CD69 Is Expressed on Platelets and Mediates Platelet Activation and Aggregation

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Summary

CD69, a surface dimer so far considered an early activation antigen restricted to lymphocytes, was found constitutively expressed on human platelets. Biochemical analysis revealed that platelet CD69 appears on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a broad 55–65-kD band, in which three 55-, 60-, and 65-kD components were detectable when nonreduced, and as two 28- and 32-kD bands when reduced, corresponding to the two disulfide-linked chains of the dimer. It therefore closely resembles lymphoid CD69, although the resolution of the three bands under nonreducing conditions is not usually seen in lymphoid cells. Moreover, as CD69 expressed on activated lymphocytes and CD3bright thymocytes, both chains are constitutively phosphorylated.

CD69 stimulation by anti-Leu-23 monoclonal antibodies induced platelet aggregation in a dose-dependent fashion. This effect was associated with Ca2+ influx and platelet degranulation, as revealed by adenosine triphosphate release. In addition, CD69 stimulation in platelets induced production of thromboxane B2 and PGE2, suggesting activation of arachidonic acid metabolism by cyclooxygenase. As observed for CD69-mediated T cell activation, platelet activation through CD69 requires molecular crosslinking. These results suggest that CD69 may function as an activating molecule on platelets, as on lymphocytes, and point toward a more general role of this surface dimer in signal transduction.

CD69 was initially detected on the surface of activated lymphocytes. Resting T lymphocytes in fact do not express CD69, but its expression may be rapidly induced by triggering of their TCR/CD3 complex (1, 2). Similarly, CD69 is induced on the surface of NK cells by interaction of IL-2 with the p75 IL-2R (3, 7). CD69 induction is extremely rapid, being detectable within 2 h from the stimulation, yet requiring new RNA and protein synthesis (1, 6). Its expression on T cells stimulated through the TCR/CD3 complex quantitatively correlates with the extent of TCR/CD3 crosslinking (6), and is strictly dependent on the activation of protein kinase C (PKC)1 and on the maintenance in time of elevated intracellular [Ca2+] levels (6).

In vivo, CD69 was found constitutively expressed and phosphorylated on CD3bright thymocytes, with a linear relationship between levels of CD69 and levels of expression of surface CD3 (5). These include all single-positive cells, and 10% of double-positive CD3bright cells. A small number of circulating large T and NK cells have also been found to be CD69+, possibly as a result of in vivo activation (3).

A physiologic ligand for CD69 has not yet been identified, but experimental evidence indicates that the molecule is capable of signal transduction (8). Anti-CD69 mAbs, in fact, induce Ca2+ influx in CD69+ T cells and, when PKC is simultaneously and independently activated, lymphokine gene expression and secretion, ultimately leading to cell prolifera-

1 Abbreviations used in this paper: DG, diacylglycerol; ECM, extracellular matrix; GaM, goat anti-mouse; PFP, plasma-free platelet; PIP2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PRP, platelet-rich plasma; TXB2, thromboxane B2; VLA, very late activation antigen.
tion (9, 10). On T cells, lymphokine gene activation by CD69 appears to be sensitive to cyclosporin A (10) and dependent on membrane expression of CD3 (11, 12). CD69+ B cells also are induced to proliferate by CD69 stimulation and simultaneous PKC activation (11).

To investigate the possible general relevance of CD69 as a signal-transducing molecule, we analyzed the expression of CD69 in nonlymphoid cells. We found that CD69 is expressed on resting platelets. The biochemical characteristics of platelet CD69 closely resemble those of lymphoid CD69. Moreover, the molecule is able to transmit a signal that results in platelet activation and aggregation. These results indicate a more generalized role for CD69 in cellular signaling and may help define possible ligand candidates.

