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Phorbol Ester-Induced Down Modulation of Tailless CD4 Receptors Requires Prior Binding of gp120 and Suggests a Role for Accessory Molecules

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The entry of human immunodeficiency virus type 1 into cells proceeds via a fusion mechanism that is initiated by binding of the viral glycoprotein gp120-gp41 to its cellular receptor CD4. Species- and tissue-specific restrictions to viral entry suggested the participation of additional membrane components in the postbinding fusion events. In a previous study (H. Golding, J. Manischewitz, L. Vujcic, R. Blumenthal, and D. Dimitrov, J. Virol. 68:1962–1968, 1994), it was found that phorbol myristate acetate (PMA) inhibits human immunodeficiency virus type 1 envelope-mediated cell fusion by inducing down modulation of an accessory component(s) in the CD4-expressing cells. The fusion inhibition was seen in a variety of cells, including T-cell transfectants expressing engineered CD4 receptors (CD4.401 and CD4.CD8) which are not susceptible to down modulation by PMA treatment. In the current study, it was found that preincubation of A2.01.CD4.401 cells with soluble monomeric gp120 for 1 h at 37°C primed them for PMA-induced down modulation (up to 70%) of the tailless CD4 receptors. The gp120-priming effect was temperature dependent, and the down modulation may have occurred via clathrin-coated pits. Importantly, nonhuman cell lines expressing tailless CD4 molecules did not down modulate their CD4 receptors under the same conditions. The gp120-dependent PMA-induced down modulation of tailless CD4 receptors could be efficiently blocked by the human monoclonal antibodies 48D and 17B, which bind with increased avidity to gp120 that was previously bound to CD4 (M. Thali, J. P. Moore, C. Furman, M. Charles, D. D. Ho, J. Robinson, and J. Sodroski, J. Virol. 67:3978–3988, 1993). These findings suggest that gp120 binding to cellular CD4 receptors induces conformational changes leading to association of the gp120-CD4 complexes with accessory transmembrane molecules that are susceptible to PMA-induced down modulation and can target the virions to clathrin-coated pits.

Entry of human immunodeficiency virus type 1 (HIV-1) into cells, which follows the binding of its envelope glycoprotein (gp120-gp41) to the cellular receptor CD4 (21), involves a multistep fusion process which is slower than in other membrane viruses such as influenza virus (19) and requires the presence of an additional target-cell membrane component(s) that has restricted species and tissue distribution (1, 2, 4, 6, 10, 16, 24, 35). Earlier studies suggested that the fusion process occurs at the plasma membrane rather than through the endocytic pathway and proceeds at neutral pH (33). However, electron micrographs of cells undergoing HIV-1 infection showed virions fusing both at the cell surface and in membrane vesicles (15), suggesting that targeting of virions to clathrin coated pits may occur even though a low pH environment is not required to trigger the fusion process.

In a previous study, we examined the fusion process of HIV-1 envelope-expressing cells with target cells that express either full-length CD4 receptors, chimeric CD4.CD8 receptors, or truncated tailless CD4 receptors (3, 28). The last two cell lines were found to be of particular interest. They both support HIV-1 viral infection, albeit at a much reduced rate compared with the full-length CD4-expressing cells (28). Furthermore, these receptors were found to be resistant to phorbol ester (PMA)-induced down modulation (3, 13). It was previously determined that phosphorylation-dependent down modulation of CD4 requires a specific structure within its membrane-proximal cytoplasmic tail, which encompasses amino acids 397 to 417 and which differs between CD4 and CD8 tails (31). Interestingly, treatment of these CD4 transfectants with PMA resulted in a dramatic reduction in their fusogenic potential in spite of the fact that no down modulation of the CD4.401 or CD4.CD8 receptors was observed (14). We postulated that the PMA-induced down modulation of a membrane component distinct from CD4 may be functioning in an accessory role during the fusion process.

In the current study, we tested the hypothesis that gp120 interaction with CD4 induces conformational changes in the receptor-ligand pair that lead to the association of CD4 with the accessory membrane component. By using the previously described transfected cell line expressing tailless CD4 molecules (CD4.401), it was found that soluble gp120 preincubation primed these cells for PMA-induced down modulation of CD4 via clathrin-coated pits. The gp120 priming effect was temperature dependent, was blocked by certain murine and human anti-gp120 monoclonal antibodies (MAbs), and did not occur in nonhuman cell lines.

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MATERIALS AND METHODS

Cells and reagents. The human cell lines CEM and HeLa and the primate cell line BS-C-1 were obtained from the American Type Culture Collection, Rockville, Md. The $CD4^-$ T-cell clone 12E1 was derived from CEM by chemical mutagenesis as previously described (18). The CD4.401-expressing recombinant retrovirus vector was described previously (3, 28). A2.01.CD4.401 and NIH 3T3.CD4.401 stable transfectants expressing tailless CD4 receptors were produced as previously described (3). The recombinant vaccinia virus vector vPE16, encoding the HIV-1 IIIB envelope (12), was provided by P. Earl and B. Moss (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.). The recombinant vaccinia virus vectors vCB-7 and vCB-2, which express wild-type or tailless human CD4 molecules, respectively, were produced by Chris Broder and Edward Berger (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.). The vCB-7 expresses full-length membrane-associated human CD4 molecules (6). The vCB-2 vector expresses truncated, membrane-associated CD4 molecules which are missing the 31 residues of the cytoplasmic tail. Both vaccinia virus vectors express CD4 under the control of the vaccinia virus compound P7.5 early-late promoter. Soluble gp120 (LAI) and fluorescein isothiocyanate (FITC)-mouse-anti-gp120 (LAI) MAb were purchased from Intracel (Cambridge, Mass.). The phorbol ester PMA and goat-anti-mouse immunoglobulin G were obtained from Sigma (St. Louis, Mo.). The MAbs OKT4A and FITC-OKT4 were from Ortho Diagnostics (Raritan, N.J.). Murine anti-CD4 MAbs L120, L122, and L71 were the generous gift of David Buck (Becton Dickinson, Franklin Lakes, N.J.). The MAb 5A8, which is specific for the second domain of CD4 (CD4D2) (22), was provided by Linda Burkly (Biogene, Cambridge, Mass.). The murine anti-gp120 MAbs 9205 (V3) and 9201 (unmapped) were purchased from Dupont (NEN Products, Boston, Mass.). The 0.5β (V3) MAb was obtained through the NIH AIDS Research and Reference Reagent Program (Rockville, Md.). The human MAb 1B1 (anti-120 CD4 binding region, CD4BR) (7) was a generous gift from H. Katinger (University of Bodenkulter, Vienna, Austria). The 684-238 MAb (V2) (23) was a generous gift from Gerald Robey (Abbott Laboratories, North Chicago, Ill.), and the human MAbs 48D and 17B (both anti-HIV-1 envelope [23, 26]) and B23 (anti-HIV-2 gp120; none cross-reactive with HIV-1 [unpublished data]) were produced in the laboratory of James Robinson (University of Connecticut, Farmington, Conn.). The 48D and 17B MAbs recognize a very complex conformational epitope, which is moderately conserved. The binding site is present on native gp120 but is better exposed after CD4 binds to gp120. Both MAbs neutralize a variety of laboratory strains (including some, but not all IIIB [LAI] clones) and some primary isolates as well.

