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Neurotoxic Activity of the HIV-1 Envelope Glycoprotein: Activation of Protein Kinase C in Rat Astrocytes

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Abstract: Envelope glycoprotein (gp120) of the human immunodeficiency virus type one (HIV-1), has adverse effects on glial cells and neurons. This study reports on the direct effect of recombinant gp120 (r-gp120) produced from different expression systems on protein kinase C, as a measure of relative neurotoxicity. Brain cells were grown *in vitro* from explants of the cerebral cortex of newborn rats, and recombinant gp120 preparations expressed in mammalian cell/vaccinia virus and insect cell/baculovirus systems were applied to astrocyte-enriched cultures. The gp120 preparations activated protein kinase C (PKC) to similar levels in these cells. Mutant recombinant gp120 lacking the amino-terminal 29 amino acids produced from the mammalian and insect cells also activated PKC to similar levels as did the full-length protein. The recombinant proteins specifically activated PKC β and ζ, suggesting that they are able to induce both Ca^{2+}-dependent and Ca^{2+}-independent isoforms of this enzyme. Alteration of PKC activity in astrocytes by gp120 indicates its ability to modulate gene expression, which is associated with the neurotoxicity of this protein**.** Furthermore, the results suggest that the deletion of the first 29 residues of NH_2-terminal end of the gp120 does not affect the functional activity of this protein with regard to modulation of signal transduction in astrocytes.

Keywords: HIV, gp120, astrocytes, baculovirus, recombinant, protein kinase C.

Introduction

Infection of human by HIV-1 is associated with a neurological syndrome termed AIDS dementia complex (ADC) which consists of cognitive /motor dysfunction accompanying encephalitis [1-4]. Cells of the monocyte-macrophage lineage including microglia are productively infected by HIV-1 in the brain, although the presence of the virus in other brain cells has also been reported [5, 6]. Replication of HIV-1 in the brain and the effects of its glycoprotein (gp) 120 are associated with the brain lesions of ADC. Expression of HIV-1 gp120 in the brain of transgenic mice was reported to have caused lesions that are similar to those in HIV-1 infected patients, including reactive astrocytosis [7]. Neuronal derangements during HIV-1 infection are therefore attributable, in part, to the direct effect of gp120 on astrocytes leading to disturbance of the functions of these cells, including homeostatic regulation of the neuronal microenvironment [8].

The objective of this study was to determine the biological activity of full-length HIV-1 gp120 and its mutant with N-terminal deletion, with respect to induction of signal transduction in astrocytes. Full-length HIV-1 gp120 expressed by the vaccinia /COS-7 cells and insect cell/baculovirus system and the deletion mutants of gp120 (lacking the NH_2-terminal 29 amino acids) expressed in COS-7 cells and insect cell/baculovirus system were applied to rat astrocytes and induction of signal transduction was determined. Both full-length and mutant preparations of HIV-1 gp120 induced PKC activity in the treated cells, suggesting that the NH_2-terminal 29 amino acids are not essential for the induction of signal transduction.

Materials and Methods

The following reagents were purchased from the companies specified: rabbit polyclonal antibodies to PKC isozymes from Life Technologies, Gaithersburg, MD; rabbit serum, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG), rabbit anti-glial fibrillary acid protein (GFAP) and phorbol-12 myristate-13-acetate (PMA) from Sigma Chemical Co, St Louis, MO; PKC enzyme assay kit from Amersham-Pharmacia Biotech, Evanson, IL; full-length baculovirus-produced recombinant gp120 from ICN, Costa Mesa, CA, Bac-to-Bac baculovirus expression kit from Invitrogen-Life Technologies, Carlsbad, CA and COS-7 and baby hamster kidney (BHK) cell lines from American Type Culture Collection (ATCC), Rockville, MD. vPE8 recombinant vaccinia virus [9] and soluble CD4 were obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH.

Expression of full-length recombinant gp120

vPE8 (r-gp120-expressing vaccinia virus) was propagated in BHK cells. Culture supernatant was cleared of debris. Virus pellet collected by centrifugation at 100, 000 g for 1 h at 4^{o}C was suspended in NET buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris hydrochloride, pH 7.4) and stored in aliquots in

liquid nitrogen. The virus was titrated in BHK cells and used to infect COS-7 cells at a multiplicity of infection (MOI) of 0.1. The cells were grown in Dulbecco's modified Eagles medium (DMEM) with 2% glutamine, 10% fetal bovine serum, 100 U penicillin, 100 μg streptomycin at 37^{o}C in a humidified atmosphere of 5% CO_2. Culture supernatant were collected 4 days post-infection, and concentrated for purification of expressed r-gp120 as explained below.

