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SIGNAL TRANSDUCTION IN SF9 INSECT CELLS: ENDOCYTOSIS OF RECOMBINANT CD4 AFTER PHORBOL ESTER TREATMENT

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In *Spodoptera frugiperda* (Sf9) insect cells infected with a recombinant baculovirus carrying a gene construct encoding human CD4 endocytosis of CD4 is induced after stimulation with phorbol-12-myristate-13-acetate (PMA). Stimulation of endocytosis with PMA reduced the amount of full-length CD4 on the plasma membrane of Sf9 cells by 50% after 2 hours. Endocytosis of CD4 is blocked after intracellular delivery by cationic liposomes of a monoclonal antibody directed against a cytoplasmic sequence of CD4. Endocytosis is also blocked by the calmodulin inhibitor W7. The PKC inhibitor H7 does not inhibit PMA-induced endocytosis. A truncated CD4, in which the last 32 C-terminal amino acids were deleted did,not respond to PMA. Our results show that PMA can stimulate the calmodulin-dependent signal transduction for endocytosis of full-length CD4 in Sf9 cells. Phosphorylation of CD4 in Sf9 cells was not detectable after PMA treatment and PKC is not required for endocytosis.

CD4, a 55 kDa glycoprotein is primarily expressed in human T helper cells, and acts as a coreceptor in the signal transduction between antigen presenting cells and T helper cells. It binds to the MHC class II on the antigen presenting cell and to a T cell receptor complex in T helper cells (1). It is involved in T cell activation through CD3/TCR and the tyrosine kinase $p56^{lck}$ (2).

In vitro studies have shown that T lymphocytes are stimulated by various phorbol esters, which have been shown to stimulate protein kinase C (PKC) (3, 4). After this stimulation CD4 is down-regulated not only in lymphocytes (5) but also in other CD4-expressing mammalian cell lines (3). A specific cytoplasmic sequence of CD4 is required for endocytosis, and mutations of several positions in the cytoplasmatic sequence block phorbol ester induced endocytosis (3, 4). Phorbol ester activation of PKC causes CD4 phosphorylation directly, which may be responsible for endocytosis of CD4 (6). The importance of PKC-mediated CD4 phosphorylation for

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ABBREVIATIONS:
H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; PMA, Phorbol-12-Myristate-13-Acetate; PE, Phycoerythrin; Sf9, *Spodoptera frugiperda*; PKC, Protein Kinase C; W7, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide.

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endocytosis has not been fully established. There is a correlation between phosphorylation of CD4 and its down regulation, but published data support contrary positions on the actual impact of phosphorylation itself (3, 4, 7). Inhibition of PKC by H7 does not block endocytosis of CD4 after phorbol ester stimulation, but it does block phosphorylation. Endocytosis was blocked only after inhibition of calmodulin in peripheral lymphocytes (7). It seems that PMA stimulates other signal transduction modes, besides the PKC related pathway (7).

We present further evidence that endocytosis of CD4 is calmodulin-dependent after PMA treatment in a cell system, which does not express any PKC activity (8). This appears to be a suitable model for the study of the influence of PMA in relation to alternate transduction pathways.

MATERIALS AND METHODS

Insect Sf9 Cell Culture:

Insect Sf9 cells (*Spodoptera frugiperda*) were obtained from Texas A&M University Entomology Department and cultured as previously described (9). Cells were cultured in Ex-cell 400 medium (JRH Biosciences, Lenexa, KS, USA) at 27°C in suspension culture to a density of 2.0 - 3.0 x 10^6 cells/ml (10).

Infection of Sf9 Cells with the Baculovirus for Full-Length and Truncated CD4

The construction of the baculovirus expression vectors for truncated and full-length CD4 has been described elsewhere (9, 11). Sf9 cells were infected with the recombinant baculovirus by using 40 ml Sf9 insect cells of 2.0 x 10^6 cells/ml, 10 ml ex-cell 400 medium and 10 ml of baculovirus with a titer of about 2.0 x 10^9 plaque-forming units/ml. The cells were cultured in spinner flask for 1 day at 27°C (10).

