HTLV-III/LAV–Neutralizing Antibodies to an *E. coli*–Produced Fragment of the Virus Envelope

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Immunization with either an *Escherichia coli* recombinant segment of the human T-cell lymphotropic virus (HTLV-III/LAV) envelope protein (gp120) or with deglycosylated gp120 envelope protein produced antibodies that neutralize HTLV-III/LAV infection in vitro. Virus neutralization titers of these antisera were equivalent to those obtained with purified native gp120 as immunogen. This localizes at least one class of neutralizing epitopes to the carboxyl-terminal half of the molecule. In addition, native gp120 prevented HTLV-III/LAV–mediated cell fusion, whereas the recombinant gp120 fragment did not. This shows that although glycosylation is not required for induction of neutralizing antibodies, it may be important for interaction with CD4, the virus receptor. A segment of the HTLV-III/LAV envelope produced in *E. coli* may be an important ingredient of a vaccine for acquired immune deficiency syndrome.

HUMAN T-CELL LYMPHOTROPIC VIRUS (HTLV-III/LAV) has been identified as the cause of acquired immune deficiency syndrome (AIDS) and one of the most pressing issues to resolve is the feasibility of a vaccine to protect against viral infection. Important proteins to consider in this regard are those associated with the viral envelope. These are derived from a precursor, gp160, that is proteolytically cleaved to generate the external envelope glycoprotein gp120 and the transmembrane envelope glycoprotein gp41 (Fig. 1) (*1–5*). Several lines of evidence support the idea that gp120 is involved in important biological processes related to the viral life cycle and that a subunit vaccine based on gp120 may be possible. (i) Antibodies that neutralize HTLV-III/LAV infection have been found in sera of people with AIDS, ARC (AIDS-related complex), as well as in asymptomatic individuals infected with the virus (*6, 7*); and antibodies exist in such sera that bind to purified gp120 (*8*). (ii) Purified gp120 elicits production of neutralizing antibodies (*9*) as does a large segment of gp120 produced in mammalian cells (*10*). (iii) Direct binding of gp120 to the T4 receptor has been demonstrated (*11, 12*), and monoclonal antibodies recognizing certain epitopes of the virus receptor prevent viral infection (*12*).

The protein backbone of gp120 represents approximately half of its apparent weight on SDS–polyacrylamide gels (*13*) with the remainder consisting of carbohydrate. This extensive degree of glycosylation might be expected to play a role in the determination and accessibility of both binding and neutralizing epitopes. We undertook the present study in an effort to discern the extent of carbohydrate involvement in both the induction of neutralizing antibodies to the virus and in binding of the envelope to CD4 as evidenced by inhibition of HTLV-III/LAV–mediated cell fusion. To achieve this, antibodies raised against native gp120, gp120 from which the carbohydrate had been enzymatically removed, and selected recombinant fragments of gp120 expressed in *Escherichia coli* were compared in various immunologic and biologic assays.

The recombinant gp120 fragment, PB1 (Fig. 1), is a fusion protein containing most of the COOH-terminal half of gp120. PB1 contains 180 amino acids encoded by the DNA sequence from the Pvu II to the second Bgl II site of the HTLV-III/LAV *env* gene (*13*) and has 30 and 24 additional non-HTLV-III/LAV amino acids on the NH_2- and COOH-termini, respectively. PB1, purified by means of a single chromatographic step (*14*), was more than 95% homogenous as judged by Coomassie blue staining of SDS–polyacrylamide gels (Fig. 2). PE3, a recombinant protein containing the NH_2-terminal half of gp120, is encoded by the DNA from the Kpn I to the first Bgl II site of the *env* gene. It is 286 amino acids long and has 43 and 11 additional amino acids on the NH_2- and COOH-termini, respectively. PENV9 consists of 300 amino acids encoded by the Bgl II to Bam HI fragment of *env* and has 54 amino acids of poliovirus protease (*15*) on the NH_2-terminus and 14 additional amino acids on the COOH-terminus. Both PE3 and PENV9 were purified to greater than 90% homogeneity.

