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EVIDENCE THAT THE SIGNAL-INITIATING MEMBRANE PROTEIN CD9 IS ASSOCIATED WITH SMALL GTP-BINDING PROTEINS

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SUMMARY: F(ab')$_2$ fragments of anti-CD9 mAb aggregate platelets by a thromboxane-dependent pathway implicating CD9 as signal initiating molecule. We demonstrate that mAbs directed against CD9, but not against GPIIb/IIIa specifically immunoprecipitate, from detergent lysates of human platelets, proteins of 25 and 26 kDa which bind [α^{32}P]GTP on nitrocellulose transfers. The binding is specific since it is blocked by GTP, but not by ATP. The GTP-binding proteins do not belong to a Mg^{2+}-sensitive subset since they are unaffected by the addition of 2μM-20 mM Mg^{2+}. The observations demonstrate that CD9 is associated with selected small G-proteins.

CD9 is a plasma membrane-located glycoprotein of broad, but selective tissue distribution (1-3). In the platelet CD9 is a major component of the cell surface (4). The function of CD9 is unknown, however accumulating evidence suggests that it is linked to the generation of intracellular signals leading to cell-cell adhesion, since antibodies to CD9 induce platelets to secrete their granules and undergo aggregation (3-10), and pre-B cells to undergo homotypic aggregation (11). We recently demonstrated that anti-CD9 mAb can induce platelet aggregation by an Fc-independent mechanism by a process requiring cyclo-oxygenase products implicating CD9 in the generation of a platelet activating signal requiring the sequential activation of phospholipase A_2 and phospholipase C (12). The CD9 proximal signal may have relevance to integrin function since we and others have presented evidence for a physical association between CD9 and the major platelet integrin GPIIb/IIIa (9,13). Signaling events are usually coupled to receptor-associated large trimeric GTP-binding proteins (14). The majority of such receptors belong to a family of seven-pass membrane glycoproteins containing a high degree of homology within their membrane-spanning regions (14,15). Only a few receptors which traverse the plasma membrane once have been demonstrated to physically associate with GTP-binding proteins (15,16). CD9 does not belong to either of these subgroups of membrane-spanning molecules, but rather traverses the plasma membrane four times (17), and none of its transmembrane regions show significant sequence homology to receptors known to be associated with trimeric GTP-

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binding proteins. A second class of GTP-binding proteins which are monomeric and of low molecular mass have been identified (18). Since a small GTP-binding protein is involved in phospholipase C-activation in the platelet (19) and it has been suggested that members of this family may regulate the assembly of protein complexes within the plasma membrane (18), we investigated the possibility that small GTP-binding proteins may associate with CD9.

MATERIALS AND METHODS

Materials: The anti-CD9 mouse mAb 50H.19 is an IgG2a (20). The IgG1 anti-CD9 mAb ALB6 and the anti-GPIIb/IIIa IgG1 mAb P2 were obtained from Daymar Laboratories.[α^{32}P]GTP (3000Ci/mmol) was purchased from New England Nuclear. Nitrocellulose membrane and the low molecular weight standards were obtained from Bio-Rad Laboratories, Ponceau S from Boehringer Mannheim and protein A-Sepharose 4B from Pharmacia. All other chemicals were purchased from Sigma.

Platelet preparation and lysis: Platelet-rich plasma was obtained from citrated blood of healthy volunteers as described earlier (4). Platelets were pelleted by centrifugation at 500 x g for 10 min at 20° in the presence of 10 ng/ml of prostacyclin and 0.1 unit/ml of apyrase (grade V). Following washing in Tyrode's/HEPES buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 4 mM NaH_2PO_4, 6 mM glucose, 20 mM HEPES (pH7.4), containing 10 ng/ml of prostacyclin and 0.1% bovine serum albumin, the platelets were suspended at 5 x 10^8 cells/ml in ice-cold lysis buffer (137 mM NaCl, 50 mM Tris/HCl (pH 7.4), 10 mM sodium iodoacetamide, 0.3% CHAPS, 0.1 mM phenylmethylsulfonyl fluoride and 1 μg each of leupeptin, pepstatin, antipain and chymostatin) and lysed under rotation for 3 h at 4°. The lysates were clarified by centrifugation at 14,000 x g for 15 min, preadsorbed with protein A-Sepharose 4B and centrifuged at 14,000 x g for 15 min.