Materials and Methods

Cells. Platelet-rich plasmas (PRPs) were obtained from healthy adult donors, 30–60 yr old. Venous blood samples were collected from Tago Inc. (Burlingame, CA) coated with rabbit anti–mouse (RaM) Ig (Dako Corp., Denmark). Immunoprecipitation was performed using anti-Leu-23 mAbs bound to RaM S. aureus. After seven washes in lysis buffer, immunoprecipitates were resuspended in 50 μl sample buffer, boiled for 5 min, and run on a 10% SDS-PAGE. Gels were fixed for 30 min in a 50% methanol, 7.5 pH acetic acid solution. 32PO4 gels were also treated o.n. with a 10% TCA, 50 mM NaH2PO4 solution. Finally, gels were dried and autoradiographed.

Platelet Functional Studies. After the last wash with Hepes buffer, platelets were resuspended at 2 × 108/ml in Hepes buffer without Hirudin and Apyrase, supplemented with 1 mM glucose, 0.2% BSA, 1 mM CaCl2, and 100 μg/ml human fibrinogen. Aggregometric assays were performed with a dual sample aggregometer (94G; Elvi, Milano, Italy) in polypropylene tubes, at 37°C, and were stirred at 1,000 rpm after adding various amounts of mAbs to PRPs, in a total volume of 0.25 ml. ATP release was measured with a luminoimeter (1241, LKB Wallac, Turku, Finland). A 50-μl work solution (80 μg/ml luciferin, 8,800 U/ml d-luciferase) was mixed with 450 μl PRPs before adding mAbs. For measurement of released thromboxane A2 (measured as TXB2) and PGE2, platelets were stimulated with mAbs in a total volume of 0.25 ml in polypropylene tubes, at 37°C, and were stirred at 1,000 rpm for 10 min, then 14 μM indomethacin was added to stop cyclooxygenase activity. Supernatants were collected, and TXB2 and PGE2 were measured by RIA (NEN, Dreieich, FRG).

Intracellular [Ca2+] Measurement. PRPs were incubated for 1 h with 4 μM Fura-2 (Calbiochem-Behring Corp.), then washed twice to obtain PFP (see above) and resuspended in Hepes buffer supplemented with 1 mM CaCl2. Aliquots of 1.5 ml were stimulated with mAbs, and fluorescence variations were monitored with a spectrofluorimeter (SMF25; Kontron, Zurich, Switzerland), set to 340 nm excitation and 510 nm emission. Fura-2 signals were calibrated according to Pollock et al. (14). Cell lysis by 50 μM digitonin allowed to determine Fmax, while addition of 10 mM EGTA in a 20-mM Tris base allowed to set Fmin.

Results

Platelets Express CD69. To assess the expression of CD69 on human platelets, PFPs were adjusted to 108/ml. Aliquots were then stained with the appropriate amounts of anti-Leu-23 and anti-EA-1 (which recognize different epitopes of the CD69 molecule), anti-Leu-3a, anti-Leu-2a, and mouse IgG1 as negative controls, and anti-gp IIb-IIIa as positive control, followed by second-step FITC-conjugated goat anti-mouse IgG. Samples were analyzed by a FACScan cytofluorimeter.
Figure 1. CD69 expression on platelets. Aliquots of PFPs were stained with anti-Leu-3a (A), anti-Leu-23 (B), anti-EA-1 (C), and anti-gp IIb-IIIa (D) mAbs, then with FITC-conjugated anti-mouse IgG, and analyzed by FACS. Staining with FITC-conjugated anti-mouse IgG only is superimposed in each panel (shaded areas).

Fig. 1 shows that CD69 was clearly detectable by both anti-Leu-23 mAb (B) and anti-EA-1 mAb (C), although its expression was ~20–30-fold lower than the gp IIb-IIIa expression (D). Control anti-Leu-3a mAb (as well as anti-Leu-2a and control mouse IgG1, not shown) did not stain platelets (Fig. 1 A). CD69 was detected on all (8/8) donors tested. Failure to reveal CD69 on platelets by previous cytofluorimetric analyses (1) may be attributed to insufficient instrument sensitivity in detecting relatively low amounts of antigen. Attempts to increase CD69 expression by PMA (10 ng/ml) or thrombin (0.5 U/ml) stimulation of PFPs resuspended in Hepes buffer supplemented with 1 mM CaCl₂ were unsuccessful (data not shown).

