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 $CD3^{bright}$ 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 Ca^{2+} influx and platelet degranulation, as revealed by adenosine triphosphate release. In addition, CD69 stimulation in platelets induced production of thromboxane B_2 and PGE_2, suggesting activation of arachidonic acid metabolism by cycloxygenase. 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 (formerly called EA-1, MLR-3, Leu-23, and AIM) is a disulfide-linked surface homodimer composed by two chains of 28 and 32 kD (1, 2). Both chains have the same 24-kD proteic backbone, but they appear to be differently glycosylated by N-linked sugars (3, 4). The dimer may be formed by the random association of the 28-kD chain with another 28-kD chain or with the 32-kD chain, as well as by the association of the 32-kD chain with another 32-kD chain. The three molecular forms appear to coexist on the surface of the same cell. Both chains are constitutively phosphorylated (1, 3, 5) and are likely to be integral membrane proteins.

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, 6). 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 maintainance in time of elevated intracellular $[Ca^{2+}]$ levels (6).

In vivo, CD69 was found constitutively expressed and phosphorylated on $CD3^{bright}$ 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 $CD3^{bright}$ 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 Ca^{2+} 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; PIP_2, phosphatidyl inositol diphosphate; PKC, protein kinase C; PLC, phospholipase; PRP, platelet-rich plasma; TXB_2, thromboxane B_2; VLA, very late activation antigen.

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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 (9:1 [vol/vol]) with ACD buffer (2.5% Na citrate, 1.5% citric acid, 2% dextrose) and centrifuged at 150 *g* for 15 min. For plasma-free platelet (PFP) preparation, PRPs were washed twice and resuspended in Hepes buffer (10 mM Hepes buffer, pH 7.4, containing 119 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.1 U/ml Hirudin [Sigma Chemical Co., St. Louis, MO], and 0.5 mg/ml Apyrase [Sigma Chemical Co.]).

Jurkat cells were cultured in RPMI 1640 (Whittaker M.A. Bioproducts, Walkersville, MD), supplemented with 1 mM glutamine, antibiotics, and 10% FCS (Biological Industries, Kibbutz Beth Haemek, Israel). To maximally induce CD69, Jurkat cells were grown overnight in the presence of 10 ng/ml PMA (Sigma Chemical Co.).

mAbs. Anti-Leu-23 (IgG1, anti-CD69), anti-Leu-3a (IgG1, anti-CD4), anti-Leu-2a (IgG1, anti-CD8), and control mouse IgG1 were kindly provided by Dr. L.L. Lanier (Becton Dickinson & Co., Mountain View, CA). Anti-EA-1 (IgG1, anti-CD69) was a gift from Dr. S. M. Fu (Oklahoma Medical Research Foundation, Oklahoma City, OK). Anti-gp IIb-IIIa (IgG1) was purchased from Pel-Freez Biologicals, Rogers, AR). Anti-Leu-23 mAb was coupled to Pandex (IDC, Portland, OR) beads as previously described (6). F(ab') fragments of anti-Leu-23 mAb were prepared by pepsin digestion and HPLC fractionation, followed by mild reduction and alkylation, according to Parham (13). Goat-anti-mouse (GaM) Ig was obtained from Tago Inc. (Burlingame, CA).

Immunofluorescence and Cytofluorimetric Analysis. PFPs were resuspended in 100 μl of PBS, and optimal amounts of mAbs were added. After 20 min on ice, they were washed twice with cold PBS, and an optimal amount of FITC anti-mouse IgG was added. After 20 min on ice, they were washed once, resuspended in cold PBS, and immediately analyzed. Analysis was performed with a FACScan cytofluorimeter (Becton Dickinson & Co.). 10^4 events/sample were collected, and data were processed by Consort 30 software and presented as single fluorescence histograms on a four-decades log scale.

Labeling, Immunoprecipitation, and SDS-PAGE Analysis. For ^{125}I labeling, 10^9 PFPs and 2×10^6 Jurkat cells were washed twice and resuspended in D-PBS without Ca^{2+} and Mg^{2+} containing 10^{-6} M KI. For cell suspension, 200 μl of a 50-μg/ml lactoperoxidase (Sigma Chemical Co.) solution and 10 μl of a 20-IU/ml glucose oxidase (Sigma Chemical Co.) in D-PBS/10^{-6} M KI solution were added. 2 mCi ^{125}I (Amersham International, Amersham, UK) was added to 0.5 ml of 10 mM glucose in the D-PBS/10^{-6} M KI solution and then mixed with the enzyme-cell suspension. Cells were kept at room temperature for 20 min with occasional mixing. Cells were then washed three times with cold PBS/0.1% azide and lysed in 0.5 ml of 0.5% NP-40 lysis buffer (0.05 M Tris-HCl, pH 8, 0.15 M NaCl, 1 mM EDTA, 0.02% NaN_3, 20 KIU/ml aprotinin) for 30 min on ice. Supernatants collected after centrifugation were immunoprecipitated.