Measurements of surface-associated CD4 and gp120 in PMA-treated and untreated cells. Surface expression of CD4 molecules was measured by flow cytometry as described before (13, 18). Cells expressing full-length or tailless CD4 (CD4.401) molecules were incubated in culture medium (200 μl) or in medium containing soluble gp120 under various conditions. After 1 wash, the volume was increased to 1.0 ml and PMA was added (100 ng/ml) for an additional 2 to 3 h. After extensive washing, the cells were stained with FITC-OKT4 or FITC-mouse-anti-gp120 for 1 h on ice in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. The stained cells were analyzed by the Epic Profile (Coulter Counter, Hialeah, Fla.). The mean fluorescence intensities in mean fluorescence units (MFU) were calculated from standard curves generated by using fluorescent beads with increasing intensities (Flow Cytometry Standards, Research Triangle Park, N.C.). The background MFU due to autofluorescence or to nonspecific binding of antibodies were subtracted from the experimental values (18).

Infection with vaccinia virus vectors and syncytium formation assays. Human and nonhuman cell lines were infected with vCB7 (full-length CD4) or vCB2 (tailless CD4) recombinant vaccinia virus vectors at 10 PFU per cell in 100 μl of RPMI 1640 medium containing 10% fetal calf serum (Sigma Chemicals) and 40 μg of cytosine β-D-arabinofuranoside (AraC; Sigma Chemicals) per ml. AraC was added to block vaccinia virus DNA replication, which restricts the expression of the CD4 cDNA to only the early vaccinia virus promoter element, and to block further induction of expression by the late promoter element, which depends on viral DNA replication. After 2 h of viral adsorption, 2 ml of medium containing AraC was added per tube. The cells were allowed to incubate for an additional 2 to 3 h to allow maximal expression of the CD4 molecules under the regulation of the vaccinia virus early promoter. These cells were used for experiments of gp120 plus PMA-induced CD4 down modulation. For syncytium formation assays, the $CD4^-$ human T-cell line 12E1 was infected with the gp120-gp41 (IIIB)-expressing vPE16 vector for 8 to 16 h. These cells were cocultured with CD4.401-expressing human or nonhuman cell lines at a 1:1 ratio (10^5 cells each) at 37°C. Syncytia were scored at 5 to 6 h of cocultures as previously described (13, 14). The effect of PMA on the fusion process was assessed by pretreatment of the CD4.401-expressing cells with PMA (100 ng/ml) for increasing times (0 to 180 min) and then by extensive washing and coculture with the vPE16-infected 12E1 cells.

Cell surface biotinylation. CEM, A2.01.CD4.401, or 3T3.CD4.401 cells were pelleted and suspended at a concentration of 5×10^7 cells per ml in RPMI with 10% fetal calf serum. Cells were incubated at 37°C with 10 μg of gp120 (Intracel, Cambridge, Mass.) per ml or medium (2×10^7 cells per sample). After 1 h, 5 ml of medium with 100 ng of PMA per ml was added to one tube with and to one tube without gp120. The cells were incubated for an additional 3 h at 37°C. The cells were washed three times with ice-cold PBS and suspended at a concentration of 5×10^7 cells per ml in PBS. A stock solution of 24 mM NHS-LC-biotin (Pierce, Rockford, Ill.) was prepared just prior to use and was added to cells at a final concentration of 0.24 mM. The cells were placed on ice for 1 h. The reaction was quenched by the addition of 20 mM Tris-HCl, pH 7.5, for 15 min.

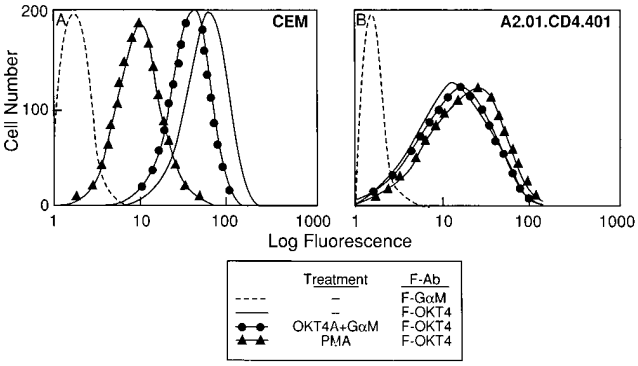

FIG. 1. Tailless CD4 receptors (CD4.401) cannot be down modulated by PMA treatment or by anti-CD4 cross-linking. CEM and A2.01.CD4.401 cells were incubated with OKT4A (60 min, 4°C) and then with goat-anti-mouse immunoglobulin G (3 h, 37°C) (●) and with PMA (100 ng/ml, 3 h, 37°C) (▲) or were left untreated (———). All groups were stained with control antibody (FITC-goat-anti-mouse) (– – –) or with FITC-OKT4 at 4°C for 1 h in the presence of 0.1% sodium azide.

Immunoprecipitation, gel electrophoresis, and blotting. The cells were washed once, and the pellet was suspended in 1 ml of lysis buffer. Lysis and wash buffers have been previously described (21). After 20 min on ice, nuclei were pelleted by spinning at $130 \times g$ for 5 min in a microcentrifuge. OKT4 (Ortho Diagnostics) was incubated with cell lysates (adjusted for the same protein concentrations) at 4°C overnight, and protein G-Sepharose (Sigma) was added for 1 h. The Sepharose beads were washed and boiled in sodium dodecyl sulfate (SDS) sample buffer containing dithiothreitol. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 4 to 15% gradient gel under the conditions described by Laemmli. The gel was blotted on nitrocellulose by using a semidry blotting system (TWEP Co., Seattle, Wash.). The blots were first reacted with streptavidin conjugated to horseradish peroxidase (HRP) and then enhanced chemiluminescence Western blotting (immunoblotting) detection reagents containing HRP substrate (Amersham, Arlington Heights, Ill.). The blots were exposed to film (X-Omat AR; Kodak, Rochester, N.Y.), and video images of bands were obtained with the GDS 5000 System (UVP Inc., San Gabriel, Calif.). Densitometry analysis was performed with SW5000 Software (UVP Inc., Cambridge, United Kingdom). Control experiments verified that the amount of light emitted was proportional to the relative amount of biotinylated CD4 in the samples.

