, 1991; Fauci, 1988; Jordan *et al.*, 1991; Ketzler *et al.*, 1990; Liu *et al.*, 1990; Maddon *et al.*, 1986; McArthur, 1987; Merrill and Chen, 1991; Price *et al.*, 1988). However, in the absence of CD4 molecules on the cell membrane of the majority of brain cells and in the light of the paucity of direct evidence for HIV infection of neurons and glial cells it has been suggested that the HIV-associated degenerative neurological abnormalities are the result of indirect mechanisms mediated by soluble factors of viral or cellular origin (Brenneman *et al.*, 1988; Dreyer *et al.*, 1990; Giulian *et al.*, 1990; Kaiser *et al.*, 1990; Pulliam *et al.*, 1991; Wahl *et al.*, 1991). These factors could be cytotoxic to subpopulations of brain cells or interfere with neuronal and glial cell functions leading to neurodegeneration.

One such factor, the HIV-1 envelope glycoprotein gp120, which is easily shed from infected cells, has been studied in the past. It was found that gp120 does

not only bind to the well-known CD4 molecules, but also to galactocerebroside (GalC) and galactosyl-sulfatide, cellular membrane components expressed on the surface of oligodendrocytes and other neural cells (Harouse *et al.*, 1991; Kuhlmann-Krieg *et al.*, 1988; Ranscht *et al.*, 1982). Polyclonal antibodies to GalC can inhibit the entry of HIV-1 in neural cell lines, suggesting that the binding of HIV to these membrane structures might be of biological significance (Bhat *et al.*, 1991; Harouse *et al.*, 1991). However, to mediate signals possibly leading to cellular dysfunction, entry of viruses into cells is not necessary, but rather the interaction of HIV-1 or gp120 with cell surface receptors may be sufficient. Indeed, it was found that treatment of neuronal cultures of rodents with gp120 together with excitatory amino acids resulted in marked increases of intracellular calcium and ultimately in neuronal cell death (Brenneman *et al.*, 1988; Choi, 1988; Dreyer *et al.*, 1990; Kaiser *et al.*, 1990; Lipton *et al.*, 1991). These observations give certainly support to the hypothesis that soluble HIV-1 gp120 may interfere not only with neuronal, but also with glial cell functions and may be associated with the development of ADC.

To study the effect of HIV-1 gp120 on human brain cells, we used the human glioma cell line D-54, which does not express CD4 molecules. Investigation of these cells revealed high-affinity binding of rgp120 to a novel trypsin-sensitive receptor. Binding of gp120 to these cells was leading to a tyrosine phosphorylation of 130 and 115 kDa proteins in the absence of any

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increase of intracellular Ca^{2+} . These data prove that binding of a HIV-1 structural protein alone to cell membrane molecules leads to signal transduction, which could be of pathogenetic significance.

MATERIALS AND METHODS

Culture of cells

The human malignant glioma cell lines D-54 and U-373 (Bigner *et al.*, 1981) were cultured in Dulbecco's minimal essential medium (DMEM) containing streptomycin (100 $\mu\text{g}/\text{ml}$), penicillin (100 U/ml), and 5% fetal calf serum (FCS) at 37° and 10% CO_2 . The CD4^+ T lymphocytic cells Molt4, H9 and C8166, and the CD4^- B lymphocytic cells BJAB were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing the same additives.

Polymerase chain reaction

For PCR, 10^5 cells were infected with HIV-1 (HTLV-III_B, a kind gift of R. Gallo; m.o.i. = 1). After 15 days (three passages), DNA was prepared and virus-specific DNA amplified with the gag-specific primers SK38 and SK39 and Taq-polymerase (Boehringer-Mannheim) as described (Ou *et al.*, 1988). DNA was separated on a 15% polyacrylamide gel and blotted on nitrocellulose. After hybridization with the end-labeled 41-bp fragment SK19 of HIV-1, filters were exposed to X-ray films (DuPont).

Preparation of RNA and Northern blot

Total cellular RNA was prepared as described (Chirgwin *et al.*, 1979). Poly-A⁺-RNA was selected on oligo-dT-cellulose (Sigma). Eight micrograms of poly-A⁺-RNA per lane was separated on 1.5% agarose gels containing 6.3% formaldehyde and blotted on Hybond N filters (Amersham) by diffusion. As hybridization probes the 0.6-kb *EcoRI/SacI* fragment of CD4-cDNA of plasmid T4-pMV6tk/neo (a gift of Dr. D. R. Littman, Columbia University, New York) and the 1.3-kb *PstI* fragment of a plasmid containing the rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA were labeled with [³²P]dCTP with a random-primed labeling kit (Boehringer-Mannheim).