For $^{32}PO_4$ labeling, 10^9 platelets were washed three times in Hepes buffer and resuspended in 2 ml of Hepes buffer. 2 mCi $^{32}PO_4$ was then delivered to the cell suspension, and cells were incubated at 37°C for 3 h. Cells were then washed three times with cold PBS/0.1% azide containing 0.1 mM Na_3VO_4, 0.4 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, and lysed in 0.4 ml of 0.5% NP-40 lysis buffer for 30 min on ice. Supernatants collected after centrifugation were immunoprecipitated.

Cell lysates were precleared three times with formalin-fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., La Jolla, 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. $^{32}PO_4$ gels were also treated o.n. with a 10% TCA, 50 mM NaH_2PO_4 solution. Finally, gels were dried and autoradiographed.

Platelet Functional Studies. After the last wash with Hepes buffer, platelets were resuspended at 2×10^8/ml in Hepes buffer without Hirudin and Apyrase, supplemented with 10 mM glucose, 0.2% BSA, 1 mM $CaCl_2$, and 100 μg/ml human fibrinogen. Aggregometric assays were performed with a dual sample aggregometer (840; Elvi, Milano, Italy) in polypropylene tubes, at 37°C, and were stirred at 1,000 rpm after adding various amounts of mAbs to PFPs, in a total volume of 0.25 ml. ATP release was measured with a luminometer (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 PFPs before adding mAbs. For measurement of released thromboxane A_2 (measured as TXB_2) and PGE_2, 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 cycloxygenase activity. Supernatants were collected, and TXB_2 and PGE_2 were measured by RIA (NEN, Dreieich, FRG).

Intracellular $[Ca^{2+}]$ 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 $CaCl_2$. Aliquots of 1.5 ml were stimulated with mAbs, and fluorescence variations were monitored with a spectrofluorimeter (SFM25; 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 F_{max}, while addition of 10 mM EGTA in a 20-mM Tris base allowed to set F_{min}.

Results

Platelets Express CD69. To assess the expression of CD69 on human platelets, PFPs were adjusted to 10^8/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.

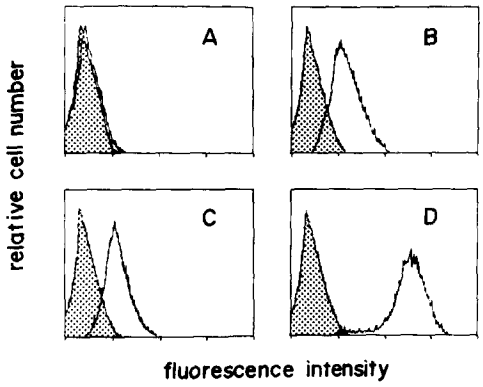

Figure 1. CD69 expression on platelets. Aliquots of PFPs were stained with anti-Leu-3a (*A*), anti-Leu-23 (*B*), anti-EA-1 (*C*), and anti-gp IIb-IIIa (*D*) mAbs, then with FITC-conjugated anti-mouse IgG, and analyzed by FACS. Staining with FITC-conjugated anti-mouse IgG only is superimposed in each 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 $CaCl_2$ were unsuccessful (data not shown).

Platelet CD69 is Biochemically Similar to Lymphoid CD69. CD69 was immunoprecipitated from PFPs radiolabeled with ^{125}I, using the anti-Leu-23 mAb, and run on a 10% SDS-PAGE. As a control, CD69 was also immunoprecipitated from

