Identification of Lysosome-Associated Membrane Protein-2 as an Activation-Dependent Platelet Surface Glycoprotein

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Platelets undergo biochemical and morphologic changes when stimulated that greatly alter their function and contribute to their role in thrombosis and hemostasis. We recently identified and cloned the cDNA for a platelet surface glycoprotein expressed on activated, not resting cells. We found that this protein, lysosome-associated membrane protein-1 (LAMP-1), is an integral membrane protein of the lysosome that translocated to the surface membrane when platelets were stimulated by a strong agonist. We now show with immuno-fluorescence flow cytometry that LAMP-2, a lysosomal membrane protein that shares approximately 30% homology with LAMP-1, is also expressed preferentially on the surface of activated platelets. Equilibrium binding studies with 125I-anti-LAMP-2 IgG showed approximately 1,100 binding sites per thrombin-stimulated platelet and less than 50 per resting platelet. Sucrose gradient ultracentrifugation fractionation of resting platelet sonicates showed that LAMP-2 colocalized with LAMP-1 and with lysosomal enzymes, and not with thrombospondin or serotonin, which are markers of the two other platelet granule compartments, a-granules and dense granules. LAMP-2 surface expression was minimal in response to platelet stimulation by weak agonists such as epinephrine and ADP. These data show that LAMP-2, like LAMP-1, translocates from the lysosomal membrane compartment to the surface membrane when platelets are activated. Regulated surface expression of these heavily glycosylated proteins may play a role in the adhesive, prothrombotic phenotype of these cells.

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MATERIALS AND METHODS

Materials. Murine monoclonal anti-LAMP-2 (H4B4) and anti-LAMP-1 (H5G11) antibodies and rabbit polyclonal anti-LAMP-2 and anti-LAMP-1 sera were gifts of Dr J.T. August (Johns Hopkins University, Baltimore, MD). These antibodies were generated from mice immunized with human LAMP proteins prepared from spleen extracts. An additional murine monoclonal anti-LAMP-2 IgG (CD3) was provided by M. Fukuda (La Jolla Cancer Center, La Jolla, CA). Monoclonal antiplatelet glycoprotein (GP) Ib (AP1) was from T. Kunicki (Blood Center of Southeast Wisconsin, Milwaukee) and anti-PADGEM (AC1.2) from Dr B. Furie (Tufts University Medical Center, Boston, MA). Murine antithrombosphandin IgG 11.4 was prepared as previously described. Rabbit polyclonal anti-β-galactosidase antibody was purchased from Cappel (Durham, NC). Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit and antimouse IgG were purchased from Tago (Burlingame, CA). Alkaline-phosphatase-conjugated goat antirabbit and antimouse IgG were purchased from BioRad (Richmond, CA), as were the colorometric substrates, 5-bromo-4-choro-3-indolyl phosphate p-toluidine salt (BCIP) and p-nitro blue tetrazolium chloride (NBT).

We now ask whether other lysosomal membrane proteins translocate to the surface after platelet activation. LAMP-1 is a member of a highly conserved small gene family made up of two members. It is approximately 30% homologous to another lysosomal membrane protein termed LAMP-2. Both proteins have the same general structure consisting of a short 10 to 11 amino acid carboxy-terminal intracytoplasmic domain followed by a classic hydrophobic transmembrane region and then a large amino-terminal extracytoplasmic region. The latter consists of two homologous domains each made up of two 36 to 38 amino acid cysteine-linked loops. These domains are separated by a pro-ser–rich region homologous to the hinge domain of IgA. The extracytoplasmic region has multiple sites for N-linked glycosylation, and contains greater than 60% carbohydrate by weight. Much of the carbohydrate consists of unusual polyactosaminoglycan residues. We now report that LAMP-2, like LAMP-1, is an activation-dependent platelet surface glycoprotein, and that surface expression results from lysosomal membrane flow and fusion.

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mmol), were purchased from Amersham (Arlington Heights, IL). Nitrocellulose (0.45 µm) was purchased from Schleicher and Schuell (Keene, NH). Sepharose 2B was purchased from Pharmacia (Piscataway, NJ). Human thrombin was a gift of Dr. J.T. Fenton (Albany, NY). D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem (San Diego, CA).