Platelet CD69 is Biochemically Similar to Lymphoid CD69. CD69 was immunoprecipitated from PFPs radiolabeled with ¹²⁵I, using the anti-Leu-23 mAb, and run on a 10% SDS-PAGE. As a control, CD69 was also immunoprecipitated from the Jurkat tumor T cell line, after overnight PMA stimulation and ¹²⁵I radiolabeling. Fig. 2 shows that, under non-reducing conditions, platelet CD69 is a diffuse 55–65-kD band, in which, however, three discrete components of ~55, ~60 and ~65 kD, corresponding to the three dimeric combinations of 28 + 28, 28 + 32, and 32 + 32 kD, were detectable (Fig. 2; lane B). Under reducing conditions, two bands of 28 and 32 kD were visible (lane F). CD69 from platelets migrated in essentially the same positions as CD69 from Jurkat (lanes D and H), suggesting close similarity between the two.

Moreover, since both chains of CD69 have invariably been shown to be phosphorylated on activated T cells, as well as on activated NK cells and thymocytes, we analyzed the phosphorylation status of platelet CD69. Resting platelets were therefore loaded with ³²PO₄. CD69 immunoprecipitated with the anti-Leu-23 mAb, and run on 10% SDS-PAGE. Fig. 3 shows that both the 28- and the 32-kD chains are phosphorylated (lane B).

Anti-CD69 mAbs Induce Platelet Aggregation and Degranulation. The functional implications for CD69 expression on platelets were therefore investigated. To evaluate a possible role for CD69 in platelet activation, we first analyzed the ability of anti-CD69 mAbs to induce aggregation. Anti-CD69 mAbs induced platelet aggregation after a lag phase that was inversely dose dependent. In the experiment shown in Fig. 4, aggregation was induced after 1.5, 2.5, and 4 min by 16 (A), 8 (B), and 4 (C) µg/ml, respectively, of anti-Leu-23 mAb. By contrast, 16 µg/ml of anti-gp IIb-IIIa mAb (D) was unable to induce aggregation.

Degranulation, usually revealed by ATP release, often accompanies and amplifies platelet activation. To assess whether CD69 triggering resulted in platelet degranulation, PFPs were stimulated with 12 µg/ml of anti-Leu-23 mAb, and ATP released in the supernatant was measured. As shown in Fig. 5, CD69 stimulation by anti-Leu-23 mAb induced significant ATP release, while control anti-gp IIb-IIIa mAb had no effect. ATP release and platelet aggregation by soluble anti-CD69 mAb were observed in 50% of the donors tested (6/12), although all donors expressed comparable amounts of CD69 on platelets. Platelets from donors that did not respond to soluble anti-CD69, however, were induced to release ATP and aggregate by maximizing CD69 crosslinking with anti-Leu-23-coupled Pandex beads (not shown).

Requirement for Crosslinking. CD69-mediated T cell acti-
Anti-CD69 mAbs Induce Ca\textsuperscript{2+} Influx. Since CD69-mediated signaling in lymphocytes includes extracellular Ca\textsuperscript{2+} influx, we tested whether anti-CD69 mAbs were able to generate an increase in intracellular [Ca\textsuperscript{2+}] in platelets (Fig. 7). PFP stimulation with 0.5 U/ml thrombin (Fig. 7 A) or with 12 µg/ml of anti-Leu-23 (Fig. 7 B) resulted in intracellular [Ca\textsuperscript{2+}] elevations that persisted for several minutes. Anti-gp IIb-IIIa (C) or anti-Leu-3a (not shown) mAbs failed to alter intracellular [Ca\textsuperscript{2+}] levels. Prior addition of 2 mM EGTA (D) completely prevented the observed intracellular [Ca\textsuperscript{2+}] elevation, suggesting that anti-CD69 stimulation was inducing extracellular Ca\textsuperscript{2+} influx.