RESULTS

Tailless CD4 molecules (CD4.401) cannot be down modulated by PMA treatment or by anti-CD4 antibody cross-linking. In previous studies with the A2.01.CD4.401 transfectant cell line, it was shown that the tailless CD4 molecules cannot be downregulated by treatment with the phorbol ester PMA (3, 14) (Fig. 1). Another treatment that leads to receptor internalization is antibody-mediated cross-linking. In order to determine the role of the cytoplasmic tail in the down modulation induced by receptor cross-linking, both cell lines were subjected either to PMA treatment or to CD4 cross-linking reagents, and the fate of the full-length and tailless CD4 receptors was determined by subsequent staining with FITC-OKT4. As can be seen in Fig. 1, wild-type CD4 molecules, expressed by CEM cells, were modulated by treatment with MAb OKT4A and then with polyclonal goat-anti-mouse serum (40 to 50% reduction in three experiments). PMA treatment of the same cell line resulted in ≥90% down modulation (Fig. 1A). In contrast, the truncated CD4 molecules, expressed by the A2.01.CD4.401 cell line, were not down modulated by either treatment (Fig. 1B). These findings suggest that the intracyto-

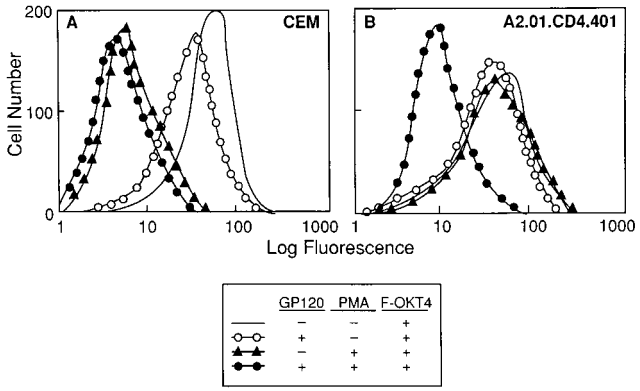

FIG. 2. gp120 pretreatment of cells expressing tailless CD4 receptors primes them for PMA-induced down modulation. CEM and A2.01.CD4.401 cell lines (10^6 cells) were treated with soluble monomeric gp120 (10 μg/ml, 30 min, 37°C [in 200 μl of culture medium]) (○), gp120 (30 min), and PMA (100 ng/ml, 37°C, 2 h [in 1 ml of culture medium]) (●); with PMA only (▲); or with medium alone (——). All groups were stained for 1 h with FITC-OKT4 at 4°C in the presence of 0.1% sodium azide.

plasmic tail of the CD4 molecules is required for its down modulation via different mechanisms. Similar results were reported by others (3, 25, 31) and seem to apply for many different membrane receptors (27, 32). Furthermore, constitutive recycling of surface CD4 molecules was also shown to be dependent on the presence of its intracytoplasmic tail (26), in agreement with recent findings that recycling of HLA class II molecules also requires intact intracytoplasmic tails in both the α and β chains of the dimers (20a).

gp120 pretreatment of cells expressing tailless CD4 molecules primes them for PMA-induced down modulation. It was previously demonstrated that T-helper-cell activation induces coaggregation of the TCR complex and CD4 molecules, requiring conformational changes in both surface receptors (9). It was also postulated that the interaction of HIV-1 envelope protein with CD4 induces conformational changes in both proteins, presumably leading to exposure of the hydrophobic gp41 N terminus and initiation of the fusion process (29). This process also involves an accessory membrane component which has not yet been elucidated. We have recently reported that PMA treatment of human cell lines induces down modulation of a membrane component (other than CD4) required for gp120-gp41-mediated cell fusion (14). It is possible that the interaction between gp120 and CD4 on cells induces association between the gp120-CD4 complex and a second membrane component that is susceptible to PMA-induced down modulation. Since truncated CD4 molecules (CD4.401) do not undergo down modulation by themselves (Fig. 1), we could test this postulate by using the A2.01.CD4.401 cells. Soluble gp120 (10 μg/ml) was added to CEM (full-length CD4) or A2.01.CD4.401 (tailless CD4) cells for 30 min at 37°C, which was followed by PMA treatment (50 to 100 ng/ml) for 3 h. It was found that while gp120 alone or PMA treatment alone induced minimal or no downregulation of the CD4.401 molecules, respectively, preincubation with gp120 and then PMA treatment resulted in a very significant down modulation of the tailless molecules (50 to 70% reduction in more than 20 experiments) (Fig. 2B). In the control CEM cells, gp120 and PMA, respectively, induced partial (20 to 30%) and substantial (70 to 85%) down modulation. The combination of both produced maximal down modulation of the wild-type CD4 molecules (≥90%) (Fig. 2A).

In the next set of experiments, we determined the kinetics and dose response of the gp120-mediated priming of CD4.401 molecules for PMA-induced down modulation (Fig. 3A, B, and C). It was found that preincubation of cells with gp120 concentrations of 10 to 20 μg/ml for 1 h primed them optimally for down modulation by PMA treatment (50 to 100 ng/ml, 2 to 3 h, 37°C). Interestingly, in most experiments, gp120 by itself induced 20 to 30% downregulation of the tailless CD4 molecules under the same regimen, while the subsequent PMA treatment resulted in 50 to 70% reduction in surface expression (Fig. 3). In all of these experiments, PMA treatment alone resulted in no down modulation of the CD4.401 molecules (Fig. 1, 2, and 3B).

Incubation of CD4.401-expressing cells with gp120 at 37°C, but not at 4°C, confers to them susceptibility to PMA-induced receptor down modulation. The ability of soluble monomeric gp120 to prime the tailless CD4 molecules for down modulation may require interaction of the gp120-CD4.401 complex with another membrane component which is susceptible to PMA-induced internalization. If such a mechanism involves lateral mobility of the complexes, it would be predicted to be temperature dependent. To test this prediction, CEM and A2.01.CD4.401 cells were incubated with gp120 (10 μg/ml) at either 4 or at 37°C for 1 h (within 1 h, the binding of gp120 to CD4 reaches saturation at the two temperatures), washed, and incubated for an additional 2 h at 37°C in the absence or presence of PMA (100 ng/ml). The cells were stained with FITC-OKT4 (to follow CD4 expression) or with FITC-mouse anti-gp120 MAb to monitor the surface-bound gp120 molecules. As seen in Table 1, preincubation of CD4.401-expressing cells with gp120 at 4°C and then transfer to 37°C, resulted in very limited down modulation of the receptors even in the presence of PMA (12%). In contrast, gp120 preincubation at 37°C resulted in 52% down modulation after the PMA treatment. A parallel reduction in surface-bound gp120 was seen under these conditions. The full-length CD4 molecules could be down modulated by PMA treatment alone (75% reduction). However, preincubation with gp120 at 37°C, but not at 4°C, resulted in maximal PMA-induced down modulation (89%), as shown also in Fig. 1. Thus, the priming effect of gp120 is temperature dependent, implying a process requiring metabolic energy and probably active lateral mobility of the molecules. These findings are similar to other receptor-ligand systems. In particular, interleukin-6 triggers the association of its receptor with a gp130 membrane component, which is required for signaling, and this association takes place at 37°C, but not at 4°C (34).

