BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 192–197 (1996)
Article No. 0034

Expression of HIV-1 Envelope Glycoprotein Alters Cellular Calmodulin

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Received November 3, 1995

Removal of parts of a known calmodulin binding site at the C-terminus of HIV-1 envelope glycoprotein, gp160, can result in diminished infectivity. We investigated whether expression of full length gp160 would result in changes in intracellular calmodulin compared to expression of gp160 truncated to remove both known calmodulin binding sites. Both Western and Northern blots demonstrated that expression of gp160 led to increased calmodulin when compared to expression of truncated gp160. The induced calmodulin was associated preferentially with a particulate subcellular fraction. Confocal immunomicroscopy confirmed the increase in calmodulin and also showed that there was enhanced colocalization of calmodulin with gp160. Understanding of the role of calmodulin in the viral life-cycle may lead to new therapeutics.

Calmodulin is intimately involved in the metabolism of T-cells, the primary target of HIV-1. Stimulation of T-cells by antigens, lectins or other activators is accompanied by an increase in intracellular calcium (1), and by increases in all calmodulin mRNA species (2), indications that calmodulin plays a part in activation. Further, trifluoperazine, a well-established calmodulin inhibitor, inhibits DNA replication in lectin stimulated lymphocytes (3), and phorbol ester stimulation results in calcium/calmodulin dependent changes in lipid metabolism (4). The various activation pathways may converge at the calmodulin dependent calcineurin control of the transcription factor NF-AT (5).

Theoretical studies have suggested that two regions of the human immunodeficiency virus-1 (HIV-1) glycoprotein gp41, the C-terminal segment of the mature, cleaved envelope glycoprotein gp160, should fall into that category of highly amphiphilic α-helix which might bind calmodulin (6,7). Subsequent evidence showed that calmodulin binds to peptides derived from these regions and to gp41 itself (8,9). Therefore, there is a strong probability that one of the intracellular interactions between HIV-1 and calmodulin is binding of calmodulin to gp41.

Experiments in which gp41 was truncated support the likelihood that calmodulin-gp41 interactions are important to the virus. Progressive truncations of the C-terminus (and therefore of the C-terminal calmodulin binding site) of gp41 from the viral strain HXB2D resulted in progressive loss of infectivity (10). Truncations deleting 67 amino acids or 147 amino acids (eliminating the C-terminal calmodulin binding site or all but three amino acids of the cytoplasmic domain, respectively) eliminated production of infective virus (10). These results are consistent with the possibility that calmodulin-gp41 interactions may be important in infectivity. Different host cells produce diverse responses to similar truncations (11,12) reinforcing the likelihood that interaction of gp41 with cellular components, such as calmodulin, influences the viral life cycle.

We have, therefore, investigated whether expression of gp160 which contains calmodulin binding sites alters calmodulin relative to expression of gp160 which does not. The data presented here indicate that expression of full length gp160 leads to markedly increased calmodulin relative to expression of truncated gp160 lacking the known calmodulin binding sites. This increased calmodulin colocalizes to a large degree with gp160.

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0006-291X/96 $12.00

MATERIALS AND METHODS

Cell culture. H-9 cells were grown in RPMI 1640 supplemented with 15% fetal calf serum in T75 flasks.

Immunocytology processing. Transfected H-9 cells were distributed into six well plates containing 22mm square cover slips. After a brief time to allow the cells to sink they were fixed for 1.5 minutes at room temperature by adding an equal volume of 0.1M cacodylate buffer, pH7.3, containing 250mM sucrose, 3.7% formaldehyde and 0.1% glutaraldehyde. The medium-fixative mixture was then aspirated and replaced with permeabilization buffer, 0.5%BSA and 0.5% Tween 20 in PBS, for 4.5 minutes. The permeabilization buffer was replaced with blocking buffer, 0.5% BSA in PBS, and the cells stored at 4°C overnight.

Staining. The coverslips were overlaid with fluorescein-conjugated gp160 antibody and with biotin-conjugated calmodulin antibody (0.1 to 0.4ng/ml) in blocking solution and incubated 1 hour at 37°C. The cells were then incubated in PBS, pH 7.4, containing 15% glycerol, 0.1mM propyl, antifade (Molecular Bioprobes) and Texas Red linked avidin for 30 minutes at 37°C. The avidin-Texas Red solution was placed with PBS, glycerol, propyl gallate, antifade storage solution and the cells placed in the dark at 4°C used for microscopy.

