Synthesis, Processing, and Intracellular Transport of CD36 during Monocytic Differentiation*

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CD36 is an integral membrane glycoprotein expressed by several cell types, including endothelial cells of the microvasculature, erythrocytes, platelets, and monocytes. In the monocytic lineage, CD36 is expressed during the late stages of differentiation in the bone marrow, in circulating monocytes, and in some tissue resident macrophages, and it is thought to mediate the phagocytosis of apoptotic cells and the endocytic uptake of modified lipoproteins. Here we analyze the synthesis, processing, and intracellular transport of CD36 in U937 and THP-1, two human cell lines representing different stages of monocytic maturation. In both cell lines, phorbol 12-myristate 13-acetate induces the expression of CD36. A 74-kDa intracellular precursor is first synthesized that has the hallmarks of a resident protein of the endoplasmic reticulum. The precursor protein is later processed into a mature form of 90–105 kDa which is transported to the cell surface. The kinetics of processing differ significantly in U937 and THP-1. These differences are specific for the CD36, as two unrelated proteins (CD11b and CD45R) are processed and transported to the surface at similar rates in the two cell lines. A 33-kDa endoglycosidase H-sensitive glycoprotein specifically associates with the 74-kDa precursor. Coprecipitation of gp33 correlates with slow processing of CD36 precursor, suggesting that gp33 may play a role in regulating the intracellular transport of CD36, during monococyte maturation.

MATERIALS AND METHODS

Reagents and Antibodies—PMA, agaroase anti-mouse Ig, bicinechonic acid solution, actinomycin D, Staphylococcus aureus V8 proteases, and cycloheximide were from Sigma; N-glycosidase F and endoglycosidase H from Boehringer Mannheim; protein A-Sepharose CL-4B from Pharmacia Biotech Inc.; ECL Western blotting detection reagent from Amersham Corp. Acetyl avidin-biotinylated horseradish peroxidase complex and e-caproylamido-biotin-N-hydroxysuccinimide ester were from Bio-Division (Milan, Italy).

The monoclonal antibodies (mAbs) used in this study were NL07 (3), OKM5 (Ortho Diagnostics, Milan, Italy), Mo91 (20), all specific for CD36, the L31 anti-HLA class I (21); the OKMI anti-CD11b (Ortho Diagnostics), and the GAP 8.3 anti-CD45RA, B (22). The IgG fraction was prepared from 1207 rabbit anti-CD36 antisemur (20) or from rabbit anti-mouse Ig μ-chain (23) by affinity chromatography on protein A-Sepharose. HRP-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig HRP were from Dako (Glostrup, Denmark). Controls were class-matched irrelevant monoclonal antibodies or a rabbit preimmune serum.

Cell Lines—The promonocytic line U937, the monocytic line THP-1, and the myelomonocytic cell lineHL60, and the leukemic T cell line Jurkat were maintained in culture as described previously (3). Cells were plated at 1 × 10⁶/ml in Petri dishes and treated with PMA (40 nm) for different

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times. In selected experiments, after 1 h of incubation in the presence of PMA, actinomycin D (5 \( \mu \)g/ml) or cycloheximide (50 \( \mu \)M) was added to cell cultures for different times before cell lysis.

Immunofluorescence Staining of Cells—If cells adhered, as after PMA treatment, they were detached by scraping. Aggregates were disrupted by vigorous pipetting. Cells were washed twice with phosphate-buffered saline and resuspended at 1 \( \times \) 10^7 cells/ml for 30 min at 4°C with the appropriate dilutions of purified mAbs or hybridoma cell culture supernatants. Bound antibody was revealed by F(ab’)_2 goat anti-mouse Ig labeled with fluorescein isothiocyanate (Technogenetics, Milan, Italy). The cells were then analyzed on a FACScan cytometerograph (Becton Dickinson, San Jose, CA).