Expression of truncated gp120 in COS-7 cells

The gp120 in pCAS-ENV (Biogen, Cambridge, MA)m, which lacks the first 29 NH_2-terminal amino acids [10] was subcloned into pcDNA6/HisA vector (Invitrogen) using the manufacturers protocol in the kit. Ten μg of the plasmid was introduced into the cells with lipofectin (Invitrogen-Life Technologies), using the protocol of the manufacturer. The cells were seeded in 100 cm^3 plates, and maintained at 37^{o}C in humidified incubator with 5% $C0_2$ atmosphere. Recombinant gp120 was purified from concentrated culture supernatant and cell lysate using the affinity nickel column kit (Novagen, Madison, WI).

Expression of truncated gp120 in insect cell/baculovirus system

Gp120 in pCAS-ENV was amplified by the polymerase chain with 18 nucleotides at both ends of the gene as primers, and blunt ligated to pCNTR in General Contractor kit (5 Prime, 3 Prime, Boulder CO). The insert was subcloned in the multiple cloning site of the baculovirus shuttle vector, pFASTBAC (bacmid). The protocol in the BAC-TO-BAC baculovirus expression kit (Invitrogen) was used for the protein expression and purification.

Determination of CD4 binding and immunoreactivity of expressed r-gp120.

For the determination of expression of r-gp120 by the virus- infected or DNA-transfected cells, the cells were grown in media with or without [^{3}H]methionine. The culture supernatant was cleared of debris and virus particles by centrifugation at 100,000 g. Agarose beads (Sigma) were linked to soluble CD4 according to the manufacturers instructions, and used to precipitate r-gp120 from solution. The suspension of agarose bead-CD4 complex was incubated with the culture medium overnight on a rotating platform at 4^{o}C, and then washed 3 times in PBS. The bound protein was eluted in 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and analyzed in 10% PAGE [11]. For immune precipitation, aliquots of the cleared supernatant were incubated with polyclonal anti-gp120 at 4^{o}C for 6 h. The mixture was then incubated with 50% suspension of Protein A agarose beads overnight at 4^{o}C on a rotating platform. The beads were precipitated by centrifugation at 6,000 g, washed three times with phosphate buffered saline (PBS) pH 7.4, and boiled in 2x SDS-PAGE loading buffer for analysis in 10% PAGE.

Purification of vPE8/COS-7-expressed recombinant gp120

The supernatant of cell culture from vaccinia infected COS-7 cells were concentrated by passing through a filter with molecular weight cut-off of 30 kDa (Millipore Corporation, Bedford, MA). The glycoproteins in the concentrate were adsorbed onto a column of agarose-lentil lectin (Sigma) and eluted with 10% methyl mannoside. Peak elution reacting with sheep anti-gp120 polyclonal antibody was purified by ion exchange chromatography on DEAE 52 cellulose (Amersham Pharmacia Biotech) eluting with sodium chloride gradient from 0 to 0.8 M in 20 mM Tris-HCl buffer. The purified peak fraction was dialyzed against 20 mM Tris-HCL, pH 8.2 and concentrated using a filter with molecular weight cut-off of 30 kDa. Protein concentration was determined by the Bradford method [12]. Immunoreactivity of purified protein was analyzed by Western blot assay.

Rat astrocyte culture

Isolation and culture of rat astrocytes were as described previously [13]. One-day old rats were sacrificed by decapitation. The meninges were removed from the cerebral cortex and the brain cells dissociated with collagenase [14]. Cells were filtered through nylon mesh (120 μm) and cultured in glutamine-free Dulbecco's modified Eagles medium with Ham's F12 (DMEM//F12). The DMEM with pyruvate contained 4.5 mg of glucose /ml, 15 mM HEPES buffer. Culture medium contained penicillin 100 U/ml and streptomycin 100 μg/ml and 10% heat inactivated fetal bovine serum. Following viability test, cell suspension was adjusted to 6 x 10^5 cells per ml in culture medium, and 5 ml of this cell suspension were placed in 75 cm^3 tissue culture flasks, and incubated at 37^{o}C in a humidified atmosphere of 5% $C0_2$. On day 3, the medium was changed, and after another 3 days of culture, fresh medium was added to the attached cells. The flask was tightly capped, wrapped over with parafilm, fastened to a bench orbital shaker, and shaken at 250 rpm for 2 h. The supernatant was discarded and replaced with a fresh medium. The shaking procedure was repeated daily for another 3 days. The purity of the adherent cells was determined by staining for GFAP immunohistochemically. Only preparations that were at least 98% pure astrocytes were used for experiments.