**Platelet isolation.** Platelets were prepared from 20 mL fresh blood drawn in 2.7 mL acid citrate dextrose (38 mmol/L citric acid, 75 mmol/L sodium citrate, 156 mmol/L dextrose). After a low-speed spin (200g for 15 minutes), the top half of platelet-rich plasma was applied to a 25-26 cm column of Sepharose 2B pre-equilibrated with divalent cation-free Modified Tyrode’s buffer (MTB: 137 mmol/L NaCl, 0.475 mmol/L NaH₂PO₄, 2.7 mmol/L KCl, 5 mmol/L HEPES, 1.2 mmol/L NaHCO₃, 5.5 mmol/L glucose, pH 7.4) containing 0.2% bovine serum albumin (BSA). The platelet peak was pooled and, in some cases, recalculated (to 1 mmol/L).

**Immunofluorescence flow cytometry.** Platelet aliquots were treated with agonists, or buffer control, for 3 minutes at 22°C, followed, when appropriate, by the specific thrombin inhibitor, PPACK (10⁻⁴ mol/L), for 2 minutes. Primary antibody was added for 30 minutes, followed by fixation in an equal volume of MTB containing 2.2% formaldehyde for 30 minutes. The platelets were washed three times with MTB/BSA and incubated with FITC-conjugated secondary antibody for 30 minutes. After washing as before, the cells were re-suspended in 400 µL filtered phosphate-buffered saline (PBS). Ten thousand cells were analyzed for each study, as previously described, using an Epics Flow Cytometer (Coulter, Hialeah, FL) gated to exclude nucleated cells.

**Western blot.** Platelets were diluted and fixed in a solution of 1% paraformaldehyde in Hanks Buffered Salt Solution (HBSS) for 15 minutes. They were then adhered to polylysine-coated glass coverslips. The slides were divided into two groups: one group was incubated and washed in solutions of HBSS containing 0.1% saponin (for permeabilization), the other in solutions of HBSS alone. Slides were washed twice, incubated in 2% goat serum for 15 minutes, and then incubated with primary antibodies for 30 minutes. After extensive washing, slides were incubated with FITC-goat antimouse IgG for 30 minutes. Slides were washed again and viewed under fluorescence.

**RESULTS**

Murine monoclonal antibody to human LAMP-2 was used to analyze platelet LAMP-2 localization and expression. As shown in Fig 1, indirect immunofluorescence flow cytometry studies showed binding of the antibody to the surface of activated, but not resting platelets. Nonimmune IgG binding was indistinguishable from anti-LAMP-2 binding to resting platelets. No LAMP-2 antigen was detected in the suspension media of the activated platelets using an ELISA assay, suggesting that LAMP-2 was not secreted. Immunofluorescent microscopy of saponin-permeabilized resting and nonpermeabilized platelets showed intracellular fluorescence of permeabilized platelets, but no fluorescence of the nonpermeabilized cells (Fig 2). These data suggest that LAMP-2, like LAMP-1, was translocated from internal platelet locations to the surface upon stimulation. Immunoblot analysis of platelet lysates (Fig 3) showed that LAMP-2 in these cells migrated as a broad band of approximately 110 to 120 Kd. This is similar to that reported for LAMP-2 purified from human liver. There was no reactivity with control nonimmune antibody.

To estimate the amount of LAMP-2 surface expression, binding of 125I-conjugated monoclonal anti-LAMP-2 IgG to activated and resting platelets was measured. Binding was time- and concentration-dependent and, as seen in a

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Fig 1. Immunofluorescence flow cytometric demonstration of platelet LAMP-2 expression. Gel-filtered platelets were treated with either thrombin (0.5 U/mL) or buffer and then incubated with murine monoclonal anti-LAMP-2 IgG, followed by FITC-conjugated goat antimouse IgG. LAMP-2 antibody bound to the surface of activated platelets (A; -) but not to resting platelets (R; --). Nonimmune IgG binding was indistinguishable from anti-LAMP-2 binding to resting platelets.