The origin of the HTLV-III/LAV DNA encoding these proteins is the BH10 clone derived from the HTLV-III_B isolate (*13*). There are 13 amino acids from the NH_2-terminus of gp120 not contained within PE3, and 13 amino acids are encoded by the HTLV-III_B genome between the COOH-terminus of PE3 and the NH_2-terminus of PB1. Overall, 455 of the predicted 481 amino acids of gp120 (94%) and 241 of the 345 of gp41 (68%) are contained within PE3, PB1, and PENV9. PB1, PE3, and

Table 1. HTLV-III/LAV–neutralizing antibodies and gp120 precipitating efficacies of goat immune sera. Neutralizing titers measuring reverse transcriptase are calculated from the assay described in Fig. 5. Neutralizing assays measuring p24 were done as described in Robert-Guroff *et al.* (*6*). A titer of 0 means that no detectable difference between immune and preimmune sera was observed. Results from sera of two different goats immunized with PB1 and PENV9 are shown. N.T., not tested. gp120 was purified and radioimmune precipitation assays of ^{125}I-labeled gp120, labeled by means of chloramine-T, were performed as in Robey *et al.* (*9*). All sera dilutions in the radioimmune precipitation assays were 1:100. All preimmune goats precipitated approximately 0.9% of labeled gp120.

Immunogen	Percentage of total labeled gp120 precipitated	HTLV-III/LAV–neutralizing titers	
		Indirect immunofluorescence of p24	Reverse transcriptase
PB1	19	1:25	1:25
PB1	18	1:60	1:20
gp120	29	1:50	1:30
DG-120	37	1:50	1:30
PE3	38	N.T.	0
PENV9	50	N.T.	0
PENV9	31	N.T.	0
Preimmune goat	0.9	—	—

N.T., not tested.

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PENV9 react in Western analyses with antibodies in sera from selected AIDS patients.

Endoglycosidase F (Endo F) was used to remove the bulk of the carbohydrate residues from gp120 (*16*) that had been purified by affinity chromatography (*9*). This treatment converts the molecule to a species (DG-120) that migrates at 58 to 60 kD (Fig. 3). This is approximately the predicted size of the nonglycosylated polypeptide (*13*). The 58- to 60-kD product was excised from an SDS–polyacrylamide gel and used as the immunogen. The size heterogeneity of Endo F–treated gp120 may have been due to incomplete digestion or to heterogeneity of the HTLV-III$_B$ virus pool from which gp120 was isolated. The HTLV-III$_B$–producing H9 cell line contains more than one integrated provirus (*13*).

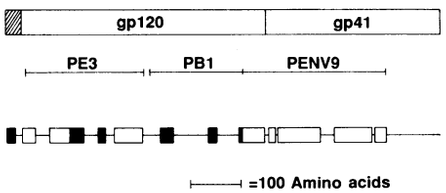

Fig. 1. Location of proteins PB1, PE3, and PENV9 in relation to gp120 and gp41. The envelope is synthesized as a precursor, gp160, that is cleaved to give gp120 and gp41. The secretion signal sequence is hatched. The location of the variable and conserved regions (*4*) are shown as darkened or open boxes, respectively.

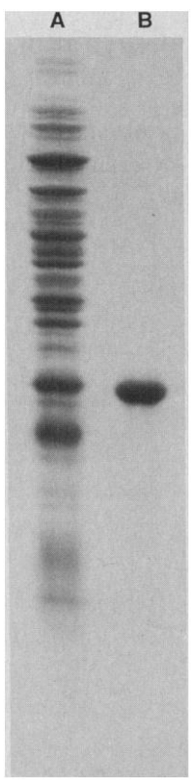

Fig. 2. Purification of PB1, PE3, and PENV9. A Coomassie blue–stained SDS–polyacrylamide gel of (A) a total cellular lysate containing PB1 and (B) purified PB1. We purified PB1 using a procedure of cell lysis with lysozyme and glass bead disruption, centrifugation, extraction of the protein from the cell pellet with 6*M* guanidine hydrochloride, dialysis into 8*M* urea, and carboxymethyl-Sepharose chromatography (Pharmacia) in the presence of 8*M* urea. PB1 precipitates when the urea is removed by dialysis, and the precipitated protein was used as the immunogen. PE3 and PENV9 were purified in a manner similar to PB1. Two goats and two rabbits were immunized with 2 and 1 mg of PB1, respectively, in Freund's complete adjuvant and boosted after 21 and 42 days. Boosts were with 1 mg of protein in Freund's incomplete adjuvant. Immunizations with PE3 and PENV9 were done with essentially the same protocol except that 1.5 mg and 100 μg of PENV9 were used for the initial immunizations and boosts, respectively.