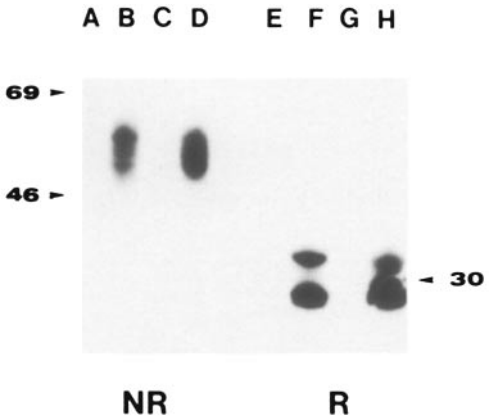

Figure 2. Biochemical analysis of platelet CD69. PFPs (lanes *A*, *B*, *E*, *F*) and Jurkat cells (lanes *C*, *D*, *G*, *H*) were labeled with ^{125}I, then cell lysates were treated with *S. aureus*/RaM-bound control IgG1 (lanes *A*, *C*, *E*, *G*) or anti-Leu-23 mAbs (lanes *B*, *D*, *F*, *H*). Immunoprecipitates were run on 10% SDS-PAGE under nonreducing (lanes *A–D*) and reducing (lanes *E–H*) conditions.

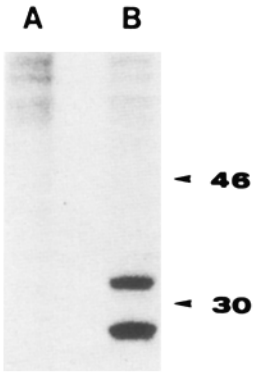

Figure 3. Phosphorylation of platelet CD69. PFPs were washed extensively in Hepes buffer before loading with $^{32}PO_4$. Cell lysates were treated with *S. aureus*/RaM-bound control IgG1 (lane *A*) or anti-Leu-23 mAbs (lane *B*). Immunoprecipitates were run on 10% SDS-PAGE under reducing conditions.

the Jurkat tumor T cell line, after overnight PMA stimulation and ^{125}I radiolabeling. Fig. 2 shows that, under nonreducing 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 $^{32}PO_4$, 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-

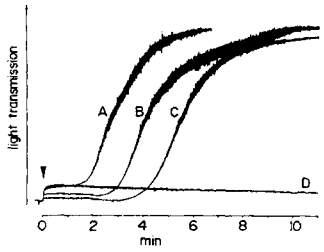

Figure 4. Induction of platelet aggregation by anti-CD69 mAb. Anti-Leu-23 mAb, at a final concentration of 16 (*A*), 8 (*B*), and 4 (*C*) μg/ml, and anti gp IIb-IIIa mAb at 16 μg/ml (*D*) was added to PFP aliquots at time 0. Changes in light transmission, due to cell aggregation, were continuously recorded by a dual sample aggregometer.

vation requires extensive crosslinking of CD69 molecules in most donors (9, 10). To further analyze the requirement for crosslinking in CD69-mediated platelet activation, experiments were performed comparing monovalent F(ab') fragments to intact IgG, in donors that responded to the soluble mAb. Fig. 6 shows that anti-Leu-23 F(ab') fragments were unable to induce ATP release (and platelet aggregation; not shown). When a second-step GaM was added, however, F(ab') fragments induced ATP release in amounts comparable with those induced by the intact mAb. Taken together, these results indicate that optimal molecular crosslinking is crucial also for CD69-mediated platelet activation.

Anti-CD69 mAbs Induce Ca^{2+} Influx. Since CD69-mediated signaling in lymphocytes includes extracellular Ca^{2+} influx, we tested whether anti-CD69 mAbs were able to generate an increase in intracellular [Ca^{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^{2+}] elevations that persisted for several minutes. Anti-gp IIb-IIIa (*C*) or anti-Leu-3a (not shown) mAbs failed to alter intracellular [Ca^{2+}] levels. Prior addition of 2 mM EGTA (*D*) completely prevented the observed intracellular [Ca^{2+}] elevation, suggesting that anti-CD69 stimulation was inducing extracellular Ca^{2+} influx.

CD69 Stimulation Activates the Cycloxygenase Pathway. Arachidonic acid metabolism by cycloxygenase is a major biochemical pathway leading to the formation of potent medi-

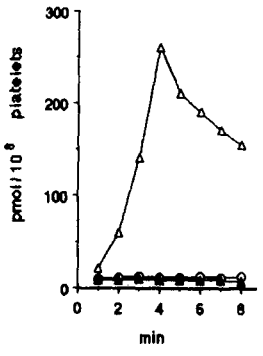

Figure 5. Release of ATP from platelets by CD69 stimulation. Aliquots of PFPs were stimulated with 12 μg/ml anti-Leu-23 mAb (Δ), 12 μg/ml anti-gp IIb-IIIa (O), or left unstimulated (▲), and ATP release in the supernatant was measured by a luminometer. Amounts of released ATP are plotted vs. time elapsed from the stimulation (time 0).