Confocal microscopy. Confocal images were obtained using a Sarastro scanning laser confocal microscope with Nikon optics. Photomultiplier settings and laser intensity were set with positive cells and IgG controls. They were then kept constant throughout an entire experimental series. Each image is representative of a minimum of 6 fields from a minimum of 3 experiments.

Antibodies. Dupont-NEN 9824 anti-gp160 was coupled directly to fluorophore using fluorescein isothiocyanate (FITC) (13). Our highly specific calmodulin monoclonal antibody (14) was biotinylated via a Succinimide reaction (15) using NHS-biotin (Pierce Chemical). For controls, nonimmune IgG's of the appropriate isotype were prepared identically.

Vectors. Both wild-type pFN (labelled pFNwt in the following text, and wt in the figures) and pFN which codes for truncated gp160 (in the text labelled pFNΔ147 and in the figures labelled Δ) have been described previously (10). The truncated gp160 (called gp160Δ147) produced in pFNΔ147 transfectants lacks 147 C-terminal amino acids, retaining only 3 cytoplasmic amino acids after the generally accepted transmembrane helical region. It consequently also lacks both known calmodulin binding sites. pFN is a non-infectious proviral construct which contains the entire genome of the HXB2D strain of HIV-1 except for 300 bp of the reverse transcriptase which have been deleted to render it inactive. It thus effectively replicates the viral context for most biological processes.

Transfection. Transfections of H-9 cells were carried out using DEAE-dextran (10). Relative transfection efficiencies for vectors expressing full length gp160 or truncated gp160 by cotransfection of pCH110 (16) and counting cells positive for β-galactosidase activity or by observing at low power fields of cells stained for gp160 (exterior epitope) 16 hours after transfection. There was no detectable difference in transfection efficiency between pFNwt and pFNΔ147.

Cell fractionation. The fractionation scheme was derived from that of Milankov and DeBoni (17). After repeated trituration of cell lysates and centrifugation to remove nuclei, the supernatants were combined to form the complete non-nuclear fraction. This was centrifuged at 100,000 xg to yield particulate and soluble (cytosolic) fractions.

Western blots. The technique for Western immunoblot of calmodulin has been optimized for our antibody (14). The monoclonal antibody (0.5mg/ml) was applied for one hour at room temperature. After three to five washes this was followed by alkaline phosphatase linked goat anti-mouse secondary antibody for one hour at room temperature, five washes in PBS-0.05% Tween 20, and alkaline phosphatase development.

Northern blots. Total RNA from approximately 2 X 10^7 H-9 cells was extracted by the single step, guanidinium thiocyanate-phenol-chloroform method (18). Denaturation of the RNA and overnight incubation of the blot were performed at 65°C. Densitometry was performed using a Lynx scanning densitometer, reversing the 28S ethidium bromide image to obtain RNA quantitation for normalizing the densities in each lane.

RESULTS

Western blots of cells and cell fractions from pFNwt and pFNΔ147 transfections were obtained at two time points post-transfection, 16 and 48 hours (Fig. 1). Analysis of whole cell lysates (LYS) showed that transfection of H-9 cells with pFNwt (wt) measurably increases calmodulin expression relative to transfection with pFNΔ147 (Δ). This was not evident at 16 hours. At 48 hours the difference was not as pronounced, apparently because the pFNΔ147 transfected cells have also begun to produce increased amounts of calmodulin. The differential effect of gp160 truncation on calmodulin concentrations appeared to be primarily localized in the particulate fraction (PEL), while the cytosolic fraction (SUP) showed little difference. It should be noted that, while the cytosolic fraction appears to have more calmodulin than the whole cell lysate, it has been treated considerably differently. Alterations of buffers and detergents may alter the sensitivity of the antibody probe, rendering comparisons between different fractions inaccurate. Comparisons between different transfections within the same fraction are of course valid.