SDS-PAGE. In some experiments endoglycosidase treatments were performed on Western blot—3 \( \times \) 10^6 cells were washed twice with tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) resuspended at 10^6/ml, and lysed with Nonidet P-40 (final concentration 1%) for 30 min at 0°C in the presence of protease inhibitors. After centrifugation for 15 min at 12,000 \( \times \) g, aliquots of the lysates were resolved by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) as described previously (24) on vertical slab gels of 7.5, 10, or 12% acrylamide. In each experiment samples were normalized so as to contain the same amount of total protein determined by bichinchoninic acid assay (Sigma). Proteins were electron-transferred (2 h at 60 V) to nitrocellulose sheets. The latter were preincubated 1 h at 20°C with 5% bovine serum albumin in TBS, incubated with HRP rabbit anti-mouse Ig (1:1000) for 1 h, and processed for ECL according to the supplier’s instructions.

The molecular mass standards included lysozyme (14 kDa), trypsin inhibitor (21 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), BSA (69 kDa), phosphorylase b (97 kDa), \( \beta \)-galactosidase (116 kDa), and myosin (200 kDa), and were revealed by red Ponceau staining.

Cell Surface Protein Labeling with Suddenimidobiotin—About 5 \( \times \) 10^6 cells were washed twice in labeling buffer (150 mM NaCl, 100 mM Hepes, pH 8.0) and resuspended in 0.5 ml of the same buffer. Suddenimidobiotin was added at the final concentration of 0.25 mM, and cells were incubated at 20°C for 30 min in the presence of 0.002% sodium azide. The cells were then washed twice with 3% fetal calf serum in phosphate-buffered saline and lysed as described previously.

Endogenous Labeing—For pulse-chase experiments, cells were preincubated for 15 min in methionine/cysteine-free medium supplemented with 2% dialyzed fetal calf serum and 1 mM glutamine and labeled at 2 \( \times \) 10^7/ml for 25 min with 500 \( \mu \)Ci/ml Tran[35S]label (ICN Radiochemicals, Milan, Italy), specific activity >800 Ci/mmol. Pulse-labeled cells were then washed and incubated in regular culture medium for various time periods before lysis and immunoprecipitations.

Immunoprecipitation—After a preclearing step (2 h at 4°C) with the appropriate dilutions of purified mAbs or normal rabbit serum adsorbed to protein A-Sepharose, cell lysates were immunoprecipitated with protein A-Sepharose beads coated with the 1207 anti-CD36 rabbit serum and the A-Sepharose bead coating, the 1207 anti-CD36 rabbit serum and the normal rabbit serum adsorbed to protein A-Sepharose, cell lysates were immunoprecipitated with protein A-Sepharose beads coated with the

RESULTS

CD36 Surface Expression Is Induced by PMA Treatment—As determined by flow cytometry, THP-1 in culture have undetectable levels of CD36 on the cell surface and are induced by PMA to express low levels of CD36 (Fig. 1). In contrast, U937 cells constitutively express low levels of CD36, which increase significantly after 48 h of PMA treatment (Fig. 1). Treatment induces morphological changes in both cell lines that resemble the progression in differentiation steps of monocytic cells into macrophages, consisting mainly in adhesion to plastic, a change in morphology, and a change in the lipid content (26, 27). This band, that likely corre-
kDa is observed in some experiments in unstimulated THP-1 cells, but not in U937 (Fig. 2b). The identity of this band, which disappears a few hours after PMA stimulation and is not evident in pulse-chase assays (see below), was not investigated further.

Noteworthy, PMA does not induce significant changes in the HLA class I molecule steady state levels (Fig. 2, bottom panels). Hence, induction of surface proteins does not appear to be a general phenomenon generated by PMA. The kinetics of appearance of p74 suggests that it might represent a precursor of CD36 whose synthesis is activated by PMA. Immunoprecipitation and pulse-chase experiments were thus designed to verify this possibility.