Inhibition of clathrin-coated pits formation by hypertonic medium prevents gp120-induced CD4 down modulation. Internalization of many different surface receptors, especially after binding of their ligands, involves interaction between the cytoplasmic tails of the receptors with plasma membrane-associated adaptin molecules (e.g., AP-2), which in turn induces the oligomerization of clathrin molecules and the formation of clathrin-coated pits which internalize. This process was also shown to be temperature dependent (26, 31). In order to determine if gp120 induces CD4 down modulation via clathrin-coated pits, we used hypertonic medium, which was previously shown to prevent normal assembly of coated pits (17, 25). As depicted in Fig. 4, in the presence of hypertonic buffer (which did not affect the cell viability during the experiment), neither gp120 alone nor gp120 with subsequent PMA treatment induced significant down modulation of the tailless or full-length CD4 molecules. It was previously shown that PMA-induced down modulation of wild-type CD4 occurs via clathrin-coated pits and is sensitive to hypertonic medium (25, 26). Here, we

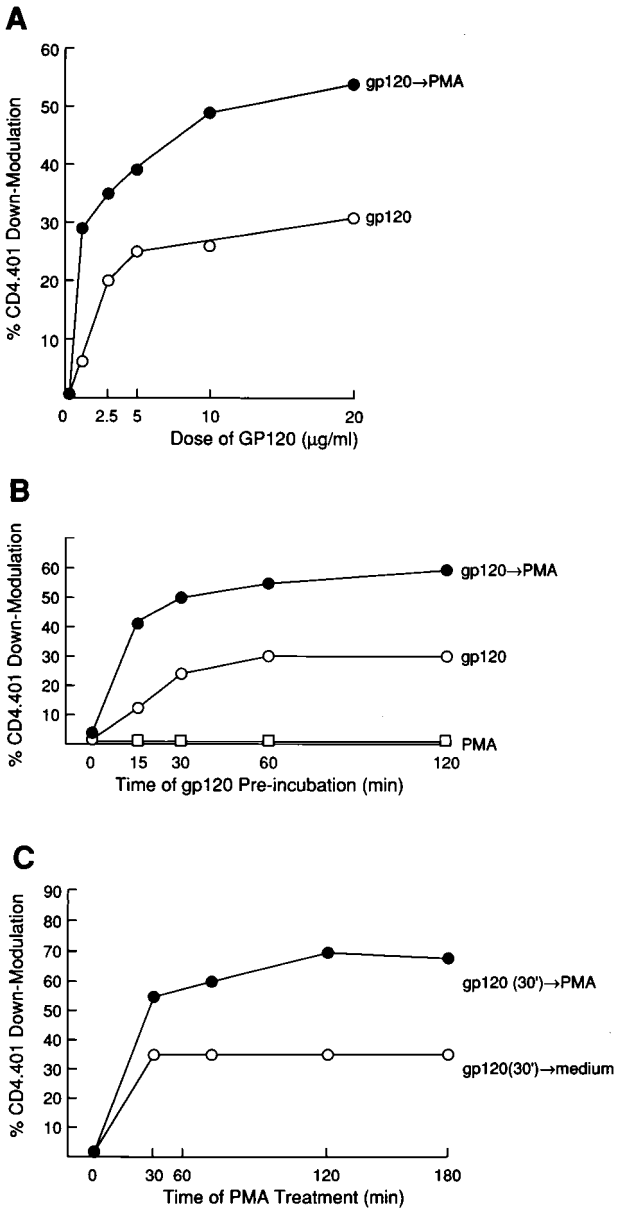

FIG. 3. PMA-induced down modulation of CD4.401 receptors following gp120 pretreatment. (A) Dose response of gp120 priming of A2.01.CD4.401 cells for PMA-induced down modulation of their tailless CD4 receptors. Soluble gp120 at increasing concentrations (1.25 to 20 μg/ml) was added to 10^6 A2.01.CD4.401 cells in 200 μl of culture medium. After washing, the cells were resuspended in 1 ml of medium and incubated in the presence (●) or absence (○) of PMA (100 ng/ml) for 2 h. (B) Kinetics of gp120 priming of CD.401 receptors for PMA-induced down modulation. A2.01.CD4.401 cells were preincubated with gp120 (10 μg/ml, in 200 μl of culture medium, 37°C) for increasing time periods (0 to 120 min) and then incubated for 2 h with 1 ml of medium (○) or PMA (100 ng/ml) (●). Control groups were incubated for 3 h in medium alone or medium containing PMA (□). (C) Kinetics of PMA-induced down modulation of CD4.401 receptors following gp120 pretreatment. A2.01.CD4.401 cells (10^6 per group) were preincubated with gp120 (10 μg/ml, 30 min, 37°C) in 200 μl of culture medium and then treated with PMA (100 ng/ml in 1 ml) (●) or medium alone (○) for increasing time periods (30 to 180 min). All groups were stained with FITC-OKT4 (or control antibody), and MFU were determined as described in Materials and Methods. The percentage of down modulation was calculated after subtraction of background fluorescence as follows: 100 − [(MFU of treated cells/MFU of untreated cells) × 100].

TABLE 1. Incubation of CD4.401-expressing cells with gp120 at 37°C (but not at 4°C) confers on them susceptibility to PMA-induced receptor down modulation[a]

Cell line	Presence or absence (°C) of:		Surface expression (MFU [10^3])	
	gp120	PMA	FITC-OKT4	FITC MAb anti-gp120
CEM	−	− (37)	59.6	0
	+ (4)	− (37)	51.9	34.1
	+ (4)	+ (37)	13.0	8.5
	+ (37)	− (37)	49.9	30.1
	+ (37)	+ (37)	6.6	5.8
	−	+ (37)	15.3	0
A2.01.CD4.401	−	− (37)	26.0	0
	+ (4)	− (37)	25.0	18.5
	+ (4)	+ (37)	23.0	18.0
	+ (37)	− (37)	24.5	18.9
	+ (37)	+ (37)	12.5	8.9
	−	+ (37)	27.0	0

[a] CEM and A2.01.CD4.401 cells were incubated with gp120 (10 μg/ml) for 1 h on ice or at 37°C and then washed. The cells were resuspended in 1 ml, transferred to an incubator at 37°C, and treated with (or without) PMA (100 ng/ml) for an additional 2 h at 37°C. After extensive washing, the cells were stained with anti-CD4 and anti-gp120 reagents and analyzed as described in Materials and Methods.

show that the gp120-induced down modulation of tailless CD4 may also proceed via clathrin-coated pits.

