Phase specific association of heterotrimeric GTP-binding proteins with the actin-based cytoskeleton during thrombin receptor-mediated platelet activation

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Abstract Subcellular distribution of heterotrimeric GTP-binding proteins during thrombin receptor-mediated platelet activation was examined, revealing two phases of translocation to the cytoskeleton. A part of Gi2α and Gsα shows first phase translocation to the low-speed pellet (15000 × *g* pellet) within 1 min after activation, suggesting involvement in platelet shape change or granule secretion. In the second phase, Gi2α, Gsα, Gqα, and Gβ translocate to the low-speed pellet, depending on platelet aggregation. These translocations correlated with the reorganization of the actin-cytoskeleton and were inhibited by cytochalasin D. Reconstitution experiments also revealed that G proteins are associated with the actin-cytoskeleton during platelet activation.

Key words: Platelet activation; Cytoskeleton; G protein; $p60^{c-src}$; GPIIbIIIa

1. Introduction

Platelets respond to several agonists, resulting in rapid shape change, granule secretion and aggregation. The platelet is, therefore, a suitable cell to assist understanding of the transmembrane signal transduction. Numerous intracellular molecular events during platelet activation have been reported: enhancement of phosphatidylinositol turnover and their key enzymes, increasing Ca^{2+} influx, elevation of tyrosine kinase activities, and reorganization of the cytoskeleton [1–4]. Of particular interest is the finding that several signal transducing molecules such as $p60^{c-src}$, phosphatidylinositol-3-kinase, phosphatidylinositol-4-kinase, phosphatidylinositol-4,5-kinase, diacylglycerol kinase, phospholipase C, phospholipase A2, and small GTP-binding proteins, rap 1B, rap 2B, rho, rac, and cdc42Hs [5–8] translocate to the reorganized cytoskeleton during the activation processes. These findings suggest that the platelet cytoskeleton may act as a functional network connecting signal transducing molecules. However, the significance of such connection remains unclear.

Heterotrimeric GTP-binding proteins (G proteins) composed of α, β, and γ subunits are involved in transmembrane signal transduction in a variety of cells. In platelets, a family of G proteins has been reported to mediate inhibition of adenylyl cyclase, and activation of phospholipase C and phospholipase A2 [9]. There is, however, no information regarding the cytoskeletal connection of G proteins in platelets. In this paper, we performed sequential analysis of G protein translocation during thrombin receptor-mediated platelet activation. As a result, we obtained the first evidence that G proteins display phase-specific translocation to the actin-based cytoskeleton; translocation of Gi2α and Gsα correlates well with the reorganization of the actin-based cytoskeleton causing a shape change, and translocation of Gqα, Gβ, Gi2α, and Gsα is associated with platelet aggregation.

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Abbreviations: G protein, guanine nucleotide-binding protein; GP, glycoprotein; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Materials

Anti-G protein antisera, AS/7, RM/1, QL, and SW/1 were purchased from Du Pont. Monoclonal antibody against $p60^{v-src}$ was purchased from Oncogene Science. Monoclonal anti-GPIIb antibody, which can be applied to immunoblotting under nonreducing conditions, was produced and provided by Seikagaku Corporation (Tokyo, Japan). Thrombin receptor (TR) peptide, SFLLRN, was synthesized by the solid-phase synthesis technique [10]. Cytochalasin D and deoxyribonuclease I (DNase I) were purchased from Sigma. RGDS peptide was obtained from Peptide Institute. Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt [11], and finally purified by gel filtration.

2.2. Preparation of human platelets

Washed human platelets were prepared as described [12] and finally suspended in 140 mM NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.1% glucose, and 10 mM HEPES (pH 7.4) at 5×10^8/ml and incubated at 37°C for 1 h prior to experiments.