Immunofluorescent staining and flow cytometry

To quantify surface molecule expression by flow cytometry, cells were harvested with Ca^{2+} - and Mg^{2+} -free PBS. To partially remove surface proteins, 2×10^5 cells/tube were incubated with 1 ml 0.1% trypsin in PBS for 10 min at room temperature, centrifuged, the supernatant aspirated, and washed once with 1 ml medium containing 10% FCS and two times with 1 ml

Ca^{2+} - and Mg^{2+} -free PBS containing 0.2% BSA (FACS-buffer). For rgp120-binding studies, cells were incubated with 200 μl 1 $\mu\text{g}/\text{ml}$ rgp120 (strain III_B; MRC Directed AIDS Programme) for 1 hr on ice, washed three times with FACS-buffer and stained with anti-gp120 and FITC-conjugated second antibodies. The following monoclonal antibodies (mAbs) were used: mouse IgG, (Dako) as negative control; anti-CD4 mAb OKT4 (Ortho Diagnostics); anti-MHC class I mAb W6/32; anti-GalC mAb (Boehringer-Mannheim); anti-galactosyl-sulfatide mAb O4 (a gift of M. Schachner, ETH-Zürich, Switzerland); anti-gp120 mAb (DuPont); FITC-conjugated rabbit anti-mouse Ig mAb (Dako).

Labeling of rgp120 with ¹²⁵I and binding studies

Labeling of rgp120 (strain III_B; MRC Directed AIDS Programme) was performed by lactoperoxidase with single-reaction enzymobead radioiodination reagent (Bio-Rad). rgp120 (5 μg) in 75 μl 0.2 M phosphate buffer, pH 7.2, 25 μl containing 1.0 mCi Na^{125}I (Amersham) and 25 μl containing 1% beta-D-glucose, were added to 50 μl rehydrated enzymobead reagent. The reaction mixture was incubated for 25 min at room temperature, directly applied to a gel filtration column (Sephadex-G25), and eluted with PBS into ice-cold tubes. Binding studies were performed with fractions containing rgp120 labeled to a specific activity of approximately 1200 cpm/pmol in 5 ml PBS containing 0.5% BSA for 2 hr at 4°. Cells were washed three times quickly with ice cold PBS 0.5% BSA and cell-bound radioactivity and supernatants counted in a gamma-counter (LKB).

Chemical crosslinks

For crosslinking 5×10^6 cells were harvested with Ca^{2+} - and Mg^{2+} -free PBS and incubated for 2 hr on ice with 0.1 μg [¹²⁵I]rgp120 in 100 μl . After washing four times with PBS, cells were resuspended in 100 μl PBS. The crosslinkers disuccinimidyl-suberate (DSS, stock: 50 mM in DMSO, Pierce) and bis-(sulfosuccinimidyl)-suberate (BS³, stock: 50 mM in PBS, Pierce) were added to final concentrations of 0.2 mM, 1 mM, and 5 mM, and the suspensions incubated 1 hr at room temperature. Cells were lysed by addition of 1 volume lysis buffer (1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM PMSF), and centrifuged for 10 min at 10,000 g. Supernatants were separated by polyacrylamide gel electrophoresis with 6% sodium dodecylsulfate (SDS-PAGE) and dried gels exposed to X-ray films (DuPont) for 7 days.

Fura-2 loading and Ca²⁺ measurements

For determination of intracellular Ca²⁺ 10⁷ D-54 cells were harvested, washed with buffer A (150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.4 mM MgSO₄, 25 mM glucose, and 25 mM HEPES, pH 7.3), and resuspended in buffer A. Fura-2 AM (1 mM in dimethylsulfoxide) was added to a final concentration of 5 μM. After incubation for 1 hr at 36°, the cells were washed twice and resuspended in buffer A. Assays were carried out at 32° with a modified 4-8202 Aminco-Bowman spectrofluorimeter (Silver Spring, MD) fitted with a magnetic stirrer and a thermostated cuvette holder. Intracellular fura-2 fluorescence intensity was measured at two excitation wavelengths (340 and 385 nm) and continuously recorded at 500 nm. Cells were treated with 0.01–1.0 μg/ml rgp120 and for control with 10 U/ml thrombin (Sigma). At the end of each individual trace, cells were permeabilized with 0.1% Triton X-100 to yield the fluorescence signals of high Ca²⁺. Then 8 mM EGTA/50 mM Tris was added to give the fluorescence signals for minimal Ca²⁺ concentration (<1 nM). Ca²⁺ values were calculated according to the equation given in Grynkiewicz *et al.* (1985) using the 340/385 nm ratio of fluorescence intensities and the Ca²⁺-dye dissociation constant ($K_d = 224$ nM).