PMA pretreatment of cells prevents subsequent gp120-induced down modulation of CD4. If the down modulation of the tailless receptors requires their association with another membrane component that is sensitive to PMA-induced internalization, it should be possible to deplete the cell surfaces of these accessory molecules by chronic PMA treatment prior to the addition of gp120 and thus to prevent the subsequent down modulation of the tailless CD4 molecules. As can be seen in Table 2, incubation of A2.01.CD4.401 cells with gp120 for 1 h and then 3 h of treatment with PMA (100 ng/ml) resulted in

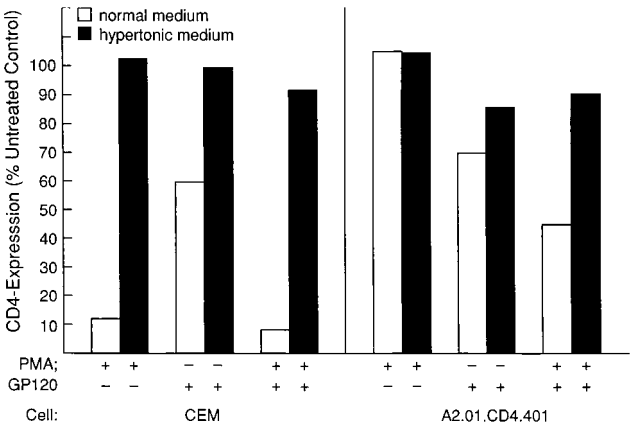

FIG. 4. Inhibition of clathrin-coated pit formation by hypertonic medium prevents gp120-induced or gp120-plus-PMA-induced CD4 receptor down modulation. CEM (wild-type CD4) and A2.01.CD4.401 (tailless CD4) cells were treated with gp120 (10 μg/ml, 1 h, 37°C), gp120 and then PMA (100 ng/ml, 2 h, 37°C), or PMA (3 h) in normal culture medium or in hypertonic medium. Control groups were incubated in the same media without any treatments. All groups were washed extensively and stained with FITC-OKT4 in PBS containing 1% BSA and 0.1% sodium azide. Data are presented as percentages of untreated controls as follows: (MFU of treated cells/MFU of untreated cells) × 100.

TABLE 2. PMA pretreatment of CD4- and CD4.401-expressing cells prevents gp120-induced down modulation of their CD4 receptors

Cell line	Treatment[a]	CD4 Expression	
		MFU (10^3)	% Control
A2.01.CD4.401 (tailless)	None	30.2	100
	PMA	30.2	100
	Overnight PMA→gp120	28.0	93
	gp120	20.0	66
	gp120→PMA	9.3	30
CEM (wild-type CD4)	None	113.3	100
	PMA	17.7	15
	Overnight PMA→gp120	16.7	14
	gp120	74.0	65
	gp120→PMA	8.0	7

[a] CEM and A2.01.CD4.401 cells were incubated overnight in medium alone or with PMA (20 ng/ml). After extensive washings, the cells were incubated with gp120 (10 μg/ml) for 2 h. Other groups were incubated with gp120 and/or treated with PMA for 2 h as indicated.

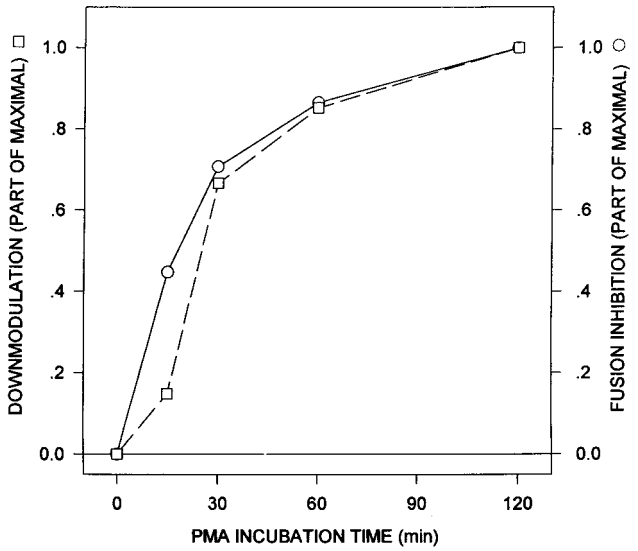

FIG. 5. Correlation between down modulation of tailless CD4 and fusion inhibition by PMA. A2.01.CD4.401 cells were treated with PMA (100 ng/ml) alone for increasing time periods (0 to 120 min) and then mixed with vPE16-infected 12E1 cells (expressing gp120-gp41) at a 1:1 ratio. Fusion inhibition was measured by scoring of syncytia 6 h afterward as previously described. The maximal fusion inhibition observed (set as 1) was 100%. In parallel, A2.01.CD4.401 cells were pretreated with gp120 (10 μg/ml) for 1 h at 37°C and then treated with PMA for the indicated time periods. Down modulation of the CD4.401 receptors was determined by staining of treated (and untreated) cells with FITC-OKT4. Maximal down modulation in this experiment (set as 1) was 65%.

70% down modulation of the CD4 molecules. In contrast, if the cells were pretreated with PMA (20 ng/ml) overnight, the subsequent addition of gp120 for 2 to 3 h did not result in significant CD4 down modulation. Together, these results suggest that soluble monomeric gp120 may induce the association of surface CD4 molecules with a second membrane component that is sensitive to PMA down modulation via clathrin-coated pits.

Correlation between down modulation of tailless CD4 and fusion inhibition by PMA. It was not certain at this point that the membrane component that facilitates the down modulation of tailless CD4 is the accessory component required for HIV-1 cell fusion. However, since both membrane components were found to be sensitive to PMA-induced down modulation, it was possible to compare the kinetics of the two phenomena in parallel. A2.01.CD4.401 cells were preincubated with gp120 or control medium for 1 h. All cells were then treated with PMA for increasing times (0 to 120 min). After washing, the cells treated with control medium were cocultured with vPE16-12E1 targets (expressing gp120-gp41) and scored for syncytium formation 6 h later. The cells preincubated with gp120 were stained with FITC-OKT4 to determine the degree of CD4.401 down modulation. As can be seen in Fig. 5, the kinetics of PMA-induced fusion inhibition and the kinetics of PMA-induced down modulation of gp120-pretreated cells were superimposed, supporting the idea that the component(s) involved in the two processes could be the same.

gp120 plus PMA treatment does not induce down modulation of tailless CD4 molecules in nonhuman cell lines. In an attempt to correlate further the CD4 down modulation with the fusion process, we tested the ability of gp120 plus PMA to induce CD4 down modulation in human and nonhuman cell lines. The vaccinia virus vectors vCB7 and vCB2 express full-length and tailless human CD4 molecules, respectively. Human HeLa cells, mouse fibroblast L cells, and monkey BS-C-1 cells (noninfectible with HIV-1 [6]) were infected with the recombinant vaccinia virus in the presence of AraC (to block expression by late promoter elements). After 4 h, the cells were treated with gp120 alone, PMA alone, or gp120 and then PMA. As can be seen in Table 3, full-length CD4 molecules expressed by either HeLa, L cells, or BS-C-1 cells infected with the vCB7 vector were readily down modulated following PMA treatment. The tailless CD4 molecules expressed by vCB2 vector on HeLa cells could also be down modulated by gp120 preincubation and subsequent PMA treatment. In contrast, similar treatment of L cells or BS-C-1 cells expressing tailless CD4 molecules did not result in any down modulation. All transfectants and recombinant vaccinia virus vector-infected cell lines were also tested in the syncytium assay. In agreement with previous work (6, 10), the murine cell lines and monkey BS-C-1 line were not fusogenic irrespective of the CD4 receptors expressed on their surfaces (data not shown).