Goats were immunized with 2 mg of PB1, 1.5 mg of PE3, 100 μg of PENV9, or 50 μg of DG-120 and boosted as described (Figs. 2 and 3). The immune sera were tested for reactivity with HTLV-III/LAV proteins from virus-infected cells on Western blots and with purified native gp120 by radioimmune precipitation. Antibodies from animals immunized with either PB1, PE3, PENV9, or DG-120 precipitate 20 to 50% of the total labeled gp120 (Table 1). The efficacies of precipitation are equivalent to those of antisera of goat, horse, or rhesus monkeys immunized with native gp120 (*9*). In addition, sera from HTLV-III/LAV–infected individuals reacted to a similar extent in a Western analysis with gp120 and DG-120 (Fig. 4) or in enzyme-linked immunosorbent assays (ELISA's) with the respective antigens. This indicates that at least some epitopes on gp120 responsible for antibody binding by human sera are independent of carbohydrate.

HTLV-III/LAV infection was assessed with the H9 clone of the HT cell line (*17*). Production of the virus was monitored by expression of either the p24 core protein or reverse transcriptase. The supernatant virus (the HTLV-III$_B$ isolate) and diluted sera were preincubated and added to H9 cells. After 5 days, p24 was measured by indirect immunofluorescence with a p24-specific monoclonal antibody (*6*) or reverse transcriptase activity was measured after 7 to 10 days (*8, 9*). The amount of viral proteins present in a particular assay varies according to the time of cell harvest. Antisera to gp120, DG-120, and PB1 completely blocked infection at a dilution of 1:8, and no effect was seen at a 1:128 dilution (Fig. 5). In contrast, anti-PE3 and anti-PENV9 sera do not block viral infectivity even at a 1:8 dilution, even though both contain antibodies capable of precipitating gp120 to an extent comparable to gp120 sera.

Neutralizing titers with these antisera (Table 1) are expressed as the serum dilution at which virus infection is 60% of that with preimmune serum. Although neutralizing titers varied somewhat, depending on whether p24 or reverse transcriptase was monitored, the titers of anti-PB1 sera were equivalent to those of anti-gp120 and anti–DG-120 sera and also to those of antisera from horses or rhesus monkeys immunized with gp120. Neutralizing titers of antibodies from HTLV-III/LAV–infected people vary widely (*6, 7, 9*), and the anti-PB1 neutralizing titers were comparable. These results demonstrate that glycosylation of the envelope protein is unnecessary for an HTLV-III/LAV–neutralizing humoral immune response and that glycosylation is not required to hold the molecule in a conformation necessary to elicit neutralizing antibodies.

To further evaluate the role of carbohydrate on gp120, the ability of gp120 and the three recombinant fragments to prevent HTLV-III–mediated cell fusion was investigated. The gp120 protein is thought to play a central role not only in infection by the

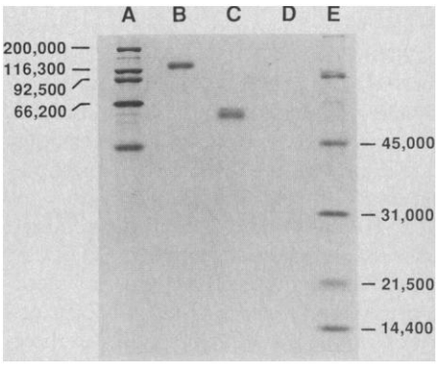

Fig. 3. Treatment of HTLV-III$_B$ gp120 with endoglycosidase F (Endo F). Molecular weight markers are shown in lanes A and E, gp120 in lane B, Endo F–treated gp120 (DG-120) in lane C, and Endo F in lane D. Purified gp120 (4 μg) in 0.05*M* sodium acetate, *p*H 5.5, and 0.025% SDS, was incubated with 0.05 unit of endoglycosidase F (Boehringer Mannheim) for 2 hours at 37°C. After digestion, the sample was analyzed on a 12% SDS–polyacrylamide gel. The gp120 used was purified from HTLV-III/LAV–infected cells by affinity chromatography and dialysis against water (*9*). After Endo F treatment, DG-120 was purified by SDS–polyacrylamide gel electrophoresis and 50 μg in Freund's complete adjuvant was used to immunize one goat. The animal was boosted twice with 50 μg of protein according to the regimen described for the immunizations described in Fig. 2.