Assay of PKC activity in cytosolic preparation

Cultured astrocytes were treated with insect cell/baculovirus-expressed r-gp120 (0.5 μg /ml) or 1 μM of PMA for 30 min and washed in PBS. The cells were disrupted in lysis buffer [50 mM Tris-HCl, pH 7.5, 10 mM EGTA, 1% Nonidet P-40, 0.3% (w/v) 2-mercaptoethanol, 1 mM phenyl methyl sulphonylfluoride (PMSF), 25 μg/ml leupeptin, 0.1mg/ml aprotinin and 50 μg/ml trypsin inhibitor] and homogenized at 4^{o}C. PKC activity in the supernatant of cell homogenate after centrifugation at 10,000 g was determined using a kit procedure (Amersham-Pharmacia Biotech).

Assay of PKC activity in fixed cells

Cultured astrocytes were treated with 0.5 μg /ml of r-gp120 or 1 μM of PMA, for 30 min. For estimation of PKC activity induced, cultured cells were fixed in 0.25% paraformaldehyde for 1 h at 4^{o}C. After washing in phosphate-buffered saline (PBS), the cells were permeabilized with 0.02% Tween 20 at 37^{o}C for 10 min. They were then washed three times in cold PBS and incubated with rabbit polyclonal antibody to PKCβ or PKCζ for 30 min. After washing, the cells were incubated with goat anti-rabbit IgG-FITC conjugate. Control cells were treated only with normal rabbit serum and goat anti-rabbit IgG-FITC. Cells were scanned for fluorescence in a cell sorter (Coulter, Miami, FL). The area of peak fluorescence was measured for each test. The value obtained in the test with r-gp120 was expressed as a percentage of the peak fluorescence induced in experiment with PMA.

Results

The immunoreactivity and CD4 binding of each protein expressed was first ascertained. To determine immunoreactivity, lectin-bound protein, which had been enriched by ion exchange chromatography was analyzed by SDS-PAGE and Western blotting, and then reacted with anti-gp120 polyclonal antibody. Distinct protein of 120kDa was obtained for the expressed full-length r-gp120 expressed in COS-7 cells. This was similar in size to the insect cell /baculovirus-expressed protein (Fig. 1). The mutant proteins were approximately 98 kDa (data not shown). To determine the biological property of the recombinant protein with respect to CD4 binding, the expression was done in [^{3}H]methionine-labeled cells. Proteins precipitated from the culture medium by CD4 were analyzed by SDS-PAGE. Both full-length r-gp120 expressed by mammalian cells and insect cells (data not shown) and the mutant proteins (Fig. 2) bound to CD4.

The full-length r-gp120 preparations produced from two different sources were of similar molecular weights. Similarly, the mutant r-gp120 preparations from different expression systems had similar molecular weights, as indicated in Figs. 1 and 2. Addition of HIV-1 r- gp120 protein to purified astrocytes at concentrations from 0.1 μg/ml to 1.0 μg/ml did not induce cell death. This was confirmed by trypan-blue staining of the cells.

Induction of PKC activity

To determine induction of signal transduction in cells stimulated with PMA and r-gp120, PKC was estimated in a mixture of the cytosolic and particulate fractions of the cell homogenate. PKC activity was significantly ($p<0.05$) increased in cells stimulated by PMA and r-gp120 than in control cells (Fig. 3). At the concentrations of the inducers used, the stimulation of PKC activity by r-gp120 (0.5 μg/ml) was significantly ($p<0.05$) higher than that by PMA (1 μM).