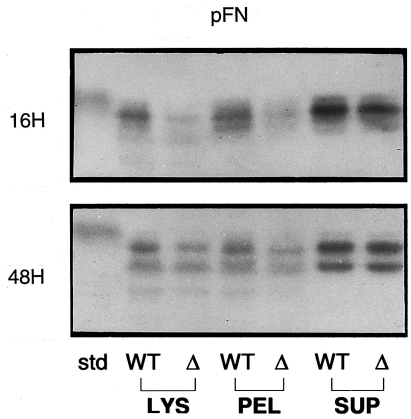

FIG. 1. WESTERN BLOTS OF CALMODULIN IN WHOLE CELL LYSATE MEMBRANE AND CYTOSOL FRACTIONS FROM pFNwt AND pFNΔ147 TRANSFECTED H-9 CELLS. Sixteen (labelled 16H) and 48 (labelled 48H) hours post- transfection the cells were lysed and fractionated as described in Materials and Methods into lysates (labelled LYS), pellets (PEL) and supernatants (SUP). Cells transfected with pFNwt are labelled WT, those with pFNΔ147, Δ. Equal amounts of protein were loaded in each lane of the gel. Following SDS-PAGE, calmodulin was detected by a Western blot. Note that calmodulin often appears as two bands in these gels, a well documented effect that is dependent upon the calcium concentration in the electrophoresis buffers.

The results in Fig. 1 were corroborated by Northern blot analysis (Fig. 2). Calmodulin has three different genes and five different transcripts (19). Only two transcripts, 1.7 kb and 4.0 kb, appeared in the blots. At 16 hours the 1.7 kb transcript from cells transfected with pFNwt (wt) was demonstrably more abundant than the 1.7kb transcript from cells transfected with pFNΔ147 (Δ). The ratios of 1.7kb transcript density to 28S RNA density from the same lane were: control (mock transfected cells), 0.28; pFNwt transfected, 0.57; pFNΔ147 transfected, 0.26. There was no density detectable for the 4.0 kb transcript. At 48 hours the disparity between 1.7 kb transcript amounts in wild-type and mutant transfectants was still evident. The ratios were: pFNwt transfected, 0.94; pFNΔ147 transfected, 0.49. Interestingly, at 48 hours the 4.0kb calmodulin transcript appears in the mRNA from pFNwt transfectants, while it does not appear in the mRNA of pFNΔ147 transfectants (pFNwt transfected, 0.79; pFNΔ147 transfected, 0).

In cells transfected with pFNwt had increased calmodulin which was largely colocalized with (Fig. 3, wt, green corresponds to gp160, red to calmodulin and yellow to coincidence of the two).

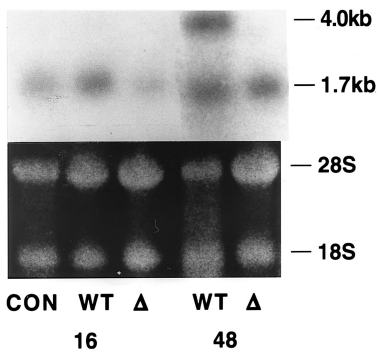

FIG. 2. DIFFERENTIAL CALMODULIN mRNA EXPRESSION IN H-9 CELLS TRANSFECTED WITH pFNwt AND pFNΔ147. At 16 and 48 hours post-transfection H-9 cells were collected and Northern blots performed as described in Materials and Methods. The top panel shows calmodulin mRNA. Bands appear at 1.7 and 4.0kb. The bottom panel shows the total mRNA (ethidium bromide stained) from which the top panel blot was transferred.