Immunoprecipitation of surface CD4 molecules from cells treated with gp120 and PMA. In addition to monitoring the fate of the CD4 molecules by staining with FITC-OKT4 MAb and then by flow cytometry, we conducted immunoprecipitation experiments of full-length and tailless CD4 molecules from CEM, A2.01.CD4.401, and NIH 3T3.CD4.401 cell lines. Cells were incubated with gp120 (10 μg/ml) or medium and then treated with PMA. The treated and untreated cells were biotinylated in order to monitor surface receptors. After cell lysis, the OKT4-immune precipitates were blotted onto nitrocellulose filters and reacted with streptavidin-HRP and then with chemiluminescence Western blot detection reagents containing HRP substrate. Only surface molecules (biotin labeled) are detected by this procedure. As can be seen in Fig. 6, PMA treatment of CEM cells resulted in 90% depletion of surface wild-type CD4 molecules. In contrast, similar treatment of A2.01.CD4.401 or 3T3.CD4.401 cells resulted in either a modest increase of surface CD4.401 molecules (120% of control in A2.01.CD4.401 cells) or no change in surface expression of the tailless molecules. Incubation of A2.01.CD4.401 cells with gp120 at 37°C led to 40% reduction, and gp120 plus PMA treatment resulted in >60% reduction of surface expression of the tailless molecules. In contrast, no significant down modu-

TABLE 3. gp120 plus PMA treatment does not induce down modulation of tailless CD4 molecules in nonhuman cell lines[a]

Cell line and vector	CD4 molecule	Treatment	CD4 expression (% control)
HeLa			
vCB-7	Full length		100
		gp120	88
		gp120 → PMA	17
		PMA	20
vCB-2	Tailless		100
		gp120	78
		gp120 → PMA	60
		PMA	98
L cells			
vCB-7	Full length		100
		gp120	90
		gp120 → PMA	15
		PMA	26
vCB-2	Tailless		100
		gp120	114
		gp120 → PMA	97
		PMA	95
BS-C-1			
vCB-7	Full length		100
		gp120	89
		gp120 → PMA	12
		PMA	19
vCB-2	Tailless		100
		gp120	150
		gp120 → PMA	150
		PMA	100

[a] Human HeLa cells, mouse fibroblast L cells, and primate BS-C-1 cells were infected with recombinant vaccinia virus vector vCB7 (full-length CD4) or vCB2 (tailless CD4) at 10 PFU per cell in the presence of AraC (40 μg/ml). After 4 h, the cells were preincubated with gp120 (10 μg/ml) (or medium control) and then incubated for 2 h in the presence or absence of PMA (100 ng/ml).

lation of the CD4.401 receptors was seen in the murine 3T3.CD4.401 transfectants under the same conditions. These results were in full agreement with the flow cytometry analyses (Table 3 and data not shown) and suggested that a species barrier may exist. Thus, the PMA-induced down modulation of full-length CD4 molecules proceeds equally well in human and murine cells, suggesting a mechanism that is dependent only on the cytoplasmic tail of the CD4 molecule itself, while the assisted down modulation of truncated, tailless CD4 receptors depends on interaction with a second membrane component which may be species specific.

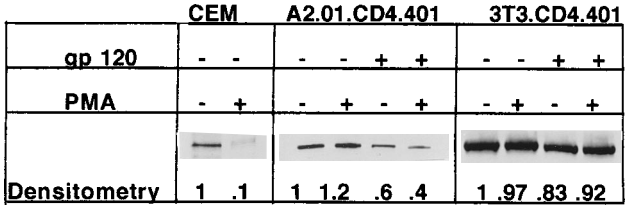

FIG. 6. Immunoprecipitation of surface CD4 molecules from cells treated with gp120 plus PMA. A2.01.CD4.401 (human) and NIH 3T3.CD4.401 (mouse) cells were preincubated with PBS or gp120 (10 μg/ml) for 1 h and then incubated for 3 h with PMA (100 ng/ml) or PBS alone. CEM cells were incubated for 3 h with PBS or PMA in PBS. For labeling of surface-associated molecules, intact cells were biotinylated with NHS-LC-biotin. Cell extracts were prepared, and the CD4 molecules were precipitated with OKT4 antibodies and then with protein G-Sepharose beads. The immune precipitates were resolved by SDS-PAGE and blotted onto nitrocellulose filters. The Western blots were reacted with HRP-streptavidin and then with enhanced chemiluminescence detection reagent containing HRP substrate.

TABLE 4. gp120-plus-PMA-induced down modulation of A2.01.CD4.401 molecules can be partially blocked by anti-V3 MAb

Cell line	gp120	PMA	MAb[a]	% CD4 down modulation
A2.01.CD4.401	+	+		66
	+	+	OKT4A (CD4-CR2)	2
	+	+	L120 (CD4)	69
	+	+	L122 (CD4)	68
	+	+	L71 (CD4-CR3)	40
	+	+	5A8 (CD4-D2)	66
	+	+	1B1 (CD4BR)	0
	+	+	684 (V2)	67
	+	+	0.5β (V3)	33
	+	+	9205 (V3)	37
	+	+	9201	66
	+	+	9205 + L71	29
CEM	+	−		25
	+	−	OKT4A	0
	+	−	9205 (V3)	3
	+	−	9201	26
	−	+		85
	−	+	OKT4A	83
	−	+	9205 (V3)	84
	−	+	9201	85

[a] MAbs were added at 10 to 50 μg/ml either to cells (anti-CD4 MAbs) or to 10 μg of gp120 per ml (anti-gp120 MAbs). After 60 min of incubation at 37°C, the cells, gp120, and MAbs mixtures were combined and incubated for an additional 2 h in the presence (or absence) of PMA (100 ng/ml).

Blocking of gp120-plus PMA-induced down modulation of CD4.401 molecules by human and mouse MAbs against CD4 or gp120 epitopes. In an attempt to identify regions within the gp120 or CD4 molecules which are involved in the down modulation of the tailless CD4 molecules, we screened a panel of human and murine MAbs (generated in different laboratories) for their abilities to block this phenomenon. In these experiments, anti-gp120 MAbs were preincubated with gp120, and the anti-CD4 MAbs were preincubated with the CD4-expressing cells for 60 min at 4°C and then the cells were added to the gp120-antibody mixture, which was transferred to 37°C for 1 h. PMA was then added for an additional 2 h, and the level of CD4 expression was determined as before. As expected, MAbs that block gp120-CD4 binding (murine OKT4A and human 1B1 MAbs) completely prevented the CD4.401 down modulation (Table 4). In addition, partial blocking was consistently seen with antibodies against gp120-V3 loop (MAbs 0.5β and 9205) and against the CDR3-like region in the first domain of the CD4 molecule (MAb L71). The combination of MAbs 9205 and L71 gave more pronounced (but not complete) reversal of the down modulation. Interestingly, other MAbs that were reported to have neutralizing effects against HIV-1, such as MAb 5A8 (directed to a CD4 domain 2 epitope) and the gp120-V2-specific MAb 684, did not block the gp120-plus-PMA-induced down modulation. Other MAbs against CD4 and gp120 (some of which are listed in Table 4) also had no blocking effects. In CEM cells, none of the antibodies blocked the PMA-induced down modulation of wild-type CD4 molecules. In contrast, both OKT4A and anti-V3 MAb (9205) blocked the modest down modulation of the wild-type CD4 molecules induced by gp120 alone.