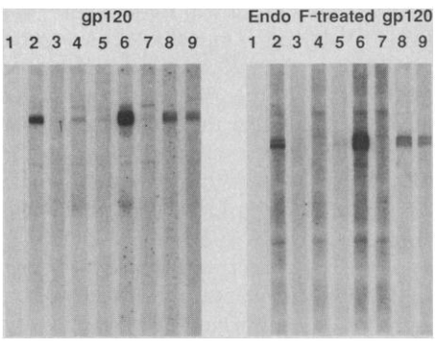

Fig. 4. Western blot comparison of human sera reactivity to gp120 and endoglycosidase-treated gp120. Purified gp120 and deglycosylated gp120 (0.4 μg each per lane) were electrophoresed on 10% SDS–polyacrylamide gels and transferred to nitrocellulose. Sera were diluted 1:100 and reacted with the Western strips. Lane 1 is normal human sera and lanes 2 through 9 are sera from AIDS, ARC, or asymptomatic HTLV-III/LAV–infected individuals.

virus but in the formation of multinucleated giant cells by cell fusion (*12, 17, 18*) and this process is mediated by attachment of gp120 to a portion of the CD4 molecule (*18*). We developed an assay system in which CD4-bearing CEM cells fuse with HTLV-III–infected cells to yield giant cells enumerable by microscopic examination within a 24-hour period (Fig. 6B). Purified gp120 completely inhibited the fusion process (Fig. 6C) presumably by saturating the CD4 molecules on the uninfected cells and preventing their attachment to cell-associated gp120 on the infected cell partners. Concentrations of gp120 as low as 2 n*M* were sufficient to block cell fusion over a 4-day period. None of the recombinant fragments demonstrated such activity even at molar concentrations 50-fold greater than the effective concentration of gp120 (Fig. 6, D, E, and F).

These results indicate that the requirements for induction of neutralizing antibodies can be distinguished from those involved in virus/receptor interactions and focus attention on the role of carbohydrate in these functions. The role of the carbohydrate on gp120 and other cell and viral surface proteins is not firmly established. The general finding is that *N*-linked glycosylation is not required for glycoprotein binding to receptors or for antigenic integrity (*19–22*) but does confer protease stability to the polypeptide. For example, the carbohydrate of murine retrovirus components dramatically influences immunoreactivity in heterologous species (*23*). It is possible that the extensive glycosylation of gp120 may block potential neutralizing epitopes.

PB1 contains 180 of the 481 amino acids of gp120 (37%) and 12 of the 24 potential *N*-linked glycosylation sites (*13*). Even though it is less than half the size of the protein component gp120, PB1 contains epitopes that allow complete neutralization of HTLV-III/LAV infection. The finding that PE3 and PENV9 elicit antibodies that bind strongly to the envelope proteins but that do not neutralize the virus suggests that neutralizing epitopes are not located in the NH_2-terminal half or the extreme COOH-terminal half of gp120 or in the NH_2-terminal two-thirds of gp41. These findings do not rule out, however, the possibility that these regions contribute to the tertiary structure of other neutralizing epitopes.

Nucleotide sequences of several HTLV-III/LAV isolates show amino acid sequence variability between viral isolates from different individuals and between sequential isolates from one individual (*4, 24–26*). This variability is clustered in gp120, and recent evidence indicates that neutralizing antibodies are virus type-specific or group-specific (*27*). For example, animal antisera to gp120

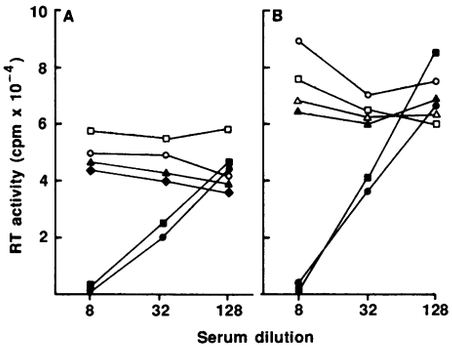

Fig. 5. Neutralization of HTLV-III/LAV infection with immune goat sera. (A) Sera to gp120 or DG-120 were diluted and assayed for virus neutralization. Preimmune sera (□ and ○) and immune sera (■ and ●) were from goats immunized with gp120 and DG-120, respectively, and immune sera from two goats immunized with PENV9 (▲ and ◆). (B) Sera to PB1 and PE3 assayed for virus neutralization. Preimmune sera (○, □, and △) and immune sera (●, ■, and ▲) were from two goats immunized with PB1 and a goat immunized with PE3, respectively. Diluted sera was mixed with HTLV-III_B and incubated 30 minutes at 37°C, and the mixture was added to H9 cells. After 7 to 10 days in culture, virus was measured by reverse transcriptase activity (*8, 9*). Similar virus neutralization results were observed with rabbit sera to these proteins.