Detection of phosphotyrosine by Western blot

To determine tyrosine phosphorylation, 5 × 10⁵ cells were incubated with rgp120 in 1 ml complete culture medium, aspirated and dissolved in 300 μl lysis buffer (200 μl PBS plus 100 μl 200 mM Tris-HCl, pH 6.8, 5% SDS, 15% glycerol, 10% β-mercapto ethanol, 0.01% bromophenol blue), and applied to 8% SDS-PAGE (100 μg/lane). A semidry Western blot on nitrocellulose filters (Schleicher & Schüll) was performed as described (Kyhse-Andersen, 1984) and the background blocked with 1% BSA and 1% gelatine in TNA (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.01% NaN₃) overnight. Anti-phosphotyrosine monoclonal antibody PY20 (ICN Flow) diluted 1:1000 in blocking buffer was applied for 2 hr. After washing for 3 × 5 min with TNA and 5 min with TNA containing 0.05% NP-40, filters were incubated with ¹²⁵I-labeled anti-mouse IgG (Amersham) diluted 1:350 in blocking buffer for 2 hr. Filters were washed 6 × 10 min with TNA and overnight with TNA containing 0.05% NP-40 and exposed to X-ray films (DuPont). Sodium orthovanadate (20 μM) was present in all solutions.

RESULTS

The human glioma cell line D-54 is CD4-negative

For our studies on rgp120-cell interactions we selected D-54 glioma cells, because these cells are

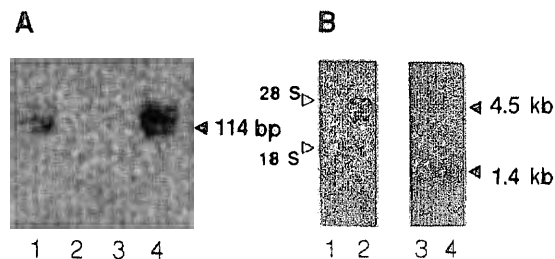


Fig. 1. Uptake of HIV-1 by D-54 cells (PCR analysis) and lack of CD4-specific mRNA in D-54 cells (Northern blot). (A) The uptake of low amounts of HIV-1 by D-54 cells was measured by polymerase chain reaction (PCR). DNA from D-54 cells was amplified with the HIV-1 gag-specific primers SK38 and SK39, the amplification products blotted on nitrocellulose and hybridized to a radioactively labeled gag-specific probe. The 114 bp HIV-1 gag-specific DNA fragment was detected in D-54 cells 15 days after infection with HIV-1 (lane 1), but not in uninfected D-54 cells (lane 2) and in buffer without DNA (lane 3). The HIV-1-infected CD4⁺ lymphocytes H9 were used as positive control (lane 4). Since cellular DNA was amplified, uptake and reverse transcription of viral RNA occurred in D54 cells. (B) Poly A⁺-RNA from cell lines was blotted on Hybond N filters and hybridized to a CD4-specific radioactively labeled probe. CD4-specific mRNA was not detected in D-54 cells (lane 1). In contrast, the CD4⁺ lymphocytes C8166 expressed CD4 mRNA at 4.5 kb (lane 2). The amount of intact RNA on the filter was controlled by hybridization with a GAPDH-specific probe revealing similar signals for RNA from D-54 (lane 3) and C8166 cells (lane 4). The position of 28 S and 18 S RNA is indicated as size marker.