Immunoprecipitation, SDS-PAGE and [α^{32}P]GTP-binding: Proteins from preadsorbed lysates obtained from 5 x 10^7 platelets were immunoprecipitated with 3 μg of affinity-purified mAb for 4 h, and extracted with protein A-Sepharose 4B. Following extensive washing of the beads (5 x in lysis buffer containing 500 mM NaCl and 1 x in 10 mM Tris/HCl (pH6.8)), the proteins were separated under reducing conditions by SDS-PAGE (4) in 5 to 20% gradient gels. Proteins were electrophoretically transferred to nitrocellulose membranes in 25 mM Tris, 192 mM glycine (pH 8.3), 20% methanol at 30 V for 16 h. For the detection of GTP-binding proteins (21) the nitrocellulose transfers were soaked in binding buffer (50 mM Tris/HCl (pH 7.5), 0.3% Tween-20 and, unless otherwise stated, 1 mM $MgCl_2$) and incubated for 30 min in 10 ml of binding buffer per 40 cm^2 containing 0.3 nM [α^{32}P]GTP. The nitrocellulose membranes were then extensively washed in binding buffer, dried and radiolabeled bands visualized by autoradiography using Kodak X-OMAT X-ray film. Molecular weights of the GTP-binding proteins were determined in relation to molecular mass standards included in each gel and visualized by staining with 0.02% Ponceau S following blotting to nitrocellulose membranes. Specific binding was determined by including non-radiolabeled nucleotides in the binding buffer.

RESULTS

Association of [α^{32}P]GTP-binding activity with immunoprecipitated CD9: When detergent-platelet lysates were tested for [α^{32}P]GTP-binding activity on nitrocellulose blots, five distinct bands could be distinguished ranging between 20 and 27 kDa, the bulk of the radiolabel being associated with proteins in the 25 to 27 kDa-range (Fig.1, lane 1). Following immunoprecipitation of CD9 with mAb 50H.19, consistently (n=14) two [α^{32}P]GTP-binding bands of proteins of 25 and 26 kDa were found, both containing approximately the same radiolabeling capacity (Fig.1, lane2). Two [α^{32}P]GTP-binding proteins of identical electrophoretic mobility were also detected by anti-CD9 mAb ALB6 (n=3, data not shown). An immunocomplex with mAb P2, prepared under identical conditions, was included as a control. No proteins labeled by [α^{32}P]GTP were detected following

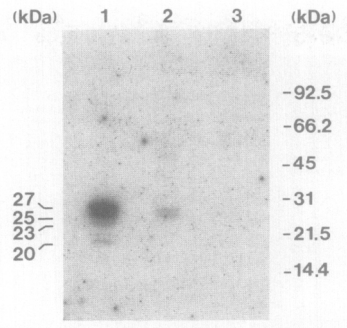

Fig. 1. Immunocomplexes of CD9 contain [α^{32}P]GTP-binding activity: Proteins from lysates from 5 x 10^6 platelets without further treatment (lane 1) or following immunoprecipitation from lysates from 5 x 10^7 platelets with mAb 50H.19 (lane 2) or with mAb P2 (lane 3) were separated by SDS-PAGE in a 5 - 20% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The binding of [α^{32}P]GTP was assessed as outlined in the MATERIALS and METHODS section. Molecular masses were determined by comparison to protein standards which were stained with Ponceau S following the electrophoretic blotting. Autoradiography was performed for 14 h.

immunoprecipitation with this antibody (n=5) (Fig.1, lane3). These observations suggest that CD9 may be associated with GTP-binding proteins.

Specificity of [α^{32}P]GTP-binding: Since GTP-binding proteins are highly specific for the nucleotide, attempts were made to competitively inhibit binding of [α^{32}P]GTP to nitrocellulose-blotted proteins by inclusion of increasing concentrations of non-radiolabeled GTP or ATP. 100 nM to 1 mM GTP was found to completely abrogate [α^{32}P]GTP-binding both to mAb 50H.19-immunoprecipitated proteins and to the proteins of the platelet lysates (Fig.2 A), whereas ATP had no effect (Fig.2 B).