Flow Cytometric Determination of CD4 in the Plasma Membrane of Intact Sf9 Cells:

Expression of CD4 constructs on the plasma membrane was measured 24 h after infection by flow cytometry (Coulter Epics, Miami, FL, USA). Sf9 cells were labeled with a saturating concentration of anti Leu3a-phycoerythrin (Becton Dickinson, San Jose, CA, USA). In general, 300,000 cells, pelleted in an Eppendorf microcentrifuge (15 sec/14000 rpm) were resuspended in 50 µl fresh Ex-cell 400 medium containing 0.6 µg anti Leu3a-PE (3 µg/ml). After 20 min incubation at 4°C, 250 µl of fresh Ex-cell 400 medium was added and the fluorescence measured by flow cytometry.

C6 Antibody and Non-Specific IgG_{2b} Antibody Delivery by Liposomes to Sf9 Cells:

C6 antibody was generated against a cytoplasmic sequence of full-length CD4 and it was produced by Organon Teknika Biotechnology Res. Inst. (Rockville, MD, USA) as an IgG_{2b} mouse isotoype and stored in a stock solution of 2.7 mg/ml (9). Non specific IgG_{2b} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Large unilamellar vesicles were prepared from a mixture of 5 µmol 1, 2-dioleoyl-3-trimethylammonium-propane, and 5 µmol of 1, 2-dioleoyl-sn-glycerol-3-phosphoethanolamine (Avanti Polar Lipids, Alabaster, AL, USA). Vesicles were made by reverse-phase evaporation (12), using multiple extrusions through two stacked 0.08 µm polycarbonate membranes (Poretics Corp., Livermore, CA, USA) within a hand held extrusion device (Liposofast, Avestin, Ottawa, Canada). Vesicles were prepared in 150 mM NaCl, 10 mM HEPES, 0.1 mM EDTA (pH 7.4). Antibody delivery vesicles were prepared with 2.0 mg/ml of antibodies and were used without separation of unencapsulated material. 2.0 x 10^5 cells/ml PBS were labeled with 10 nM of lipid for 10 min at 4°C. Cells were washed and cultured as described before.

Endocytosis of CD4 after Phorbol-12 Myristate-13 Acetate (PMA) Treatment of Sf9:

Infected Sf9 cells in the early logarithmic phase of infection were used for endocytosis measurements. They were incubated with 100 nM PMA, cultured at 27°C and labeled as described above for the flow cytometry detection. In control experiments, cells were incubated without PMA. Values after PMA treatment were normalized by the control value.

Incubation of Sf9 Insect Cells with W7, H7, and PMA for Endocytosis Measurements:

For measurements with the calmodulin inhibitor W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide) or the PKC inhibitor H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) (Calbiochem, San Diego, CA, USA) cells were incubated for 30 min at 27°C

with 25 μM of the inhibitors and then for 30 min with PMA at a final concentration of 100 nM. Detection was the same as above and values were normalized by control values.

RESULTS

Endocytosis of CD4 after PMA Treatment

Endocytosis of two different CD4 constructs was studied in Sf9 cells after baculovirus infection. Sf9 cells in the early logarithmic phase of infection (24-30 hours after infection with full-length CD4; Fig. 1) respond to PMA treatment but not after 60 hours or later. The amount of full-length CD4 in the membrane of Sf9 cells was reduced by 50-60% after 2 hours, as compared with control experiments (Fig. 2). The truncated CD4 construct did not respond to PMA treatment (Fig. 2). The results indicate that PMA treatment can induce endocytosis of full-length CD4, but not of a truncated CD4 lacking the last 32 amino acids at the C-terminal domain.

Neither CD4 construct was phosphorylated, as indicated by ^{32}P-assay (data not shown). Phosphorylation of CD4 thus appears not be the signal for endocytosis of CD4 and PMA is not activating PKC in Sf9 cells, which, as it was reported do not express PKC (8).