highly responsive to external stimuli like cAMP-analogs (Bigner *et al.*, 1981) and might therefore be good candidates to investigate signal transduction. We first assessed these cells for susceptibility to HIV-1 and presence of CD4 molecules. Like the neuronal cell lines SK-N-BE and IMR-32 and the glioblastoma line U-373 (Li *et al.*, 1990; Harouse *et al.*, 1989), we found that the glioblastoma line D-54 was nonproductively infected with HIV-1. In the absence of production of infectious virus and the development of a cytopathogenic effect (CPE), HIV-1-specific DNA could be detected in D-54 cells by polymerase chain reaction (Fig. 1A) as well as p24 core antigen, although these cells did not contain CD4-specific mRNA or truncated RNAs (Fig. 1B). In addition, we confirmed by immunofluorescent staining and flow cytometry that these cells, in contrast to the T cell line Molt4, did not express CD4 on their surfaces (Fig. 2A, 2B). Obviously, binding and entry of HIV-1 into D-54 cells occurred by a CD4-independent mechanism.

D-54 glioma cells express galactosyl-sulfatide

GalC and galactosyl-sulfatide have been found to bind HIV-1 gp120 as purified substances and on the surface of neural cells (Bhat *et al.*, 1991; Harouse *et al.*, 1991). Therefore, we determined the expression of

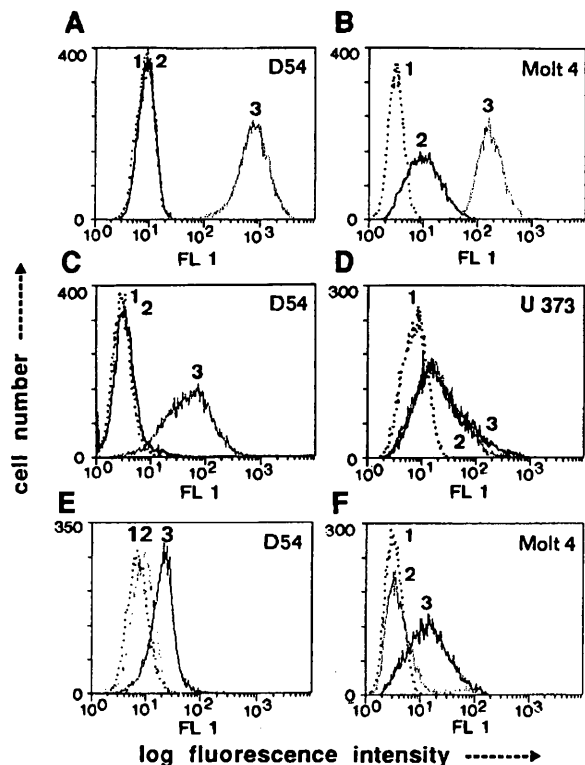


Fig. 2. Expression of surface molecules and binding of rgp120 to CD4-negative human glioma cells determined by flow cytometry. (A) CD4 was not detectable on the surface of D-54 cells: negative control (signal 1), CD4 (signal 2), major histocompatibility complex (MHC) class I as positive control (signal 3). (B) As control, CD4 was detectable on Molt4 cells: negative control (signal 1), CD4 (signal 2), MHC class I (signal 3). (C) D-54 cells did not express GalC, but galactosyl-sulfatide: negative control (signal 1), GalC (signal 2), and galactosyl-sulfatide (signal 3). (D) U-373 cells expressed GalC and galactosyl-sulfatide: negative control (signal 1), galactosyl-sulfatide (signal 2), and GalC (signal 3). (E) Detection of rgp120 bound to D-54 cells by anti-gp120 antibodies: negative control (signal 1), rgp120 on trypsin-treated D-54 (signal 2), and rgp120 bound to D-54 cells (signal 3). (F) CD4⁺ Molt4 cells did bind rgp120: negative control (signal 1), rgp120 on trypsin treated Molt4 (signal 2), rgp120 bound to Molt4 cells (signal 3).

GalC and galactosyl-sulfatide on D-54 cells by immunofluorescent staining with monoclonal antibodies and flow cytometry. D-54 cells were not stained with antibodies to GalC, but expressed galactosyl-sulfatide on their surfaces (Fig. 2C). As control we stained U-373 cells, which expressed GalC and galactosyl-sulfatide (Fig. 2D). We now investigated whether galactosyl-sulfatide is the only molecule on D-54 cells capable of binding gp120, or if a further class of binding sites might be expressed on these cells.