Since a 24 kDa [α^{32}P]GTP-binding protein from human platelets was reportedly inhibited by elevated Mg^{2+} concentrations, whereas a 27 kDa protein was not (21) we investigated the binding of [α^{32}P]GTP to proteins on nitrocellulose blots in response to varying concentrations of $MgCl_2$. We found that the [α^{32}P]GTP-binding capacity of the 25 and 26 kDa proteins precipitated by mAb 50H.19 did not vary in the presence of Mg^{2+} concentrations ranging between 2 μM and 20 mM (Fig.3, 1a - 5a). In contrast, in whole platelet lysates we observed that the two faster migrating proteins of 20 and 21 kDa only bound [α^{32}P]GTP at Mg^{2+} concentrations above 2 μM (Fig.3, 1b - 5b). EDTA (2 mM) added in combination with 1 mM $MgCl_2$ drastically reduced the binding of all GTP-binding proteins, whereas EGTA had no effect (Fig.3, 6a,b and 7a,b).

DISCUSSION

We report here the co-immunoprecipitation of two small GTP-binding proteins of 25 and 26 kDa with the signal-initiating membrane protein CD9, but not with the major platelet integrin GPIIb/IIIa (Fig.1). Since CD9 and GPIIb/IIIa are reported to be present on human platelets at 45,000 and 46,000 copies respectively (6,22), and the immunoprecipitations were performed with saturating concentrations of mAbs under identical conditions, the difference in precipitation of [α^{32}P]GTP-binding proteins is inherent to the antigen and not to their abundance. The failure to detect

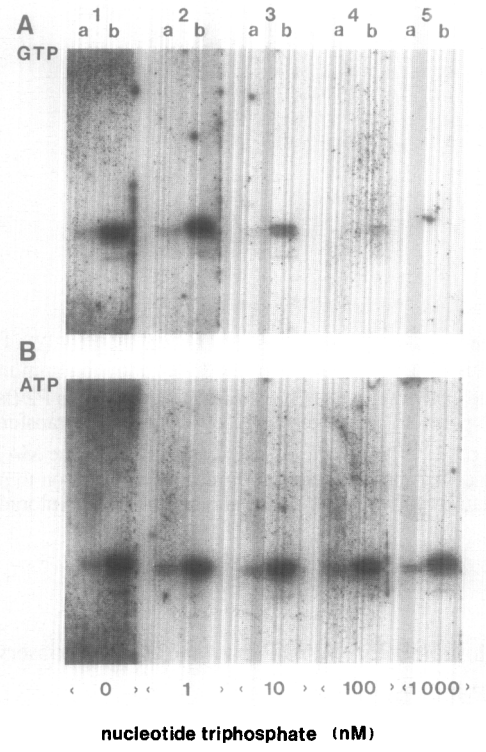

Fig.2. Effect of unlabeled nucleotides on the binding of [α^{32}P]GTP: Proteins from lysates immunoprecipitated with mAb 50H.19 (a) or from untreated lysates (b) were separated by SDS-PAGE and blotted to nitrocellulose paper. Strips were cut out and treated with [α^{32}P]GTP in the presence of indicated concentrations of unlabeled GTP (panel A, groups 1 - 5) or of unlabeled ATP (panel B, groups 1 - 5). The autoradiograms were developed after 14 h exposure.

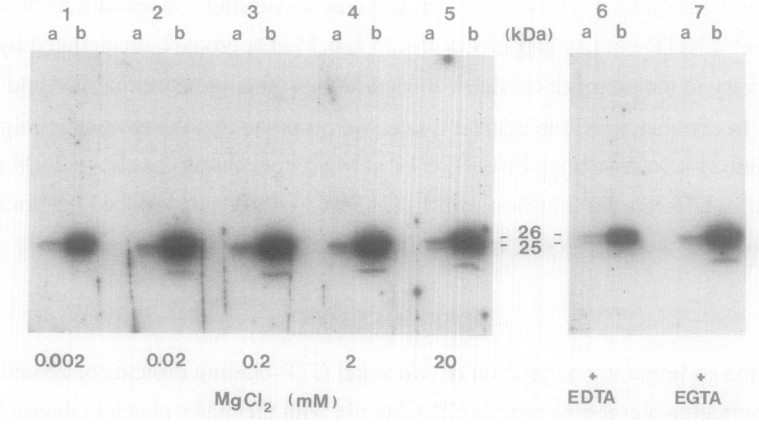

Fig. 3. Effect of Mg^{2+} concentration and chelating reagents on the binding of [α^{32}P]GTP: Aliquots of mAb 50H.19-immunoprecipitated proteins (a) or of untreated lysates (b) were separated by SDS-PAGE and blotted onto nitrocellulose paper. [α^{32}P]GTP-binding was performed on strips in the presence of indicated concentrations of $MgCl_2$ (groups 1 - 5) or in the presence of 1 mM $MgCl_2$ and either 2 mM EDTA (group 6) or 2 mM EGTA (group 7). Molecular masses were estimated as described in figure 1. Autoradiography was performed for 22 h.