Representative equilibrium binding isotherm (Fig 4), reached saturation between 0.5 and 1.0 µg/mL. In this study, nonspecific binding at maximum input antibody concentration was 8.7% (184 cpm). Scatchard analysis (inset) of these data showed 1,126 ± 318 binding sites per activated platelet. The number of anti-LAMP-2 binding sites expressed on activated platelets varied among individual donors as well as from day-to-day with the same donor, ranging from 700 to 1,800 binding sites per platelet. Resting platelets expressed little or no LAMP-2 on their surfaces. As shown in Fig 5, when compared with platelets maximally stimulated by thrombin, an average of 2.3% of binding was seen on resting platelets (range, 0 to 40 molecules per platelet).

We have previously shown that, unlike α-granule and dense granule secretion, platelet lysosome release, measured as LAMP-1 surface expression, did not follow stimulation by the weak agonists ADP or epinephrine. In addition, platelet acid hydrolase release, which is presumably from lysosomes, has been studied by several investiga-

Fig 2. Immunofluorescence demonstration of intracellular LAMP-2 expression in resting platelets. Intact platelets or platelets permeabilized with saponin (0.1%) were fixed in parafomaldehyde and adhered to polylysine-coated glass coverslips. These were then incubated with murine monoclonal anti-LAMP-2 IgG (CD3) at 40 µg/mL followed by FITC-conjugated goat antimouse IgG. Immunofluorescent microscopy showed antibody binding only to permeabilized platelets (A), and not intact cells (B). Nonimmune IgG did not bind. Shown is one area containing approximately 50 dispersed platelets at a magnification of ×1,800.
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Fig 4. 125I-anti-LAMP-2 IgG binding to thrombin-stimulated platelets. Gel-filtered platelets were stimulated with 0.5 U/mL thrombin for 10 minutes at 22°C. Increasing concentrations of [125I]murine monoclonal anti-LAMP-2 IgG were then added to 200 μL aliquots for 30 minutes at 22°C. Bound radioactivity was then measured after centrifugation through silicone oil. Data are expressed as specific binding, i.e., that which was inhibited with a 20-fold excess of unlabelled antibody. Inset shows Scatchard analysis of these data.

A series of sucrose density gradient plateau fractionation studies was performed to investigate further the intracellular localization of LAMP-2 in resting platelets. Platelets were sonicated in such a way as to only disrupt their outer plasma membrane, and centrifuged through a 30% to 60% continuous sucrose gradient. A typical banding pattern was observed, as described previously. Two narrow, low density “membrane” bands (fractions 2 and 4) were clearly distinguished from an intermediate density band (fraction 6) and two high density bands (fractions 8 and 10). These bands were collected along with the intervening regions (fractions 1, 3, 5, 7, 9, and 11 through 13), and subjected to protein and ELISA analyses. In one experiment, platelets were loaded with [14C]-hydroxytryptamine, and radioactivity in the fractions detected by scintillation counting. Figure 6A shows colocalization of β-galactosidase, a lysosomal enzyme, and LAMP-1, a lysosomal membrane protein, with LAMP-2 in fraction 6. GP Ib, a marker of the external platelet plasma membrane, did not cosediment with LAMP-2 and was found in fraction 4. Figure 6B shows the distribution of [14C]-hydroxytryptamine, a dense granule

Fig 5. Agonist dependence of platelet surface LAMP-2 expression. Gel-filtered platelets (200 μL) were incubated with thrombin (0.5 U/mL), ADP (2.5 μmol/L), epinephrine (0.5 mmol/L), or buffer for 10 minutes at 22°C. Anti-LAMP-2 IgG was then added for 30 minutes at 22°C without stirring and bound antibody detected as is Fig 1.

Thr None ADP epi

1 2 3 4 5 6 7 8 9 10 11 12 13

FRACTION NUMBER

Fig 6. Sucrose density gradient centrifugation of sonicated platelets. (A) Colocalization of β-galactosidase (○), a lysosomal enzyme, and LAMP-1 (△), a lysosomal membrane protein, with LAMP-2 (○). Proteins were detected by ELISA, with specific antibodies, followed by an alkaline-phosphatase-conjugated second antibody. The data are expressed as rate of color development (a function of bound antibody) over time. (B) The distribution of [14C]-hydroxytryptamine (serotonin) (○), a dense granule marker, as measured by scintillation counting. (C) The distribution of TSP (□), an α-granule marker, measured by ELISA, as in (A).
marker. Greater than 47% of the total counts recovered were found in one high-density fraction, fraction 10. Figure 6C shows the localization of the α-granule marker, TSP. Greater than 90% of the total TSP was found in fractions 9 and 10. This biochemical pattern of α-granule and dense granule contents is similar to that reported by others. From these data, colocalization of LAMP-2 with lysosomal proteins, and clear differentiation from the two other granule pools, we conclude that LAMP-2 is lysosomal in resting platelets.