Blocking of Endocytosis

We delivered into the cytoplasm the C6 monoclonal antibody, which binds to the cytoplasmic sequence of full-length CD4 (9). We studied whether this antibody can influence endocytosis of full-length CD4. The monoclonal C6 antibody, delivered into the cytoplasm blocked endocytosis of full-length CD4 in the presence of PMA (Fig. 3). After delivery of a nonspecific antibody into the cytoplasm of Sf9 cells, endocytosis of full-length CD4 was not inhibited and the amount of CD4 in the membrane was reduced to 50-60% of controls. The binding between full-length CD4 and the C6 antibody can reduce the mobility of CD4 and create clusters (9), but it appears that these clusters do not enter coated pits. Apparently, endocytosis of CD4, bound by an antibody of 150 kDa, is prevented by simple steric hindrance. Alternatively, it is possible that the antibody covers a signal sequence on the CD4 cytoplasmic domain essential for endocytosis (9).

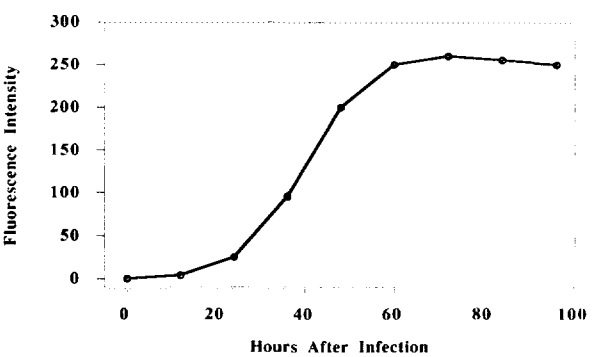

Fig. 1. Expression of full-length CD4 on Sf9 insect cells after baculovirus infection. CD4 was measured in a flow cytometer after Leu-3a-PE labeling.

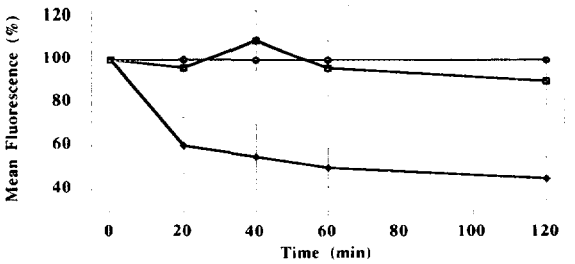

Fig. 2. Endocytosis of full-length (◆) and truncated (■) CD4 constructs in Sf9 cells after PMA treatment. Cells were infected with a baculovirus containing the gene for full-length and truncated CD4 and measured in a flow cytometer after Leu3a-PE labeling. Mean fluorescence values were normalized by dividing through control values (full-length and truncated CD4 without PMA (●)) and multiplication by 100. Curves represent the mean ± SD (n = 6).

Blocking of the Calmodulin Signal Transduction Pathway

A block of the calmodulin signal transduction pathway in Sf9 cells, as described in previous measurements (9), can inhibit full-length CD4 clustering. This obstruction also seems to be related to the blockade of endocytosis of this CD4 construct. The calmodulin inhibitor W7 blocks PMA induced endocytosis of full-length CD4 in Sf9 cells (Fig. 4). These results confirm our measurements related to the clustering phenomena (9). Clustering is the preliminary step for endocytosis of CD4 and blockade of clustering prevents endocytosis.

Incubation with the PKC inhibitor H7 did not alter the PMA-induced endocytosis of CD4 (Fig. 3). The inhibitors alone were not able to induce CD4 endocytosis (data not shown), and the viability of Sf9 cells were for all experiments between 88% and 94%, as determined by flow cytometry and trypan blue.