Binding of rgp120 to cell surfaces

To roughly estimate the amount of purified CHO cell-derived recombinant gp120 of HIV-1 (strain IIIIB) capa-

ble of binding to D-54 cells, we first used immunofluorescent staining methods and analysis by flow cytometry. After stringent washing, low, but significant levels of rgp120 binding to D-54 cells remained detectable, when compared to CD4⁺ Molt4 cells (Fig. 2E, 2F). In addition, we made the interesting observation that the specific binding of rgp120 was decreased after treatment of both cell lines with trypsin (Fig. 2E, 2F). Since galactosyl-sulfatide is not sensitive to trypsin, these results suggested the presence of a proteinous receptor for gp120 on the surface of D-54 cells.

To determine the exact binding properties of rgp120 to D-54 cells, rgp120 was labeled with ¹²⁵Iodine (Fig. 3A). The affinity constant for the rgp120-D-54 cell interaction was assessed by whole cell binding assays. Providing ¹²⁵I-labeled rgp120 up to a concentration of 100 nM, we could not reach the level of saturation (Fig. 3B), but the binding of ¹²⁵I-labeled rgp120 (400 fM/10⁶ cells) was specifically inhibited by 50% with approximately 20 nM unlabeled rgp120 (not shown). Intermediate saturation of high-affinity binding sites of rgp120 on D-54 cells was detected already at a free concentration of 300 to 500 fM rgp120, whereas at higher concentrations low-affinity binding increased. Scatchard analysis of these data revealed binding sites with a dissociation constant (K_d) of approximately 1.2×10^{-10} M for high-affinity and 3.2×10^{-9} M for low-affinity binding sites (Fig. 3C). The abundance of receptor sites on D-54 cells was determined to be approximately 7×10^4 /cell for high affinity receptors and more than 10^6 /cell for lower affinity receptors (average of eight experiments). The flow cytometry data suggest that at least one of these receptors is trypsin sensitive and therefore of proteinous nature, whereas the other is likely to be galactosyl-sulfatide.

Crosslinking of rgp120 to cell surface molecules

To investigate the nature of the trypsin-sensitive gp120 receptors on D-54 cells, we performed crosslinking experiments with several chemical crosslinkers. Using the crosslinkers disuccinimidyl suberate (DSS) and bis(sulfosuccinimidyl) suberate (BS³), we detected one labeled ligand-receptor complex at approximately 300 kDa with D-54 cells, but not with Molt4 cells (Fig. 4). The intensity of this 300-kDa band (Fig. 4, lanes 3-8) was relatively low, as compared to cross-linked CD4 molecules on the surface of Molt4 cells (Fig. 4, lanes 9-11). Increasing concentrations of cross-linkers led to high molecular weight complexes with D-54 and Molt4 surface molecules on top of the gel (Fig. 4, lanes 5, 8, 11). The 300-kDa complex is unlikely to consist of cross-linked rgp120 molecules themselves, since we did not observe bands of dimerized gp120 with a molecu-

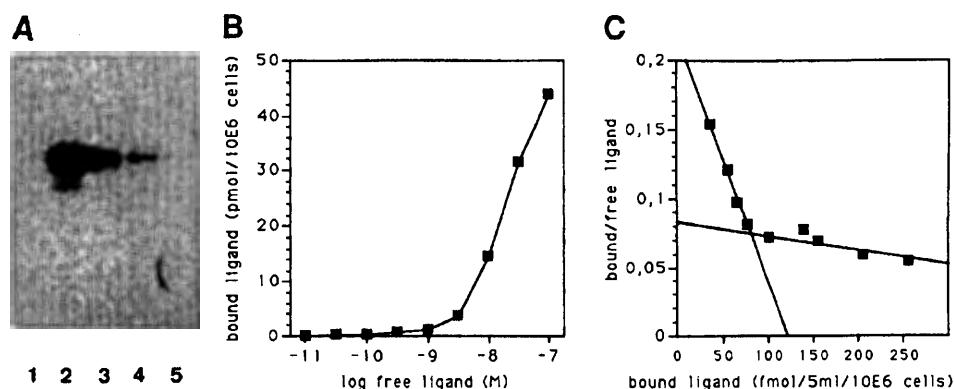


FIG. 3. Labeling of rgp120 and analysis of binding to D-54 cells. (A) Purified ^{125}I -labeled rgp120 visualized after separation on 10% SDS-PAGE by autoradiography. Fractions 2 and 3 from the gel filtration column (lanes 2 and 3) were used for binding studies. (B) To measure binding of rgp120 to D-54 cells, 1×10^6 cells in several experiments were incubated with 20 fM to 100 nM ^{125}I -labeled rgp120. The logarithmic binding curve of one representative experiment with 1×10^6 D-54 cells is shown. (C) Scatchard analysis of low concentrations of rgp120 binding to D-54 cells. Binding sites with equilibrium dissociation constants (K_d) of approximately 1.2×10^{-10} M and 3.2×10^{-9} M were found for high- and low-affinity classes of receptors. High-affinity binding corresponded to approximately 7×10^4 binding sites/cell and low-affinity binding to more than 10^6 binding sites/cell.