**DISCUSSION**

Secretion from membrane-bound cellular storage pools in response to agonist stimulation is a general characteristic of many cells, including platelets. In his widely accepted membrane flow hypothesis, Palade suggested that during secretion, granule integral membrane proteins become incorporated into the external plasma membrane. Studies of P-selectin (GMP140), a platelet α-granule integral membrane protein, provided evidence that a membrane fusion event does indeed occur during platelet activation and secretion. The biologic importance of this mechanism of phenotypic surface transformation by the activated platelet was shown by the recent demonstration that P-selectin is a "receptor" on activated platelets that mediates adhesion to leukocytes. We recently reported that LAMP-1, an integral lysosomal membrane protein, also translocates to the plasma membrane during an activation and secretion event, suggesting that lysosomal membrane fusion also occurs during platelet secretion. Nieuwenhuis et al. have also reported that CD63, a 53-Kd platelet activation antigen, is also a transmembrane lysosomal protein. Data reported here using highly specific monoclonal antibodies suggest that lysosomal membrane flow and fusion also leads to surface expression of a second, highly glycosylated protein, LAMP-2. However, the number of LAMP-1 and LAMP-2 molecules estimated on the surface of activated platelets (<2,000 of each) is substantially less than that reported for P-selectin or CD63. However, these data confirm the existence of lysosomal membrane fusion during platelet activation and identify another activation-dependent surface protein that may have a functional role during thrombosis.

These data also support the concept that platelet stimulation by "strong agonists" such as thrombin and collagen is mediated by different intracellular events than stimulation by "weak agonists" such as ADP and epinephrine. We previously showed that surface expression of LAMP-1 required platelet stimulation by a strong agonist. We have now studied LAMP-2 surface expression in response to different agonists and found expression was also maximal in response to thrombin and nearly absent on platelets stimulated with high concentrations of the weak agonists ADP or epinephrine. α-Granule secretion occurred under these circumstances, suggesting that lysosome secretion and membrane flow is coupled to a different stimulus-response pathway than α-granule secretion.

While there is no known function for either of the LAMP proteins, the amino acid sequence of LAMP-1 is nearly identical to two leukemia cell glycoproteins: GP130/P2B, from the highly metastatic tumor cell line MDAY-D2, and a differentiation marker in hematopoietic cells. In addition, the N-linked polyolactosaminoglycans carried on both lysosomal proteins have been correlated with increased metastatic potential. The sialylated polyolactosamines carried by LAMP-1 and LAMP-2 are also potential substrates for α(1-3) fucosylation, leading to the generation of sialylated Lewis and sLe antigens. Recent reports have shown that the sLe antigens are ligands for some members of the selectin adhesion molecule family (E-selectin and the L-selectin antigen). Although a minor fraction (<2%) of LAMP protein is associated with the plasma membrane of most nucleated cells, presumably as a result of selective exchange of lysosomal and plasma membranes, our studies are the first to show significant and regulated cell surface expression on normal adult cells. As previously noted, increased surface expression of LAMP has been observed on transformed cells of high metastatic potential and, interestingly, on embryonic cells. Activated platelet surfaces share certain functional characteristics with these cells, such as enhanced adhesiveness and protease activity. It is thus possible that the regulated expression of LAMP-1 and LAMP-2 on the surface of activated platelets may play a role in these functions. A deglycosylated form of LAMP has been shown to bind collagen and the fibronectin adhesion peptide RGD, further suggesting a possible role in adhesion.

In summary, we report the identification and characterization of a previously unknown platelet activation marker. This protein translocates from the lysosomal membrane, and has characteristics of an adhesive molecule. Identification of proteins, like LAMP-1 and LAMP-2, that are preferentially expressed on activated cells may aid in the design of novel diagnostic and therapeutic approaches to diseases such as atherosclerosis and thrombosis.

**REFERENCES**

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