p74 Is the CD36 Precursor and Is Differentially Processed in the Two Cell Lines—Pulse-chase experiments were performed with THP-1 and U937 stimulated for 12 h with PMA (Fig. 3). In both cell lines, p74 is the most abundant form detected after a 25-min pulse (lanes 1 and 6). In U937, p74 rapidly converts into the endo-H-resistant forms of 90–105 kDa. After chasing 14 h, virtually all p74 has been converted in the mature form, demonstrating a clear precursor-product relationship. Also THP-1 cells synthesize p74 (lane 6); however, in these cells the conversion into gp90–105, the form that can be found on the cell surface, is much slower than in U937. Densitometric tracings of the autoradiograms reveal that 75% of CD36 has been processed in U937 after 2 h, while only 35% in THP-1. These results correlate well the fact that, as drawn by immunofluorescence and Western blots (Figs. 1, and 2), there is very little CD36 on the surface of THP-1 after 12 h of PMA stimulation. Moreover, in THP-1 neither p74 nor mature CD36 are present after a 14-h chase (lane 10), suggesting that they undergo intracellular degradation. Hence, the low expression of CD36 on the surface of THP-1 largely depends on posttranslational events. In all likelihood, the absence of the 90–105-kDa form reflects a slow transport through the Golgi apparatus. The differences could be due to a generalized slower rate of transport in THP-1, or rather a specific feature of CD36. To discriminate between these two possibilities we analyzed the maturation of two other proteins, the constitutively expressed CD45R and the PMA-inducible molecule (CD11b).

The PMA-induced accumulation of gp74 depends on de novo synthesis and dimerization of CD11b antigen is similar in THP-1 and the PMA-inducible molecule (CD11b). The kineticsof procession and pulse-chase experiments. Cells treated for 12 h with PMA were endogenously labeled with [35S]methionine/cysteine, then washed and incubated in regular culture medium for various chase-time periods before lysis and sequential immunoprecipitations with 1207 anti-CD36 rabbit serum, OKM1 mAb anti-CD11b, and GAP 8.3 mAb anti-CD45RA and B; see “Materials and Methods.” The arrow indicates the co-immunoprecipitated 33-kDa protein, the bracket indicates the mature CD36 protein, and the arrowhead indicates the gp74. Molecular mass markers are as indicated under “Materials and Methods.”

Moreover, in THP-1 neither p74 nor mature CD36 are present after 14 h of chase (Fig. 3c, lanes 3 and 8).

gp74 is Antigenically Related to gp90–105 and Is Not Expressed on the Plasma Membrane—Consistent with the results of Fig. 2b, similar amounts of p74 and gp90–105 are detected by Mo91 in the lysates of THP-1 cells that were treated with PMA for 48 h (Fig. 4, Total Lys). A rabbit antiserum raised against CD36 molecules (Fig. 4, 1207), but not preimmune serum (Fig. 4, Ctrl), is able to precipitate both p74 and gp90–105, confirming that the two molecules are antigenically related.

When intact THP-1 cells are labeled with biotin prior to cell lysis, only the gp90–105 band is decorated by avidin (see the right lane), suggesting that p74 is not expressed on the cell surface. The faint band with an apparent molecular mass of 74 kDa detected by avidin in the immunoprecipitate (1207) might be due to the biotin labeling of the intracellular precursor from a few dead cells permeabilized during incubation. A weak band of 130-kDa molecular mass is present in the immunoprecipitated material (1207). This band, that was never detected in the total lysates, is likely to originate from artifactual cross-linking by succinimidobiotin.

If p74 were a precursor of mature CD36, treatment with N-glycosidase F, an enzyme that cleaves all types of N-linked glycosidic residues, should yield a single band of about 57 kDa (28). As shown in Fig. 5 (lanes 1 and 2) this is indeed the case. In PMA-differentiated U937 cells, both the gp90–105-kDa protein band and the one of 74 kDa yield a unique protein band of 57 kDa when N-linked sugars are removed. Treatment with endoglycosidase H allows one to distinguish high mannos (sensitive) from complex sugars. As evident from lanes 3 and 4, gp74 is sensitive to this glycosidase, while gp90–105 is largely resistant. The partial digestion of gp90–105 is probably due to incomplete processing of some of the CD36 glycans. The PMA-induced accumulation of gp74 depends on de novo synthesis and dimerization of CD11b antigen is similar in THP-1 and the PMA-inducible molecule (CD11b).
mRNA and protein synthesis. Consistent with gp74 being a precursor of CD36, both actinomycin D (a mRNA synthesis inhibitor) and cycloheximide (an inhibitor of protein synthesis) prevent the accumulation of the gp74 induced by PMA treatment (not shown).