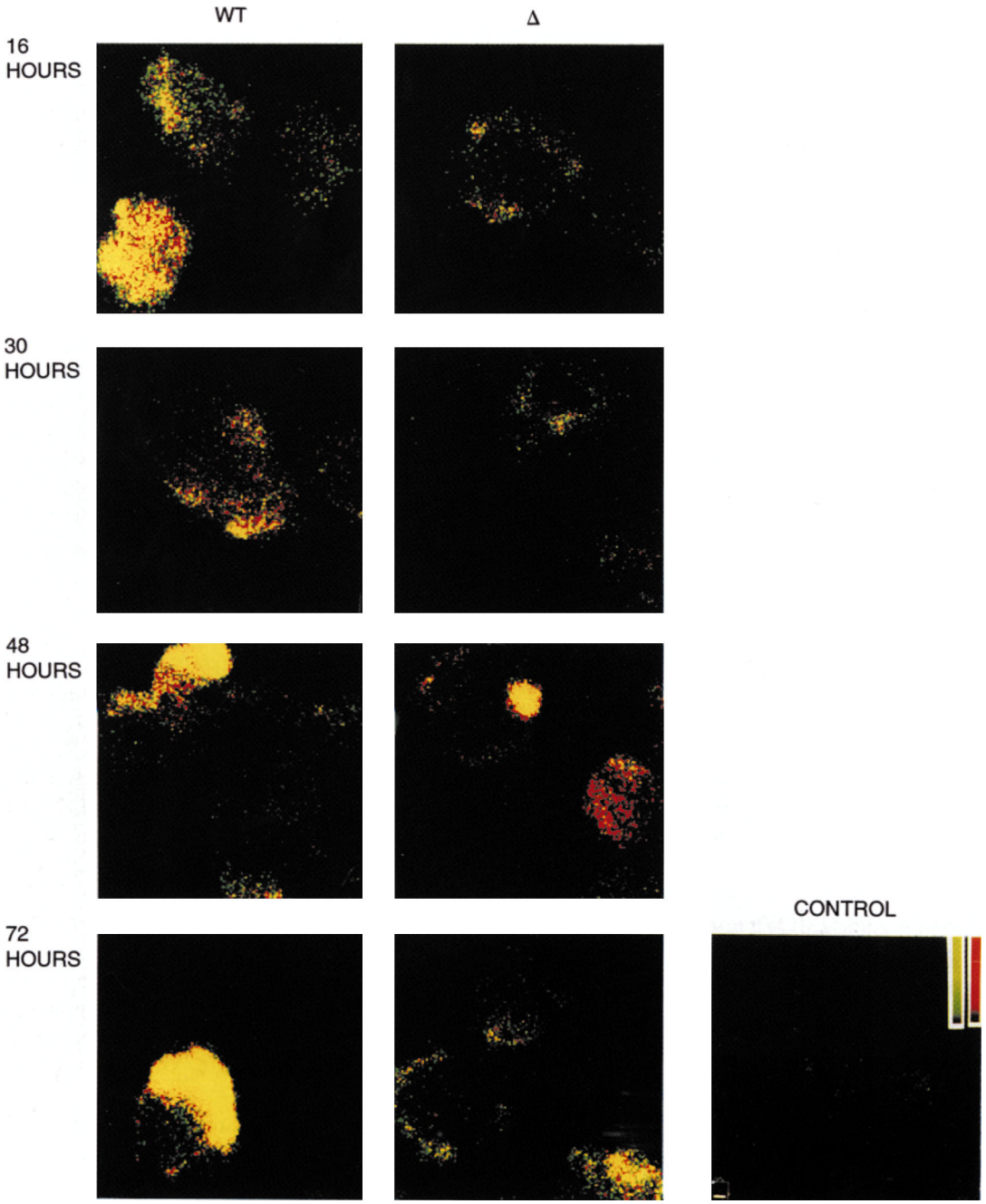

FIG. 3. COLOCALIZATION OF CALMODULIN AND gp160 IN H-9 CELLS TRANSFECTED WITH pFNwt OR WITH pFNΔ147. At 16, 30, 48, and 72 hours after transfection with pFNwt or pFNΔ147 cells were fixed, permeabilized, blocked and stained with fluorescein conjugated gp160 antibody and biotinylated calmodulin antibody (Materials and Methods). The cells were then exposed to avidin-Texas Red for 30 minutes at 37°C. Shown are single optical sections demonstrating the overlap (yellow) of the red (calmodulin) and green (gp160) staining (pFNwt transfectants in the left column (wt), pFNΔ147 transfectants in the right (Δ)). The panel labelled control shows pFNwt transfected cells stained with IgGs matched with the labelled reactive antibodies in concentration and fluorophore density. Pseudocolor scales and the 1 micron marker are shown in the control panel.

On the other hand cells transfected with pFNΔ147 (Fig. 3, Δ) showed little calmodulin induction at early times (Fig. 3, Δ, 16 and 30 hours). At later times in PFNwt transfectants the colocalized regions often resembled caps or oversized distorted caps with little calmodulin or gp160 which is not colocalized (Fig. 1, wt, 48 and 72 hours). In contrast in PFNΔ147 transfectants at later times cells exhibited small patches of overlapping calmodulin and gp160 (Fig. 3, 48 hours, Δ) or areas primarily of gp160 antigenicity with only small amounts of coincident calmodulin (Fig. 3, 72 hours, Δ).