[α^{32}P]GTP-binding proteins in association with GPIIb/IIIa is consistent with the inability to detect CD9 in P2-immunoprecipitates and provides further evidence that mAb P2 recognizes an epitope which is not present on the CD9-GPIIb/IIIa complex (9). The observation that only two bands of [α^{32}P]GTP-binding proteins were precipitated by anti-CD9 mAbs, whereas in unfractionated platelet lysates five radiolabed bands could be discerned (Fig. 1), suggests that CD9 is selectively complexed to certain small GTP-binding proteins. The question of specificity of labeling by the radionucleotide was addressed by attempting to block the binding of [α^{32}P]GTP with unlabeled nucleotides. GTP reduced the binding of [α^{32}P]GTP to the mAb 50H.19-precipitated proteins of 25 and 26 kDa as well as to all the GTP-binding proteins from unfractionated platelets in a dose-dependent manner with complete abrogation of radiolabeling between 100 nM and 1 μM (Fig.2 A), a concentration well within the reported range for small GTP-binding proteins (21,23). This finding together with the observation that ATP (up to 1 mM) had no effect on the binding of radiolabeled GTP (Fig.2 B) indicates that the 25 and 26 kDa proteins are GTP-binding proteins. Bhullar and Haslam (21) showed inhibition of [α^{32}P]GTP-binding of a 24 kDa platelet protein to elevated Mg^{2+} concentrations. The mAb 50H.19-precipitated proteins of 25 and 26 kDa were different proteins, since their binding of [α^{32}P]GTP did not change when $MgCl_2$ was present at concentrations between 2 μM and 20 mM (Fig.3).

We observed that the 25 and 26 kDa CD9-associated GTP-binding proteins required immunoprecipitation from ten times the concentration of platelets used to detect [α^{32}P]GTP-binding proteins in the unfractionated platelet lysate. However, this does not necessarily indicate that their [α^{32}P]GTP-binding capacity is relatively low. The number of small GTP-binding proteins found in human platelets is steadily increasing. So far eight have been clearly identified: the c-ras, the rap1A, 1B and 2B, the rac1, ralA, G25K and the cytosolic c25KG (23-29), ranging in molecular mass between 20,000 and 29,000, and as many as twelve [α^{32}P]GTP-binding proteins of 24 to 27 kDa have been visualized by separating platelet membrane proteins by two-dimensional SDS-PAGE (28). Under our separation conditions we detect only five bands of [α^{32}P]GTP-binding proteins in unfractionated platelet lysates. It is therefore likely that some of these bands contain two or more specific GTP-binding proteins. The anti-CD9 mAb immunoprecipitated 25/26 kDa proteins may represent selected components of the bands observed in the unfractionated lysates. The efficiency of [α^{32}P]GTP-binding on nitrocellulose transfers is known to vary among GTP-binding proteins. Of the small GTP-binding proteins the ral proteins retain the highest degree of GTP-binding, whereas others vary between low efficiency and total loss of binding (21,27,28). Alternatively, the 25/26 kDa proteins may represent low efficiency GTP-binding proteins. A final possibility is that only a fraction of the 25/26 kDa GTP-binding proteins present in the total lysate exist in an immunocomplex with CD9.

We consistently observed two GTP-binding proteins in immunocomplexes with anti CD9 mAbs. Since the CD9 molecule has a molecular mass of 22,000 to 27,000, estimated by SDS-PAGE (20), it migrates well within the range of the two [α^{32}P]GTP-binding proteins in our gel system. However, as judged by its primary structure (17), CD9 does not contain any consensus sequences for GTP-binding. Thus our data strongly suggest that CD9 is tightly associated with one or two small GTP-binding proteins. We are currently investigating the nature of the association.

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