Recently, several laboratories generated MAbs against either CD4 or gp120 which bind better to the CD4-gp120 com-

TABLE 5. Blocking of gp120-plus-PMA down modulation of CD4.401 receptors by human MAbs 48D and 17B

Preincubation (4°C)[a]	Treatment (37°C) with PMA	% CD4.401 down modulation
CD4.401 + gp120	−	18
CD4.401 − gp120	+	0
CD4.401 + gp120	+	55
(gp120 + 1B1) → CD4.401	+	6
(gp120 + 9205) → CD4.401	+	30
(gp120 + 9201) → CD4.401	+	55
(gp120 + 48D) → CD4.401	+	46
(gp120 + 17B) → CD4.401	+	40
(gp120 + B23) → CD4.401	+	56
(CD4.401 + gp120) → 1B1	+	45
(CD4.401 + gp120) → 9205	+	27
(CD4.401 + gp120) → 9201	+	55
(CD4.401 + gp120) → 48D	+	12
(CD4.401 + gp120) → 17B	+	0
(CD4.401 + gp120) → B23	+	53

[a] MAbs (50 μg/ml) were preincubated for 1 h at 4°C with gp120 (10 μg/ml), and the mixtures were added to A2.01.CD4.401 cells (CD4.401). Alternatively, the MAbs were added to A2.01.CD4.401 cells which had been preincubated with gp120 (10 μg/ml) for 1 h at 4°C. The preincubation mixtures were then transferred to 37°C for an additional 1 h and then treated with PMA for 2 h.

plex than to the individual proteins (8, 12a, 23). Presumably, such antibodies recognize either combinatorial epitopes expressed by the complex or conformationally dependent new epitopes that are induced by the gp120-CD4 interaction. Two such antibodies are the human MAbs 48D and 17B. These MAbs were shown to recognize a complex conformational epitope which is moderately conserved among laboratory strains and primary isolates. The binding site is present on native gp120 but is better exposed after CD4 binding (23). Neither MAb binds to a mutant gp120 with a deletion of the entire V3 region. However, binding was completely restored in the presence of soluble CD4 (36). Both MAbs could neutralize a variety of laboratory strains (including some, but not all IIIB-LAI clones) (23) and some primary isolates as well (28a). In the experiment depicted in Table 5 (representing three independent experiments), several anti-V3 MAbs as well as 48D and 17B were incubated with gp120 for 1 h at 4°C, and the mixtures were then added to A2.01.CD4.401 cells, transferred to 37°C for an additional 1 h, and treated for 2 h with PMA. Alternatively, the cells were first incubated with gp120 for 1 h at 4°C, transferred to 37°C, mixed with MAbs for 1 h, and then treated for an additional 2 h with PMA. As can be seen in Table 5, the human MAb 1B1, which binds to the CD4 binding region on gp120, could block the CD4.401 down modulation only if it was preincubated with gp120 prior to its addition to the cells. The anti-V3 MAb 9205 could reduce the down modulation from 55 to 30% (if preincubated with gp120) or to 27% (if added to cells preincubated with gp120). The 48D and 17B MAbs could block the down modulation only marginally if they were preincubated with gp120 at 4°C (46 and 40% down modulation, respectively). However, if these MAbs were added to cells that were preincubated with gp120, they could very effectively block the subsequent PMA-induced CD4 down modulation (from 55 to 12 and 0%, respectively). These data support the idea that following gp120-CD4 interactions, the complex undergoes conformational changes that expose new epitopes on both proteins. These conformational changes may facilitate the interaction of the gp120-CD4 complex with an additional transmembrane component(s), most likely containing an intracytoplasmic tail, which renders the entire complex susceptible to PMA-induced down modulation via clathrin-coated pits (Fig. 7). It is not clear yet whether the regions recognized by MAbs 48D and 17B are involved in a secondary interaction with the

FIG. 7. Hypothetical model for gp120-induced association between surface CD4 receptors and an accessory component which contains intracytoplasmic tail and is sensitive to down modulation via clathrin-coated pits. gp120 induces conformational changes in CD4.401.

conformationally changed CD4 molecules or with the second component itself.

DISCUSSION

HIV-1-envelope mediated cell fusion is a complex process that depends not only on the high-affinity binding of gp120 to its cellular receptor CD4, but also on the participation of an additional, not yet identified cellular component(s) that assists the fusion process during the postbinding stages (1, 2, 4, 6, 10, 24, 35). The accessory component(s) was shown to be absent from most nonhuman tissues and also to be differentially expressed in different human tissues. Recently, it has been suggested that the accessory component may be nonprotein molecules (e.g., glycolipids), which are present in human erythrocyte plasma membranes (11). Although they are quite compelling, it is difficult to reconcile these findings with the rather complicated species and tissue restrictions of HIV-1 cell fusion. It is conceivable that both protein and nonprotein components are involved in the fusion process and must be present in the target cells in appropriate proportions or possibly colocalize in membrane patches which also include CD4 following interaction of the viral receptors with the viral oligomeric envelope glycoprotein.

In our studies, we used T-cell transfectants expressing truncated CD4 molecules (28). Such tailless molecules are resistant to down modulation induced by either antibody-mediated cross-linking or phorbol ester treatment. Furthermore, the constitutive low-level recycling of CD4 molecules in nonlymphoid cell lines (such as HeLa) was also diminished in cells expressing CD4 molecules missing a large part of the cytoplasmic tail (25). In a previous study, we reported that such cells are nevertheless fusogenic, with target cells expressing the HIV-1 envelope (14). However, pretreatment with PMA blocked the fusion process, although the CD4.401 expression was either unaffected or increased. Similar treatment of the envelope-expressing target cells did not interfere with the fusion process (14). These findings suggested that at least one of the accessory components required for successful HIV-1 Env-mediated fusion is susceptible to PMA-induced down modulation. In the present study, we considered the possibility that gp120 binding to CD4 induces conformational changes in these molecules which facilitate their association with the accessory membrane component. Since this component was found to be susceptible to PMA-induced down modulation, it could be a transmembrane protein with a cytoplasmic tail that allows it to interact with adaptin molecules and be targeted to clathrin-coated pits (27). If such an association occurs between tailless CD4 molecules and the putative accessory component, it could lead to their co-down modulation by subsequent PMA treatment.