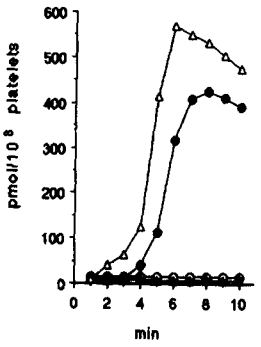

Figure 6. Requirement for CD69 crosslinking. Aliquots of PFPs were stimulated with 12 μg/ml of intact anti-Leu-23 IgG (Δ), 12 μg/ml of anti-Leu-23 F(ab') (▲), 12 μg/ml of GaM (O), or 12 μg/ml of anti-Leu-23 F(ab'), followed after 2 min by 12 μg/ml of GaM (●). Released ATP is plotted vs. time elapsed from the stimulation (time 0).

ators involved in the amplification and regulation of platelet responses (15). Cycloxygenase activation in platelets results in endoperoxides (PGG_2, PGH_2) formation, with subsequent PGE_2 generation by isomerization. TXA_2 is then produced by TXA_2-synthetase from PGH_2, and is rapidly converted to TXB_2. We measured PGE_2 and TXB_2 in the PFP supernatant after CD69 stimulation with 12 μg/ml anti-Leu-23 mAbs (Fig. 8). Both PGE_2 (Fig. 8 *A*) and TXB_2 (Fig. 8 *B*) were released in the supernatant of PFPs treated with the anti-Leu-23 mAb, while anti-gp IIb-IIIa mAb had no effect, indicating activation of cycloxygenase and TXA_2-synthetase upon CD69 stimulation.

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^{2+} influx with consequent platelet activation and aggregation.

CD69 is a disulfide-linked homodimer that has been de-

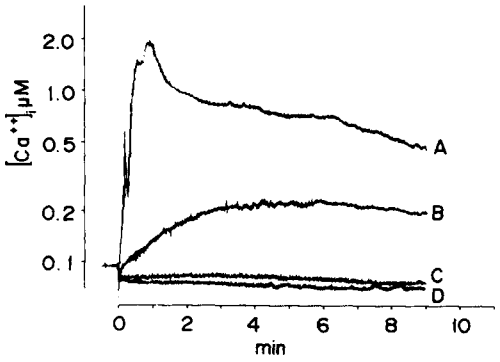

Figure 7. Induction of [Ca^{2+}] influx in platelets by anti-CD69 mAb. PRPs were loaded with Fura-2, then thrombin at 0.5 U/ml (*A*), anti-Leu-23 mAb at 12 μg/ml in the absence (*B*) or in the presence of 2 mM EGTA (*D*), and anti-gp IIb-IIIa at 12 μg/ml (*C*) were added to PFP aliquots at time 0. Changes in fluorescence emission were continuously recorded by a spectrofluorimeter. [Ca^{2+}] was measured by 50 μM digitonin (F_{max}) and 10 mM EGTA (F_{min}) calibration.

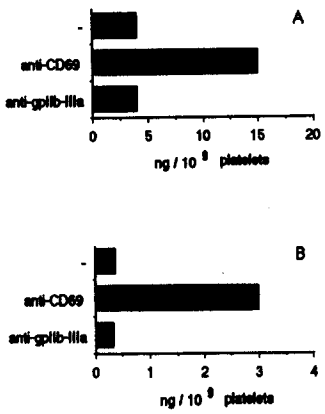

Figure 8. Production of PGE_2 and TXB_2 upon CD69 stimulation. Aliquots of PFPs were left untreated or stimulated with 12 μg/ml of anti-Leu-23 or anti-gp IIb-IIIa mAbs. PGE_2 (*A*) and TXB_2 (*B*) released in the supernatant were measured by RIA.

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^{2+} channel. Anti-CD69 mAbs, in fact, induce Ca^{2+} 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 diphosphate (PIP_2) hydrolysis and diacylglycerol (DG) formation, since CD69-induced gene activation always requires independent PKC stimulation (10, 16).