isolated from the HTLV-III_B isolate does not neutralize another HTLV-III virus strain, the divergent RF isolate, whereas neutralizing antibody from an AIDS patient blocks infectivity of both isolates (*8*). Preliminary experiments reveal that anti-PB1 sera also do not neutralize the RF isolate. The region spanned by PB1 contains three of the five variable regions and none of the three conserved regions of gp120 (Fig. 1).

It is possible that the variable regions within PB1 play a role in neutralization specificity, and the first and third of these are predicted to be strongly antigenic (*28*). Amino acid sequence variations may present an obstacle to the development of an effective vaccine, which would have to neutralize multiple virus types.

Subunit vaccines produced from recombinant proteins offer several advantages over more traditional vaccine approaches with killed or attenuated viral preparations. First, recombinant HTLV-III/LAV envelope proteins would be safe to prepare and administer, and no genetic material would be introduced by vaccination. Second, purified recombinant envelope fragments are more readily produced than purified gp120 isolated from virus-infected cells. For example, 1 mg of purified gp120 is obtained from 500 g of infected cells whereas approximately 3 g of purified PB1 can be obtained from 500 g of *E. coli*. Even though much more PB1 than gp120 was used for immunization in the experiments presented here, it is not yet determined what minimal amount of either of these proteins is needed to elicit neutralizing antibodies. Finally, because it is produced from a recombinant gene, the sequence of PB1 can be altered by in vitro mutagenesis to give a spectrum of envelope sequences. Such experiments will also allow the location of the neutralizing epitopes to be defined more precisely and could lead to the preparation of synthetic peptides bearing neutralizing epitopes.

The ability of the PB1 recombinant fragment to induce neutralizing antibody in animals, combined with its apparent lack of

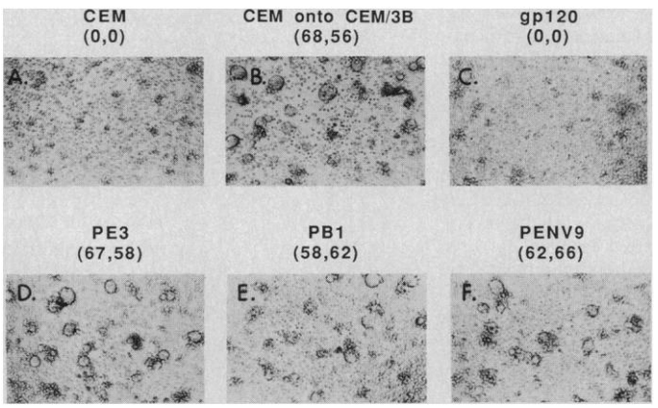

Fig. 6. Inhibition of HTLV-III/LAV–mediated cell fusion with purified gp120 but not with envelope recombinant peptides. CEM cells chronically and stably infected with HTLV-III_B were derived from an acutely infected culture of CEM cells and represent a subpopulation that were not susceptible to the cytopathological effects of the virus. The gross morphology of these cells does not differ from control CEM cells (A). About 75,000 CEM cells were mixed with 5,000 chronically infected CEM/HTLV-III_B cells in a final volume of 90 μl in 96 one-half wells (CoStar, A/2 cluster 96 wells, 1/2 area plates). Multinucleated cells are shown in (B). The test antigens were added to duplicate wells in a volume of 10 μl so that the final concentrations were 0.4 μg/ml gp120 (C), 10 μg/ml PE3 (D), 4 μg/ml PB1 (E), and 4 μg/ml PENV9 (F). The PB1 and PENV9 antigens were in SDS containing phosphate-buffered saline (PBS) so that the final concentrations of SDS in the assay were 0.0002 and 0.002%, respectively. These levels of SDS have no effect on the fusion process itself nor do they diminish the blocking activity of the purified gp120. Giant cell counts of duplicate wells are indicated in parentheses and a representative field for each treatment is shown at a 100× original magnification.