lar weight of 240 kDa and trimerized gp120 with a molecular weight of 360 kDa and also no bands between 120 and 240 kDa with D-54 cells. Furthermore, the 300-kDa complex could not be observed when D-54 cells were harvested with trypsin or treated with trypsin after harvesting (not shown). In contrast, cross-linking on Molt4 cells, which were capable of crosslinking much more ^{125}I -labeled rgp120 than D-54 cells, revealed in addition to the 180 kDa band also a weak

band at 240 kD, probably the dimerization product of rgp120. Therefore, the 300-kDa complex observed with D-54 cells is likely to consist of ^{125}I -labeled rgp120 cross-linked to a single 180-kDa molecule. Taken together, these data indicate that a trypsin-sensitive 180-kDa cell surface protein might be a high-affinity receptor for gp120.

Determination of intracellular calcium concentrations

Recent reports on the neurotoxicity of gp120 in cell culture suggested that signal transduction through an unknown surface molecule may disturb cellular functions by raising intracellular Ca^{2+} concentrations (Breneman *et al.*, 1988; Dreyer *et al.*, 1990; Kaiser *et al.*, 1990; Pulliam *et al.*, 1991). When we measured intracellular Ca^{2+} concentrations in D-54 cells in response to 0.01 to 1 $\mu\text{g}/\text{ml}$ rgp120, no increase of Ca^{2+} levels could be detected (Fig. 5A). As control, we measured a high response of Ca^{2+} increase after treatment of cells with 10 U/ml thrombin (Fig. 5B), ionomycin and Triton X-100 (not shown). Thus, intracellular Ca^{2+} concentrations were not affected by rgp120 in D-54 cells.

Activation of tyrosine phosphorylation by rgp120

Assessing a different signal transduction pathway, namely the activation of cellular tyrosine kinases, we observed a rapid induction of tyrosine phosphorylation of proteins after interaction of rgp120 with D-54 cells (Fig. 6). The phosphotyrosine-specific antibody used in this Western blot detected a predominant molecular species at 130 kDa and a minor band at 115 kDa after

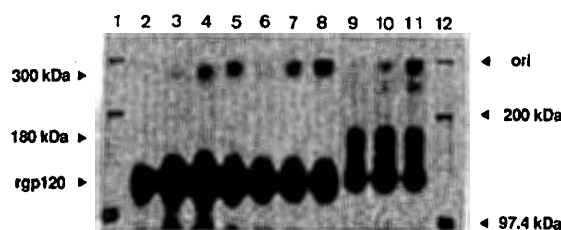


FIG. 4. Cross-linking of rgp120 to cell surface proteins of D-54 and Molt4 cells. ^{14}C -labeled marker proteins had molecular masses of 200 kDa and 97.4 kDa (lanes 1 and 12). ^{125}I -labeled rgp120 was incubated with D-54 cells (lanes 2-8) and with Molt4 cells (lanes 9-11). Incubation of D-54 with ^{125}I -labeled rgp120 in the absence of crosslinker (lane 2) did not lead to higher molecular weight complexes. Increasing concentrations (0.2 mM, 1 mM, 5 mM) of the cross-linkers disuccinimidyl suberate (DSS) (lanes 3-5 and 9-11) and bis(sulfosuccinimidyl) substrate (BS^3) (lanes 6-8) led to one ligand-receptor complex with an approximate relative molecular mass of 300 kDa (lanes 3-8). The crosslink of the CD4 molecule (60 kDa) on Molt4 cells with rgp120 was used as positive control giving a complex of 180 kDa (lanes 9-11). A diffuse band of approximately 240 kDa was also seen with Molt4 cells, which is likely to consist of the dimerization product of rgp120. Unspecific high molecular weight complexes at the top of the gel accumulated with increasing concentrations of crosslinkers (lanes 5, 8, 11).