A 33-kDa Endo-H-sensitive Glycoprotein Is Associated with the gp74 Protein Precursor—As evident from Fig. 3a, a protein with a molecular mass of 33 kDa (p33) is coprecipitated by anti-CD36 in both U937 and THP-1 (lanes 1 and 6). The presence of this protein seems to correlate with the inability of the 74-kDa precursor of being processed. While the 33-kDa protein is no longer immunoprecipitable after 30 min of chase in U937 cells (Fig. 3a, lanes 2 and 3) it is still detectable after 2 h of chase in THP-1 cells (Fig. 3a, lane 9).

Nonreducing gels (not shown) reveal that the p33 molecule is not disulfide-linked to CD36 precursor, while like gp74, p33 is endo-H-sensitive and is digested into a 22–24-kDa molecule (Fig. 6).

**DISCUSSION**

In the present study, the U937 promonocytic and the THP-1 monocytic cell lines have been exploited to analyze the synthesis, processing, and surface expression of CD36 during monocyte/macrophage differentiation. Some of the events that characterize this developmental program, including changes in the growth rate, adherence, and expression of surface markers can be mimicked in these cell lines by stimulation with PMA (26, 27). Compared with blood monocytes, in vitro differentiated macrophages and alveolar macrophages showed a down-regulation of CD36 surface expression (29) that is consistent with the different degree of differentiation and CD36 expression observed in the two cell lines used in the study.

The most intriguing finding that emerges from our experiments is that the intracellular processing of CD36 is faster in U937 than in THP-1. Crucial to this conclusion is the identification of different molecular glycoforms of CD36 that represent discrete stages in the biogenesis of this membrane protein. That the 74-kDa protein is a precursor of the mature CD36.

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2. M. Alessio, L. De Monte, A. Scirea, and P. Gruarin, unpublished data.
The 1207 anti-CD36 rabbit serum (R\textsubscript{u}CD36) and the anti-mouse IgM rabbit serum (R\textsubscript{u}IgM) were used to immunoprecipitate the gp74 and the gp33 from endogenously labelled U937 or radiolabelled mouse Ig \( \mu \)-chain, respectively before or after adsorption on intact human platelets.

| Antibodies | Precipitated proteins | Area<sup>a</sup> | Adsorption on intact human platelets | Area<sup>a</sup> | Inhibition |
|------------|-----------------------|-----------------|---------------------------------------|-----------------|------------|
| R\textsubscript{u}CD36 | gp74                  | 31.54           | 6.49                                  | 79.4            |            |
|              | gp33                  | 11.50           | 2.29                                  | 80.1            |            |
| R\textsubscript{u}IgM |                       | 15.20           | 14.8                                  | 3.9             |            |

<sup>a</sup> The area of specific bands resolved by SDS-PAGE was calculated by densitometric analyses and expressed in pick arbitrary units.

As in other glycoproteins, these size differences are likely to reflect different intracellular locations. The selective accessibility to biotin indicates that only the mature CD36 (gp90–105) is expressed on the plasma membrane. On the other hand, the sensitivity of gp74 to the endoglycosidase H, an enzyme that degrades the high mannose glycans characteristic of protein that did not reach the medial Golgi apparatus (30), suggests that the low molecular weight precursor is localized in an early compartment of the secretory pathway. Hence, the steady state ratio between the two bands can be taken as an indication of the efficiency of intracellular processing and transport. With this in mind, it appears that the transport of CD36 is slower in THP-1 than in U937 at all time points after PMA administration and that the rate of synthesis of the precursor has little influence on the subsequent processing events. As reported by others (31) we confirmed that PMA induces an increase in the rate of transcription and accumulation of specific transcripts, but does not alter the posttranslational events that modulate the expression of CD36 on the cell membrane in the two cell lines. At least two features seem to retard the transport of CD36 to the surface of THP-1. First, transport of the protein to the Golgi apparatus is severely impaired in these cells, as indicated by the abundance of the endo-H-sensitive gp74 and its slow conversion into the more mature forms. Second, as evident from the pulse-chase experiments (Fig. 3) and confirmed by detergent insolubility analyses (not shown), gp90–105 is degraded at a faster rate in THP-1 cells.