association with human T4 cells, has important ramifications from the standpoint of potential vaccine strategies. The binding of native gp120 as it might exist in a vaccine preparation to T4 cells could mask relevant epitopes that would otherwise be available for immunologic recognition. In addition, strong interactions with CD4 may have negative influences on various T4 cell functions as has been shown, for example, with the Leu-3 antibody suppression of soluble antigen proliferative responses (*29*). In view of this, the PB1 recombinant peptide might be a more attractive candidate for an HTLV-III/LAV vaccine than native viral gp120 even though both induce neutralizing antibodies. However, the efficacy of PB1 as a vaccine is still untested and final decisions must depend on immunizing chimpanzees that are susceptible to HTLV-III/LAV infection (*30, 31*). Even though many people exposed to HTLV-III/LAV do have neutralizing antibodies, it is unknown under what circumstances these are protective against viral infection. To what extent artificial immunization can mimic or improve natural immunity awaits further study.

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Oligodendrocyte Adhesion Activates Protein Kinase C–Mediated Phosphorylation of Myelin Basic Protein

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When isolated adult oligodendrocytes adhere to a substratum myelinogenesis occurs. Investigation of the mechanism by which this happens indicated that the oligodendrocyte-substratum interaction (i) activated protein kinase C–dependent phosphorylation of myelin basic protein and (ii) promoted the synthesis of myelin basic protein. In addition, when agents that activate protein kinase C (second messenger diacylglycerol or a tumor-promoting phorbol ester) were added to nonattached oligodendrocytes, they mimicked the influence of the substratum by inducing phosphorylation of myelin basic protein; and reagents that increase cellular adenosine 3′, 5′-monophosphate (cyclic AMP) inhibited phosphorylation of myelin basic protein. Thus, at least in vitro, the interaction between oligodendrocytes and the substratum may mediate myelinogenic events, and phosphorylation of myelin basic protein may be an early requirement in the sequence of steps that ultimately results in myelin formation.

CIRCUMSTANTIAL EVIDENCE INDICATES that cell-cell and cell-substratum interactions are key elements in central nervous system (CNS) myelination, but the origin and nature of the signals that initiate and control assembly and maintenance of myelin are unknown. In oligodendrocytes (OLG), the cells that assemble and maintain CNS myelin (*1*), myelination is preceded by the synthesis of myelin basic protein (MBP) and other myelin proteins and lipids. Myelin basic protein is phosphorylated (*2*); the functional significance of this modification is unclear, although it may be a signal for myelination (*3*). The fact that isolated OLG in culture express myelinogenic properties (*4, 5*) makes them suitable for investigating mechanisms that control myelin assembly. We have shown that, upon attachment onto a polylysine substratum, mature OLG re-enact events associated with myelinogenesis (*6–8*) and, over time in culture and without neurons, reform myelin, a process termed myelin palingenesis (*9*). We have now extended this work to investigate the mechanism by which the substratum influences myelinogenesis by observing early changes in metabolism after cell-substratum interaction. Our results indicate that the substratum transmits a signal that activates protein kinase C (PKC)–mediated phosphorylation of MBP, as well as promoting the synthesis of MBP.

Oligodendrocytes are isolated from ovine white matter (Fig. 1) and maintained in culture (*10*) as floating clusters (B3.f) or attached to a polylysine substratum (B3.fA). The appearance of live B3.f OLG, as viewed by phase microscopy, is shown in Fig. 1A. When B3.f OLG are plated onto a polylysine substratum, after having been maintained in vitro for 4 days as floating clusters, they adhere within 6 hours and undergo modifications in metabolism and morphology (*10*). Over time in culture, changes in morphology become readily apparent by light microscopy (Fig. 1, B and C). We can maintain B3.fA cultures for 120 days and longer with 99% purity, as assessed by immunocytochemical criteria (*9, 10*).

We then examined the influence of attachment on the synthesis and phosphorylation of MBP. B3.f and B3.fA cultures were labeled with $^{32}PO_4^{3-}$ for 30 minutes, MBP was immunoprecipitated, and the amount of radiolabel incorporated into MBP was assessed by autoradiography of the proteins separated by SDS–polyacrylamide gel electrophoresis (Fig. 2). The uptake of $^{32}PO_4^{3-}$ into MBP was low in B3.f cells but was considerably higher in B3.fA cultures (Fig. 2, lanes a and b, respectively). This differ-

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