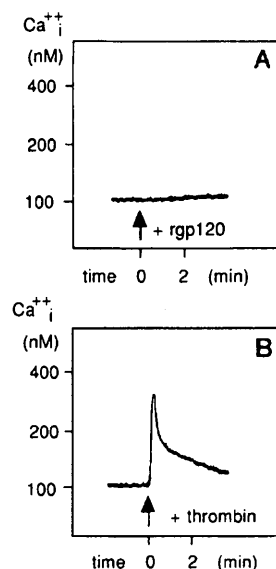


FIG. 5. Intracellular calcium concentration in human glioma cells. D-54 cells loaded with fura-2 were treated with 1 μ g/ml rgp120 (A) and 10 U/ml thrombin (B) as a positive control. The intracellular fluorescence intensity was measured at two excitation wavelengths (340 and 385 nm) and continuously recorded at 500 nm with a spectrofluorimeter. The computed intracellular Ca^{2+} concentration (vertical axis) was plotted as a function of time (horizontal axis). No response to rgp120 could be detected.

0.5, 1.0, and 10 min of incubation of cells with 1 μ g/ml rgp120 (Fig. 6, lanes 4–6). Maximal tyrosine phosphorylation was found already 0.5 to 1.0 minutes after treatment of D-54 cells with rgp120. In contrast, the lymphocytic cells Molt4 (Fig. 6, lanes 7–10) and BJAB (not shown) did not reveal tyrosine phosphorylation in response to rgp120. Tyrosine phosphorylation was also not observed after treatment of D-54 cells with antibodies to galactosyl sulfatide (not shown). Induction of tyrosine phosphorylation was completely abolished by pretreatment of the cells with trypsin (Fig. 6, lanes 11, 12), indicating that trypsin-sensitive cell surface proteins are essential for transmission of the signal.

DISCUSSION

Our results indicate that a surface molecule of approximately 180 kDa expressed on the surface of the human glioma cells D-54 might be a novel receptor for gp120, which transduces either itself, or as a receptor complex, a signal leading to tyrosine phosphorylation of two proteins with molecular masses of 130 and 115 kDa. The human glioma cells did not express the main HIV-1 receptor CD4 as mRNA or as protein on their surfaces. It has been described that several CD4⁻ cells

are susceptible to HIV-1, implicating the existence of additional HIV-1 receptors on neural cells (Clapham *et al.*, 1989; Harouse *et al.*, 1989, 1991; Kozlowski *et al.*, 1991; Li *et al.*, 1990; Mizrahi *et al.*, 1991; Schmitt *et al.*, 1990). The only known molecular species expressed on the surface of D-54 cells capable of binding gp120 was found to be galactosyl-sulfatide. A similar molecule, GalC, has been described to bind rgp120 with an affinity of 1.16×10^{-10} M (Harouse *et al.*, 1991), which is higher than the affinity of rgp120 binding to CD4 ($2-5 \times 10^{-9}$ M; Lasky *et al.*, 1987). We found that D-54 cells express two classes of binding sites with higher (1.2×10^{-10} M) and lower (3.2×10^{-9} M) affinity to rgp120. When compared with the results of Harouse *et al.* (1991), our affinity data suggest that the higher affinity receptors consist of galactosyl-sulfatide and the lower affinity receptors of the trypsin-sensitive cell surface proteins. We also determined the binding of rgp120 to trypsin-treated D-54 cells. But the resulting values of the binding assays were partially higher than of untreated cells and could not be evaluated properly. A possible explanation for these results could be that galactosyl-sulfatide epitopes are unmasked by the treatment with trypsin and led to higher binding values.

By chemical cross-linking of ^{125}I -labeled rgp120 to cell surface molecules we detected one ligand-receptor complex of approximately 300 kDa. The cross-linkers DSS and BS³ do not link lipids like galactosyl-sulfatide to proteins, since reactive amino groups are missing. Therefore, the 300-kDa complex cross-linked by DSS and BS³ is likely to consist of rgp120 and the trypsin-sensitive cell surface component binding rgp120. Since no smaller complexes of dimerization