DISCUSSION

Western and Northern blots indicate that, relative to expression of gp160Δ147, expression of wild-type gp160 in cells produces an increase in calmodulin and calmodulin mRNA (Figures 1, 2). Further, the increase in calmodulin can be detected primarily in the particulate fraction of cells transfected with pFNwt, when compared to cells transfected with pFNΔ147 (Fig. 1, 16 or 48 hours, particulate subfraction labelled PEL). The localization of induced calmodulin to a particulate fraction is consistent with the idea that binding of calmodulin to gp160 would sequester it to the exocytotic pathways travelled by newly synthesized gp160 (20). We speculate that the total cellular calmodulin increase in pFNwt transfected cells is a homeostatic response to such sequestering.

Immunocytological studies show that, when full-length gp160 is expressed, calmodulin colocalizes with gp160 in non-nuclear regions (Fig. 3). At later times in pFNwt transfectants almost all visible gp160 in a given optical slice is coincident with calmodulin antigenicity. The anaglyph images of calmodulin and gp160 are consistent with the possibility of exocytotic pathway colocalization of the molecules suggested by the Western blot analysis.

The modest but distinct colocalization of calmodulin with gp160Δ147 which sometimes appears (Fig. 1, 48 hours) might be explained in either of two ways: 1) The Δ147 truncation exposes yet another calmodulin binding site. The gp41 putative transmembrane domain is 30 amino acids long and the 12 preceding amino acids are also predominantly hydrophobic. Given that only 21 amino acids are necessary for a transmembrane helix, the final 15 amino acids of gp160Δ147, LRIVFAVLSIVNRVR, which follow the rules for certain calmodulin binding sites (21), might be available for calmodulin binding. 2) It is possible that the residual colocalization of gp160Δ147 with calmodulin is due to other calmodulin binding sites in the other viral proteins.

In lymphocytes increased calmodulin mRNA may be directly associated with cell death. Glucocorticoid induced apoptosis results in increases in three calmodulin transcripts, 1.4 kb, 1.7 kb, and 4.0 kb (22). While no 1.4 kb mRNA appears in our Northern blots, the 1.7 and later 4.0 kb transcripts are increased in the wild-type transfected cells when compared to cells expressing the deletion mutant (Figure 2). Thus the increased calmodulin mRNA in pFNwt transfected cells may point toward a mechanism underlying the HIV-1 capability for inducing apoptosis (23). In fact, in other studies we have shown that calmodulin inhibitors will reverse gp160 mediated enhancement of Fas-stimulated apoptosis in Molt-4 cells (G. Pan, W. Radding, T. Zhou, J. Mountz, and J. M. McDonald, submitted).

The colocalization of calmodulin with gp160 could provide an explanation of the tendency to reduced proliferation and/or increased cell death associated with HIV-1 infection. Under the correct calcium conditions calmodulin can activate both the membrane associated calmodulin dependent adenylate cyclase and the cytosolic phosphodiesterase (24) in vivo. Excess calmodulin, if associated with adenylate cyclase in the particulate fraction and not with the phosphodiesterase in the cytosol, would tend to increase cAMP by increasing adenylate cyclase activity. HIV-1 raises cAMP levels in T-cells (25), while reducing T-cell proliferation. Reduction of cAMP in the presence of HIV-1 apparently restores proliferative capacity (26). In thymocytes elevated cAMP can lead directly to apoptosis (27). Thus we raise the possibility that sequestering of calmodulin to cellular structures which appear in the particulate fraction in these experiments may promote apoptosis via elevated cAMP.

Calmodulin inhibitors such as melittin can also diminish production of the virus from infected cell lines (28). While their action has been attributed to interactions with viral proteins such as gag, the data presented here pose an alternative, that the inhibitors interfere with a process in the production of virus which is dependent on the gp160 induced calmodulin. The overproduction of calmodulin described here may thus participate in two of the viral functions, predisposition of the cells to apoptosis, noted above, and viral production. Elucidation of the intimate link between the virus and Ca^{2+}/calmodulin signal pathways of the cell may therefore eventually lead to points of intervention where cell death and viral propagation may be halted.

ACKNOWLEDGMENTS

This study was supported by the Center for AIDS Research/Core Support Grant #P30-AI-27767 from the NIAID. We thank Maggie McKenna, Department of Pathology, and Jill Gemmill, Neuroscience Research Center, for their technical assistance.

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