The presence of a protein that antigenically, biochemically, and functionally resembles CD69 on resting platelets is intriguing. Other molecules induced or upregulated on activated T lymphocytes have been shown to be constitutively expressed on platelets. These include members of the very late activation antigen (VLA) group of proteins of the integrin family, which function as extracellular matrix (ECM) receptors. Collagen receptor (ECMR II) platelet gp Ia-IIa (17) and fibronectin receptor (ECMR IV) platelet gp Ic-IIa (18) are identical to VLA-2 and VLA-5 respectively, expressed on long-term activated T cells (19). Moreover, platelet laminin receptor VLA-6 (20) is upregulated on activated T cells (A. Santoni, unpublished results). VLA-2, -5, and -6 may therefore function on platelets as activation-independent receptors for ECM proteins, and possibly for ECM-mediated platelet activation and aggregation, while on activated lymphocytes, they are supposed to be involved in mechanisms of endothelial adhesion, extravasation, and tissue penetration (19). VLAs on lymphocytes are acquired or upregulated several days after the stimulation, and persist on the cell surface, possibly for the entire cell life. The upregulation of CD29 (platelet gp IIa), the β_1 chain of the integrin family, shared by all VLAs, has been in fact proposed as a phenotypic marker for memory T cells in vivo (21).

PTA1, a 67-kD glycoprotein of unknown function, is also expressed by activated T cells and resting platelets. mAbs against PTA1 induce platelet activation and aggregation (22), but not T cell activation (23). On T cells, PTA1 expression is slowly upregulated during activation and still maintained several days after the stimulation (23).

By contrast, CD69 is a transiently expressed activation antigen. On T cells, CD69 is induced within 2 h after the stimulation of the TCR/CD3 complex. Experiments designed to evaluate the membrane turnover of the protein on T cells indicated that once maximally induced in vitro on the cell surface (within 18–24 h), CD69 has a t1/2 of ~24 h, if the inducing stimulus is removed (6). Cells that express CD69 without in vitro stimulation, like $CD3^{bright}$ thymocytes and a few circulating lymphocytes, are likely to have acquired their expression in vivo as a result of a recent stimulation, and in fact, progressively lose CD69 expression when cultured in vitro without stimulation (R. Testi, unpublished results). CD69 is therefore needed for a short period of time during the stimulation. It has been suggested that CD69 might be functionally associated with an IL-1R (2). However, the present data would not encourage us to generalize this hypothesis, since no effect of IL-1 or IL-1R 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, TXA_2 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 $[Ca^{2+}]$ influx and a number of activating events, which include degranulation, arachidonate metabolism by cycloxygenase with formation and release of PGE_2 and TXB_2, and finally, platelet aggregation.

A significant increase of intracellular $[Ca^{2+}]$ is generally considered sufficient to trigger most of the activation events that lead to platelet adhesion and aggregation (15). Intracellular $[Ca^{2+}]$ 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^{2+} influx) (30), mostly derived from PIP_2 metabolism. In fact, strong platelet agonists (thrombin, collagen, TXA_2, platelet-activating factor) and some weak agonists (vasopressin, epinephrine) stimulate receptors coupled to specific G_p proteins (31, 32) that activate phospholipase (PLC) and PIP_2 metabolism (33, 34). However, PLC-independent receptor-operated Ca^{2+} channels may be opened by ADP (35), and possibly also by TXA_2 (36).

Although is not clear at the moment whether CD69-induced Ca^{2+} influx is PLC independent, it may be directly responsible for a variety of $[Ca^{2+}]$-dependent events that contribute to platelet activation and aggregation.

$[Ca^{2+}]$-dependent proteases are responsible for cleavage of actin-binding proteins and of p235, involved in cytoskeleton reorganization during platelet aggregation (37). $[Ca^{2+}]$/calmodulin–dependent kinases may be directly activated by

elevated intracellular [Ca^{2+}] and control the phosphorylation of the 20-kD myosin L chain, necessary for shape change (38) and possibly contraction and secretion. Finally, PLA_2 is activated in the presence of elevated intracellular [Ca^{2+}] (39), allowing arachidonic acid release from membrane phosphatidylcholine and phosphatidylethanolamine, and subsequent activation of cycloxygenase with PG endoperoxides PGG_2/PGH_2 and PGE_2 synthesis and TXA_2 formation.

The results presented here strongly suggest that CD69 crosslinking results in the activation of the cycloxygenase pathway, since anti-Leu-23 mAbs induced PGE_2 and TXB_2 production by platelets. Arachidonic acid, the main cycloxygenase substrate, may in part be released also from phosphatidyl inositol by PLC (40), but the major source remains the PLA_2-dependent release from membrane phospholipids (phosphatidylcholine and phosphatidylethanolamine). It is therefore likely that CD69 triggering directly generates [Ca^{2+}]-induced PLA_2 activation. However, further studies are required to assess the relative contribution of PLC and PLA_2 to CD69-mediated platelet activation.

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