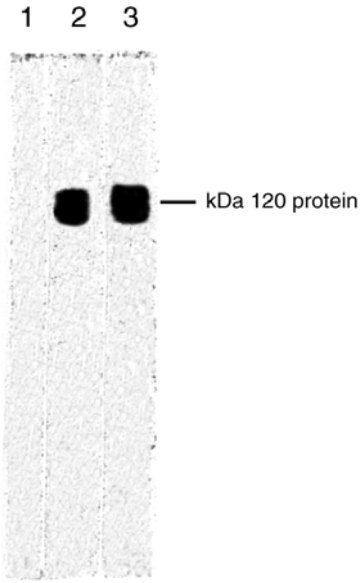

Figure 1. Immunoblot analysis of r-gp120 produced by vEP-COS-7 cells and insect cell/baculovirus system. Virus-free culture supernatant of vEP-infected COS-7 cells was run through lentil lectin-conjugated agarose bead column. Bound glycoprotein was eluted with 10% mannoside in PBS/Tris buffer, purified by DEAE 52 ion exchange chromatography, dialyzed and concentrated. Five μg of the concentrate was resolved by the SDS-PAGE and analyzed by Western blotting, using anti-gp120 polyclonal antibody. Lane 1: preparation from control cell supernatant; lane 2: preparation from vEP-infected COS cells; lane 3: purified insect cell/baculovirus system-expressed r-gp120.

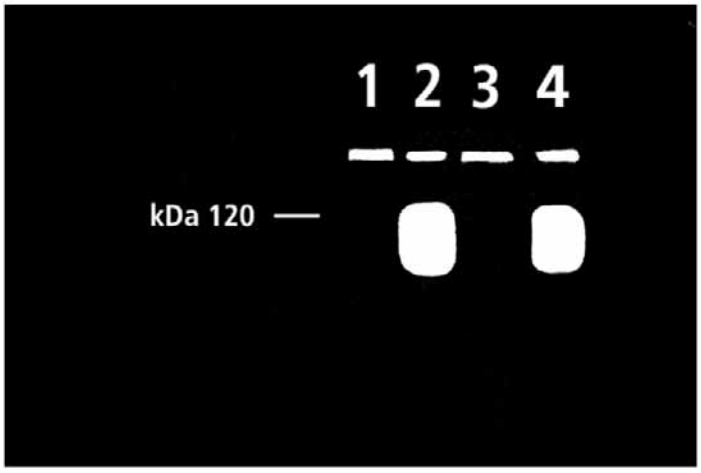

Figure 2. CD4-precipitation of [^{3}H]methionine-labeled mutant r-gp120 with deleted NH_2-terminal 29 amino acids. Cell culture supernatant was cleared of debris by ultra-centrifugation, concentrated and incubated with agarose beads conjugated to soluble CD4 overnight. After PBS washing, the beads were boiled in 2X SDS-PAGE loading buffer, and the supernatant analyzed in 10% PAGE, followed by auto-radiography of the dried gel. Lane 1: control untransfected COS-7 cells; Lane 2: mutant r-gp120 expressed in COS-7 cells; Lane 3: insect cells not infected with recombinant baculovirus; Lane 4: mutant r-gp120 expressed in insect cell/baculovirus system.