DISCUSSION

Sf9 insect cells, infected with recombinant baculoviruses are often used to express mammalian proteins (13). Compared to bacterial or yeast cells, many of the post-translational modifications of higher eucaryotes are performed in this system. It is desirable to study

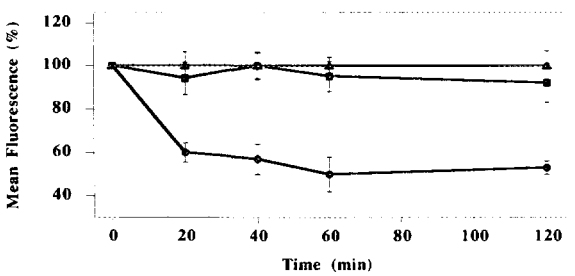

Fig. 3. Endocytosis of full-length CD4 after antibody delivery with cationic liposomes and treatment with PMA. Full-length-expressing Sf9 cells were incubated for 24 hours with cationic liposomes (▲), with non-specific IgG_{2b} encapsulated in cationic liposomes (●) and with C6 monoclonal antibody encapsulated in cationic liposomes (■). CD4 was labeled after PMA treatment with Leu3a-PE and the mean fluorescence was measured in a flow cytometer. Mean fluorescence values were normalized by dividing through control values (only liposomes (▲)) and multiplication by 100. Curves represent the mean ± SD (n = 3).

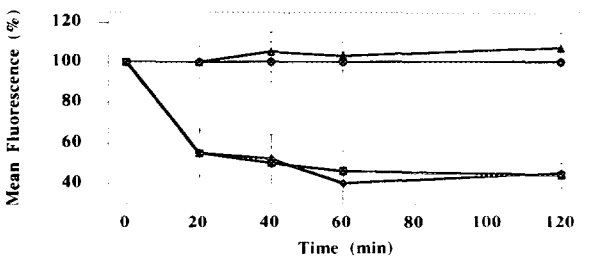

Fig. 4. Endocytosis of full-length CD4 in Sf9. Cells were infected with a baculovirus containing the gene for full-length CD4 and measured in a flow cytometer after Leu3a-PE labeling. Cells were incubated before PMA treatment (100 nM) 30 min with 25 µM W7 (▲) or with 25 µM H7 (■). Treatment of cells with 100 nM PMA only is shown (◆). Mean fluorescence values were normalized by dividing through control values (full-length CD4 without PMA (●)) and multiplication by 100. Curves represent the mean ± SD (n = 4).

mammalian proteins comparatively in a system in which other mammalian proteins thought to be co-factors are missing. PKC might be a co-factor involved in CD4 endocytosis which is not expressed in Sf9 cells (8). In our studies, Sf9 cells were used to evaluate the PMA related down-regulation of expressed human CD4. Although PKC is stimulated by PMA, there is evidence that PMA may be involved in other signal transduction pathways (7).

We have focused on PMA-induced endocytosis of CD4 in Sf9 insect cells in order to compare it with clustering of CD4 in Sf9 cells, as described elsewhere (9). Clustering, as well as endocytosis of CD4 following PMA treatment is dependent upon the cytoplasmic sequence of CD4. Both events can be stimulated for full-length CD4, but not for a truncated CD4 construct.

In previous studies, inhibition of PKC had shown variable results with respect to CD4 endocytosis. After treatment with the PKC inhibitor H7, CD4 in peripheral human T cells was not phosphorylated but endocytosis occurred after PMA treatment (7). In contrast, similar measurements showed down-modulation of CD4 on cloned human CD4+ lymphocytes (14) or on stably transfected HeLa cells (15). Nevertheless, Sf9 cells did not show any CD4 phosphorylation after PMA treatment (data not shown) and they have been described as PKC free (8). Using H7 at a non-toxic concentration ,we were unable to inhibit endocytosis of full-length CD4 or clustering of full-length CD4 in Sf9 cells (9).

Inhibition of calmodulin, a calcium binding protein, which is expressed in Sf9 cells (16) and is important for coated pit formation (17) can block clustering and endocytosis of CD4. Apparently these two steps are related and can be stimulated or blocked in the same way. These processes require an intact, freely accessible cytoplasmic domain of CD4 (no antibody binding) and calmodulin. Phosphorylation of CD4 is not required in Sf9 cells. A cluster, comprised of several CD4 molecules alone might be required in combination with calmodulin dependent coated pit formation for the endocytosis of CD4.

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