An intracellular localization of CD36 has already been reported in platelets, where some mature CD36 is stored within the \( \alpha \)-granules and can be rapidly transported to the plasma membrane upon activation (32). In uninduced monocytic cell lines, however, the intracellular pool of CD36 is small (Fig. 2). Moreover, the PMA-induced expression of CD36 is dependent on RNA and protein synthesis, suggesting that the molecule is neither recycled from intracytoplasmic stores, as observed for platelets, nor does it represent a degradation product.

Although the rate of bulk flow membrane traffic can be regulated (33), faster transport per se is not sufficient to explain the more efficient processing of CD36 in U937. Other membrane molecules, either PMA-inducible (CD11b) or constitutively expressed (CD45R), are indeed processed and transported intracellularly at similar rates in U937 and THP-1.

It is well established that individual proteins are secreted at different rates by the same cell (34). An important limiting step of intracellular transport occurs at the endoplasmic reticulum-Golgi apparatus boundary and generally reflects the rates at which the folding of the cargo protein takes place. Also integral membrane proteins are subject to the same quality control events that restrain transport to structurally mature molecules, providing an additional step for regulating gene expression during development. Perhaps the best examples of such posttranslational regulatory mechanisms come from the stage-specific expression of antigen receptors on T and B lymphocytes (see Refs. 35 and 36 for reviews). As for these multimeric proteins, subunit assembly is essential for negotiating transport to the plasma membrane; the absence of a single subunit is generally sufficient to cause retention and degradation of the other components (37). However, there are also cases in which the assembly of existing subunits is dynamically regulated. For instance, for unknown reasons B lymphocytes are unable to polymerize and secrete IgM (23). Similarly, immature thymocytes synthesize but fail to assemble T cell antigen receptor \( \alpha \)- and \( \beta \)-chains, degrading them in the endoplasmic reticulum (38). If multimeric proteins hence can be regulated at the level of assembly, only the rate of folding would modulate the transport of monomeric molecules. As most of the chaperones identified so far that catalyze the folding of nascent proteins are ubiquitous, abundant proteins, it is not easy to envisage a model that explains the different rate of transport of CD36. The latter is thought to be a monomeric receptor (see Ref. 1). However, in platelets the mature CD36 transiently associates with proteins involved in transducing signals (18), while in endothelial cells, it is present as a detergent-insoluble complex (39). These interactions may be responsible for the detergent "insolubility" of gp90–105 observed in U937 and THP-1, but it is less likely that they mediate the different processing of gp74.

An appealing hypothesis is that the observed association with gp33 may regulate the transport and processing of the CD36 precursor, determining its intracellular retention or the expression on cell surface. Like the CD36 precursor to which it is noncovalently bound, gp33, or at least the fraction that associates with gp74, is also retained in a pre-Golgi compartment, as demonstrated by sensitivity to endoglycosidase H. The association between gp33 and gp74 inversely correlates with processing of the latter. Hence, only in THP-1, where processing of CD36 is slow, does gp33 coprecipitate at extended chase times. Lastly, in U937 dissociation of gp33 correlates with the

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acquisition of endo-H resistance. The disappearance of radioactive gp33 upon chase indicates that it is mostly newly synthesized gp33 that associates with the CD36 precursors. These results suggest that gp33 interact only once with the CD36 precursor. This behavior differs from other known chaperone molecules, such as BiP, that have been shown to associate sequentially with more than one newly synthesized proteins (40). It is of note that a molecule that displays similar biochemical properties to gp33 is coprecipitated from COS cells transfected with CD36. A conservation among species and cell types would suggest a general role for this protein.

A CD36 deficiency has been described as the absence of surface expression of CD36 molecule (41, 42). Recently, it has been shown that the substitution of proline-90 to serine directly leads to CD36 deficiency, impairing the maturation of the CD36 precursor and addressing its intracellular degradation (43). It will be of interest to investigate whether gp33 associates with these mutants.

In conclusion, it appears that, during their differentiation, monocytic cell lines exploit many levels, including alterations in intracellular transport, to regulate the expression of CD36. It remains to be seen whether the regulatory mechanisms demonstrated here for cultured neoplastic cells are exploited, as postulated by others (44), also in the normal myelomonocytic maturing pathways.

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