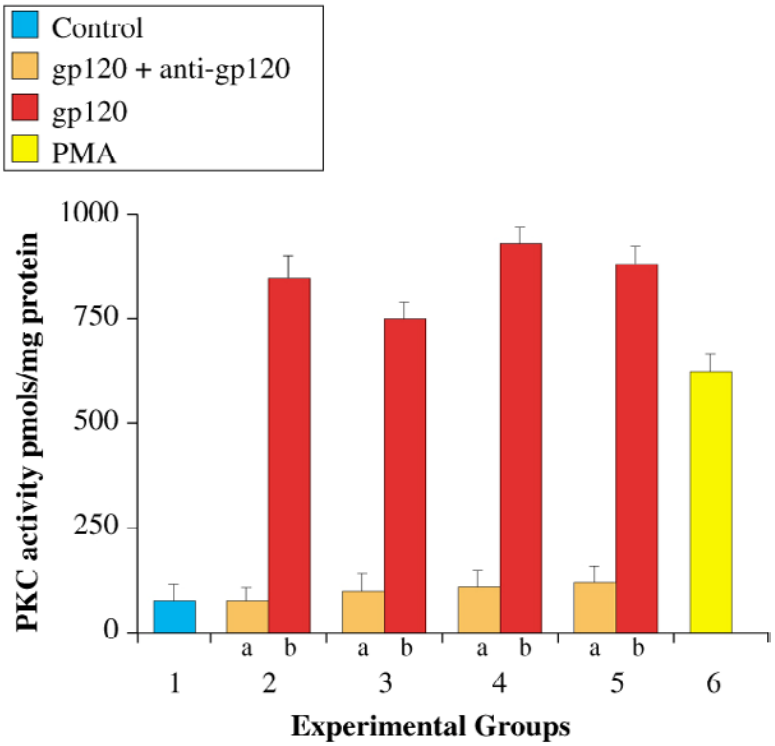

Figure 3. Effect of stimulation of astrocytes with gp120 and PMA on PKC enzymes. PMA or baculovirus system–expressed r-gp120 with or without polyclonal antibody to gp120 was applied to astrocytes in culture. PKC was assayed in the supernatant of cell homogenate, which had been centrifuged at 10,000 g. Induction of PKC by PMA was significantly less than by r-gp120 ($p<0.05$). Sheep anti-gp120 (1:20) effectively blocked the activity of r-gp120.

In order to determine if certain PKC isozymes are preferentially stimulated, the level of activity of PKCβ and PKCζ isozymes was estimated in whole cells by flow cytometry, following application of the recombinant proteins. The level of PKC activity induced by r-gp120 was expressed as a percentage of the value obtained when the activation was by PMA. Both full-length r-gp120 expressed by insect cell/baculovirus system (Bac–r-gp120) and by vaccinia/mammalian cell system (vPE8-r-gp120) were able to induce PKC activity in the rat astrocytes (Fig. 4). Both recombinant mutant r-gp120 proteins expressed by mammalian cells (COS7-r-gp120DL) and by insect cell/baculovirus system (Bac-r-gp120DL) showed equivalent ability to induced PKCβ and PKCζ activities (Fig. 5).

The levels of PKCβ and PKCζ activities induced by full-length gp120 were not significantly higher ($p>0.05$) than those by mutant r-gp120. In all tests with the r-gp120 or PMA, the activity of PKCβ induced was significantly ($p<0.05$) lower than that of PKCζ.

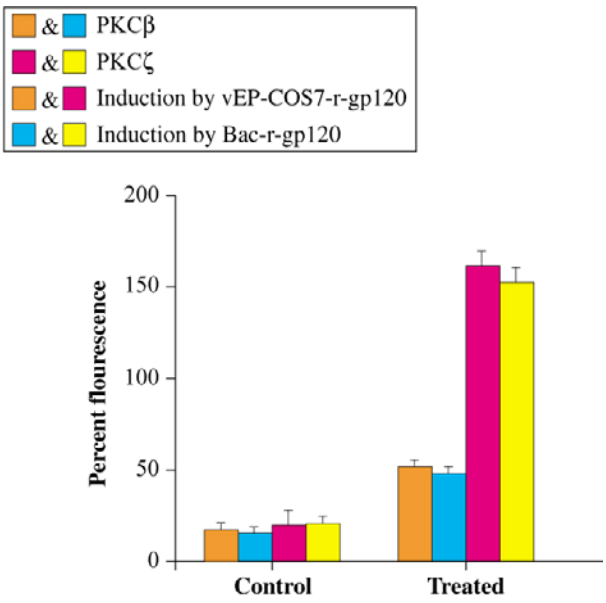

Figure 4. Effect of stimulation of astrocytes with recombinant gp120 on PKCβ and PKCζ. PMA or full-length recombinant gp120 produced from vaccinia-infected COS-7 cells (vPE8-COS7-r-gp120) or by insect cell/baculovirus system (Bac-r-gp120) was applied to rat astrocytes. PKC isozyme activity is presented as a percentage of the activity induced by PMA. Each bar represents a mean of five assays. Both protein preparations stimulated PKC to a similar level. PKCζ response was significantly ($P<0.05$) higher than that of PKCβ.