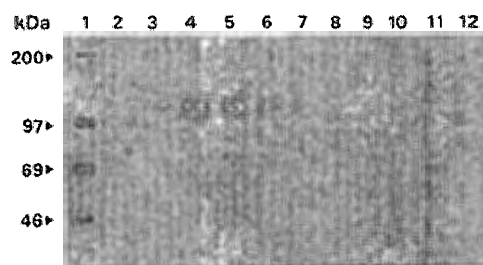


FIG. 6. Tyrosine phosphorylation induced by rgp120-treated human glioma cells. ^{14}C -labeled marker proteins had the molecular masses of 200, 97, 69, and 46 kDa (lane 1). The following lanes were loaded with the extracts of D-54 and Molt4 cells in culture medium, respectively (lanes 2 and 7); D-54 cells incubated with anti-gp120 antibody (lane 3); D-54 and Molt4 cells incubated with 1 μ g/ml rgp120 for 0.5, 1, and 10 min, respectively (lanes 4–6 and 8–11); D-54 cells preincubated with 0.1% trypsin for 10 min, washed, and then incubated with 1 μ g/ml rgp120 for 0.5 and 1 min (lanes 11 and 12). Induced tyrosine phosphorylation of 130 and 115 kDa proteins was detected only in the glioblastoma line D-54.

products or complexes of degradation products of rgp120 were detected with D-54 cells, it is likely that a single molecular species of approximately 180 kDa was cross-linked to rgp120. The low intensity of the cross-linked complex in comparison to the CD4-rgp120 complex on Molt4 cells is astonishing, since the number of CD4 molecules per cell is similar to the number of high-affinity receptors and less than the number of lower affinity receptors on D-54 cells. Because of the low intensity, one could suppose that the protein receptor corresponds to the lower abundance, higher affinity class of binding sites. However, one reason for the low intensity of the cross-linked complex could be that cross-linking of rgp120 with the protein receptor with lower affinity to gp120 is competed by the higher affinity binding of rgp120 to galactosyl-sulfatide on the surface of D-54 cells.

It was found that antibodies to the membrane lipid GalC can induce an increase of intracellular Ca^{2+} concentrations in oligodendrocytes, but only after treatment of cells with very high concentrations of antibodies ($\geq 470 \mu\text{g/ml}$; Dyer and Benjamins, 1990). We did not detect any increase of intracellular Ca^{2+} in response to rgp120 in D-54 cells. The signal transduction pathway of the observed tyrosine phosphorylation is clearly of a different nature, since antibodies to galactosyl-sulfatide did not lead to tyrosine phosphorylation, and treatment of cell surfaces with trypsin abolished the transduced phosphorylation signal. The tyrosine-phosphorylated 130- and 115-kDa proteins might be subunits of a receptor complex with the cross-linked 180-kDa rgp120-binding molecule. However, it is not possible to clearly judge whether phosphorylation is inter- or intramolecular based on the rapid kinetic of tyrosine phosphorylation in intact cells. Our control using CD4⁺ Molt4 cells did not reveal induction of tyrosine phosphorylation in response to rgp120, which is in accordance with the results of Horak *et al.* (1990) for T lymphocytes. Tyrosine phosphorylation of proteins with molecular masses in the range of 110 to 140 kDa has been described to occur after activation of many cell surface receptors on brain cells including receptors for neurotrophic factors (Soppet *et al.*, 1991; Squinto *et al.*, 1991), the interleukin-6 receptor (Hibi *et al.*, 1990), and receptors of the integrin family (Kornberg *et al.*, 1991). It will be of great interest to identify the nature of the receptor complex we found and the tyrosine kinase involved.

Programmed cell death or apoptosis is a physiological mechanism involved in normal tissue turnover during embryogenesis and adult life, especially in the brain, bone marrow and thymus (McConkey *et al.*, 1990). In AIDS patients, this mechanism has been suggested to be responsible for T helper cell depletion

(Ameisen and Caprone, 1991) and could also cause cellular dysfunction and atrophy as observed in the brain (Fauci, 1988). In addition, the HIV envelope glycoprotein gp120 has been shown to increase intracellular Ca^{2+} concentrations together with NMDA receptor agonists and to injure rodent neurons *in vitro* (Breneman *et al.*, 1988; Dreyer *et al.*, 1990; Lipton *et al.*, 1991). These mechanisms and the observed tyrosine phosphorylation in response to rgp120 in glial cells could provide a combination of signals leading to astrocytosis, impairment of myelin, and neurodegeneration as found in brains of AIDS patients. Further experiments are required to investigate the pathogenetic significance of this tyrosine kinase signal transduction pathway in neuro-AIDS.

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