**Discussion**

The data presented here indicate that human platelets constitutively express CD69, a molecule so far considered an early activation antigen restricted to lymphoid cells. Moreover, we provide evidence that platelet CD69, similarly to lymphoid CD69, may be involved in signal transduction, since CD69 crosslinking by mAbs generates Ca\textsuperscript{2+} influx with consequent platelet activation and aggregation.

CD69 is a disulfide-linked homodimer that has been de-
scribed as an antigen induced very early during lymphoid activation (1–3, 5, 8). Its expression, which requires new RNA and protein synthesis, is strictly dependent on PKC activation (4, 6, 16). In vivo, CD69 is found on thymocytes expressing high levels of CD3, possibly as a result of intrathymic TCR/CD3 engagement (5). CD69 on T lymphocytes appears to be functionally linked to a Ca++ channel. Anti-CD69 mAbs, in fact, induce Ca++ influx when properly crosslinked on the surface of CD69+ T cells (9, 10). This influx is prolonged in time and contributes, when PKC is simultaneously activated, to lymphokine gene activation. CD69-induced IL-2 and IFN-γ gene expression are in fact completely blocked by EGTA (10). On the other hand, signaling through CD69 in T cells is not likely to result in an effective activation of PKC, from phosphatidyl inositol di-phosphate (PIP₂) hydrolysis and diacylglycerol (DG) formation, since no effect of IIα or IIAR on platelets has been reported. mAbs directed against other structures present on resting platelets may directly trigger platelet activation and aggregation. CD9 (gp 24), whose ligand is unknown, can induce DG formation, degranulation, TXA₂ production, and aggregation, when bound by anti-CD9 mAbs (24–26). mAbs against CD36 (platelet gp IV), an activation-independent thrombospondin receptor, also induce PKC-dependent platelet aggregation (27).

CD69 crosslinking in platelets generates [Ca++] influx and a number of activating events, which include degranulation, arachidonate metabolism by cyclooxygenase with formation and release of PGE₂ and TXB₂, and finally, platelet aggregation.

A significant increase of intracellular [Ca++] is generally considered sufficient to trigger most of the activation events that lead to platelet adhesion and aggregation (15). Intracellular [Ca++] levels in platelets are primarily controlled by inositol 1-4-5 triphosphate (release from internal stores) (28, 29), and possibly also by inositol 1-3-4-5 tetrakiphosphate (extracellular Ca++ influx) (30), mostly derived from PIP₂ metabolism. In fact, strong platelet agonists (thrombin, collagen, TXA₂, platelet-activating factor) and some weak agonists (vasopressin, epinephrine) stimulate receptors coupled to specific G proteins (31, 32) that activate phospholipase (PLC) and PIP₂ metabolism (33, 34). However, PLC-independent receptor-operated Ca++ channels may be opened by ADP (35), and possibly also by TXA₂ (36).

Although is not clear at the moment whether CD69-induced Ca++ influx is PLC independent, it may be directly responsible for a variety of [Ca++] dependent events that contribute to platelet activation and aggregation.

[Ca++] -dependent proteases are responsible for cleavage of actin-binding proteins and of p235, involved in cytoskeleton reorganization during platelet aggregation (37). [Ca++] / calmodulin–dependent kinases may be directly activated by

Figure 8. Production of PGE₂ and TXB₂ upon CD69 stimulation. Aliquots of PFPs were left untreated or stimulated with 12 μg/ml of anti-Leu-23 or anti-gp Iib-IIIa mAbs. PGE₂ (A) and TXB₂ (B) released in the supernatant were measured by RIA.
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