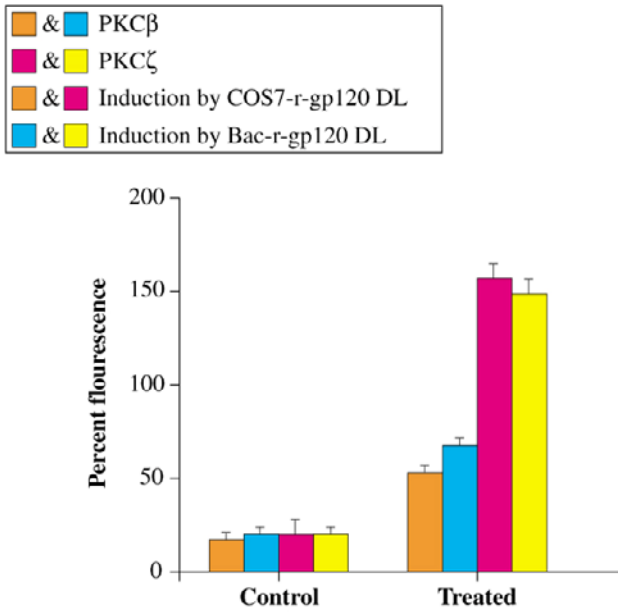

Figure 5. Effect of stimulation of astrocytes with recombinant mutant gp120 on PKC. PMA or recombinant mutant gp120 produced from COS-7 cells (COS7-r-gp120DL) or by insect cell/baculovirus system (Bac-r-gp120DL) was applied to rat astrocytes. PKC isozyme activity is presented as a percentage of the activity induced by PMA. Each bar represents a mean of five assays. Both protein preparations stimulated PKC to a similar level. PKCζ response was significantly ($P<0.05$) higher than that of PKCβ.

Discussion

Full-length r-gp120 produced in mammalian and insect cells showed similar molecular weights. Also, the two preparations of mutant r-gp120 protein with deleted NH_2-terminal 29 amino acids had similar molecular weights. This suggests that the level of glycosylation of this protein may not be widely different when produced from the two systems.

PKC is primarily activated by diacylglycerol (DAG), which together with inositol 1, 4, 5 triphosphate (IP3), is a product of the hydrolysis of inositol triphosphates, following the interaction of the cell surface receptor with a ligand (15). PMA was used as a control ligand in the present study because, like DAG, it utilizes PKC to mediate signal transduction. The present results suggest that gp120 is one of the ligands that can alter PKC activity in astrocytes, thus setting up a cascade of events leading to neuronal damage during HIV-1 infection.

The mutant protein induced PKC in astrocytes to the same level as did the full-length protein. This indicates that the deletion did not affect the portion of the protein that enables the induction of signal transduction in astrocytes. This opens a question as to the functional role of the NH_2-terminal 29 amino acids of gp120.

PKC actually consists of a number of different molecules (16). These include the conventional PKC isozymes: α, β-1, β-II, and γ which require calcium, phospholipid and diacylglycerol for their activation. The other molecules include the novel PKC isozymes δ, ε, and η, which are calcium-independent and the atypical PKC, ζ and λ. These isoforms differ in tissue distribution, substrate specificity and kinetics of activation and inactivation. It has been earlier demonstrated that gp120 does activate both the calcium-dependent and Ca-independent isoforms in monocytic cell line, U937 (17). Only two of the isozymes, PKCβ and PKCζ. were tested in the present study, primarily to determine if the recombinant gp120 and its mutant have similar effects on isoforms of PKC. The results indicate that in astrocytes, both the calcium-dependent and calcium–independent PKC isozymes are inducible by HIV-1 gp120 and the particular mutant under consideration.

When PKC is activated, it phosphorylates certain cellular proteins, resulting in cellular activation and gene expression. Induction of PKC activity is an important biochemical step during cellular activation by HIV-1. Several reports indicate that HIV-1 and its gp120 induce signal transduction in target monocytic cells. These include observation of elevated Ca^{2+} and IP3 in lymphocytes and increased levels of Ca^{2+} and IP3 in HIV-1 infected H9 cells (18, 19). It is expected that this activation is associated with the induction of gene expression, including cytokine production in those cells. Accordingly, several investigations have demonstrated that HIV–1 is capable of inducing the production of various proinflammatory cytokines and monokines in monocytic cells, including interleukin–1 (IL-1), tumor necrosis factor alpha (TNF-α), interleukin-6 and arachidonic acid (20, 21, 22, 23).

Induction of PKC by gp120 in astrocytes therefore is an indication of the ability of this viral protein to activate certain genes in these cells, including GFAP (24**)** and, most likely, cytokines**.** If inflammatory cytokines are produced by astrocytes activated by HIV-1 gp120 in vivo, they can be very toxic to neurons, and thus contribute to the pathogenesis of the brain lesions in AIDS.

In conclusion, a 29-amino acid deletion of the HIV-1 gp120 in the NH_2-terminal region did not affect certain functional properties of the protein, including binding to CD4 and induction of signal transduction in astrocytes. Recombinant gp120 produced from mammalian and insect cell systems did not differ in their ability to induce signal transduction. Work is in progress to characterize the genes activated in astrocytes following induction of signal transduction by the HIV-1 gp120.

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