The findings of this study support these predictions. Preincubation of T cells expressing the tailless CD4 molecules (CD4.401) with monomeric soluble gp120 for 30 to 60 min primed them for down modulation in the subsequent 2 to 3 h. Up to 35% down modulation was seen if the cells were simply incubated in culture medium at 37°C. However, in the presence of PMA, up to 75% down modulation of the tailless receptors was observed. Priming did not occur if the cells were incubated with gp120 at 4°C for up to 2 h prior to the PMA treatment at 37°C. Under these conditions, maximal binding of gp120 to surface CD4 receptors took place (as determined by flow cytometry), suggesting that the association of the gp120-CD4 complexes with the accessory membrane component was temperature dependent. Furthermore, if the same cells were treated with PMA overnight and subsequently exposed to gp120, no down modulation of their CD4 receptor was seen. The most likely explanation is that the prolonged PMA treatment depleted the surface of the accessory component required for the CD4.401 down modulation. These findings cannot be used as proof that the phenomenon described here is an integral part of the HIV-1 cell fusion process, especially since it was argued that HIV-1 cell entry does not require a low pH endocytic compartment (33). They may also not apply to virus internalization. However, several findings suggested the existence of common features between the Env-CD4-initiated fusion process and the gp120-induced susceptibility of tailless CD4 molecules to down modulation. First, the kinetics of PMA-induced inhibition of fusion and the kinetics of the PMA-induced CD4 down modulation in gp120-pretreated A2.01.CD4.401 cells were identical. Second, by using a murine transfectant cell line (3T3.CD4.401) or nonhuman cell lines (mouse L cells and monkey BS-C-1 cells) infected with vaccinia recombinant virus vectors expressing either full-length or truncated CD4 lacking the cytoplasmic tail, it was found that gp120-induced tailless CD4 down modulation was restricted to human (T and HeLa) cells. In the nonhuman cells, PMA treatment could efficiently induce down modulation of full-length CD4 molecules but not of the tailless CD4 molecules even after preincubation with gp120. As mentioned above, incubation of T cells expressing full-length or tailless CD4 molecules with gp120 only at 37°C resulted in 25 to 35% down modulation. In nonlymphoid cells expressing CD4 receptors, gp120 alone induced much less down modulation (0 to 15%). It is known that PMA activates several kinases (e.g., protein kinase C) that phosphorylate cytoplasmic tails of transmembrane proteins. Furthermore, binding to adaptin molecules, which in turn initiates the oligomerization of clathrin and formation of clathrin-coated pits, often requires phosphorylation of membrane receptors' cytoplasmic tails (27). It is conceivable, therefore, that in lymphoid T-cell lines, such as CEM, the accessory molecules are partially phosphorylated even prior to PMA treatment, allowing targeting of the gp120-CD4 complexes to clathrin-coated pits. PMA treatment may increase the phosphorylation state of these molecules and also induce phosphorylation of the full-length (but not of tailless) CD4 molecules, resulting in more pronounced down modulation via clathrin-coated pits.

In an attempt to identify regions within the gp120 and CD4 molecules that are involved in the association with the accessory component, we screened multiple murine and human MAbs for their abilities to block the down modulation. Interestingly, the most effective MAbs were the human MAbs 48D and 17B. These MAbs were shown to bind to a complex conformational epitope on the gp120 envelope which is better exposed after CD4 binding. It is likely that neutralization of HIV-1 with these two MAbs occurs at a post-receptor binding event, similarly to the mechanism of HIV-1 neutralization by anti-V3 MAbs. In our system, two anti-V3 MAbs blocked only partially, while the 48D and 17B MAbs blocked the PMA-induced down modulation of gp120-pretreated CD4.401 receptors almost completely. However, the blocking occurred only if the MAbs were added to cells after their incubation with gp120.

We also observed partial yet consistent blocking by a murine MAb specific for the immunoglobulin-like third complementary region (CDR3) in the CD4 first domain. This region has been the subject of much debate regarding its involvement in gp120 binding, surface CD4-induced gp120 shedding, and HIV-1 neutralization (5, 20, 30). Further experiments are required to establish a role for this region in CD4 association

with the putative accessory membrane component and/or conformationally altered V3 loop on gp120.

Together, our findings could support a model which suggests that gp120 binding to its cellular receptor induces conformational changes in the receptor-ligand complex that facilitate its association with another transmembrane molecule with a cytoplasmic tail which can readily undergo phosphorylation and contains motifs required for targeting to clathrin-coated pits (Fig. 7). This trimolecular complex may assist in the dislocation of the gp120 and exposure of the gp41 hydrophobic region. The fusion process most likely starts at the plasma membrane; however, within 30 min, virions may be targeted to clathrin-coated pits where fusion continues. Further studies will shed light on the CD4-gp120 regions involved in these interactions and assist in isolation of the accessory membrane component.

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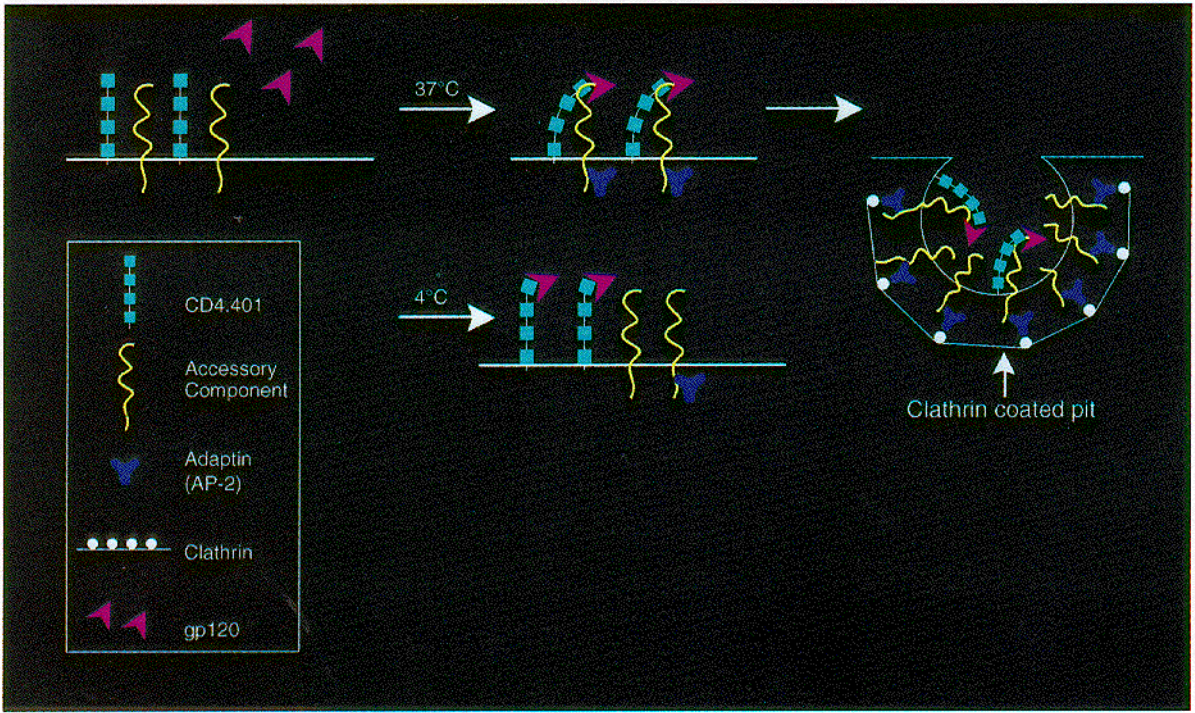
37°C
4°C
CD4.401
Accessory Component
Adaptin (AP-2)
Clathrin
gp120
Clathrin coated pit