2.3. Platelet activation and subcellular fractionation

Platelet aggregation was monitored by a lumi-aggregometer model 560-VS (Chrono-log). ATP secretion was monitored with luciferin–luciferase reagent (Chrono-log). Platelets were activated with 40 μM TR peptide at 37°C with or without constant stirring at 1000 rpm. In some experiments, platelets were preincubated with 0.5 mM RGDS peptide or 10 μg/ml cytochalasin D before activation. At indicated times, aliquots of activated platelets were lysed with half volume of ice-cold lysis buffer (3% Triton X-100, 150 mM Tris-HCl (pH 7.5), 15 mM EGTA, 75 μM 4-amidinophenylmethylsulfonyl fluoride (p-APMSF), 1.5 mM diisopropyl fluorophosphate (DFP), 30 μg/ml leupeptin, 0.35 U/ml aprotinin). Subcellular fractionation was performed according to the well-established method of Fox et al. [13] with minor modifications. The lysate was kept on ice for 10 min, and then centrifuged at 15000 × *g* for 10 min to obtain the low-speed pellet. The supernatant was further centrifuged at 120000 × *g* for 2 h to separate the high-speed pellet and the supernatant.

2.4. Actin-binding assay

The low-speed pellet as described above was sonicated with hypotonic buffer (0.2 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.1 mM DTT, 0.05% Triton X-100, 75 μM p-APMSF, 1.5 mM DFP, 30 μg/ml leupeptin, 0.35 U/ml aprotinin) and dialyzed during overnight against the same buffer. The hypotonic extract and actin monomer (0.3 mg/ml) were mixed in the presence or absence of 0.4 mg/ml

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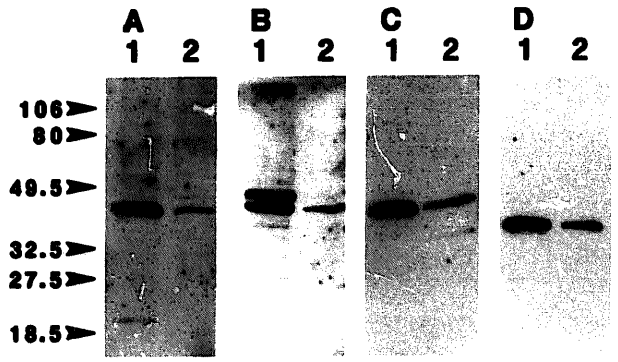

Fig. 1. Immunoblotting of bovine brain membrane and human platelets with anti-G protein antisera. Bovine brain membrane (lane 1) and human platelets (lane 2) were subjected to 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The blots were probed with 1:400 dilution of AS/7 (Gi2α, A), RM1 (Gsα, B), QL (Gqα, C), or SW/1 (Gβ, D). Positions of molecular weight standard are indicated in kD on the left.

DNase I. The buffer conditions were as follows: 20 mM MES (pH 7.0), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT, 0.05% Triton X-100. After incubation at 37°C for 30 min, the mixture was centrifuged at 100 000 × g for 30 min. The supernatants and the pellets were examined for the contents of G proteins by immunoblotting.

2.5. Others

SDS-PAGE was performed in the presence or absence of 2-mercaptoethanol by the method of Laemmli [14]. Immunoblotting was performed as described [12]. Proteins were detected using enhanced chemiluminescence system (Amersham). The blots were scanned with a flying-spot scanner model CS-9000 (Shimadzu, Kyoto).

3. Results

The specificity of the antisera used in this study was examined by immunoblotting (Fig. 1). In our experiments, AS/7, which recognizes Gi1α and Gi2α, reacted with a 41 kDa protein in human platelets (Fig. 1A, lane 2). It has been reported that platelets express all of the three members of Giα subunits [15]. Considering the prominence of Gi2α in platelets (Gi2α >> Gi3α > Gi1α) [15] and the results of two-dimensional electrophoresis of human platelets followed by immunoblotting with AS/7, we regarded the 41 kDa protein as Gi2α. RM/1 serum against Gsα recognized a 45 kDa band in platelets (Fig. 1B, lane 2), indicating that the 45 kDa form of Gsα is dominant in platelets. QL serum, specific for Gqα and G11α, reacted with a 42 kDa band and an additional faint band with a lower molecular mass in platelets (Fig. 1C, lane 2). As an undetectable amount of G11α is expressed in platelets [16], the 42 kDa band represents Gqα exclusively. SW/1 serum against common Gβ recognized a 35 kDa band in human platelets (Fig. 1D, lane 2).

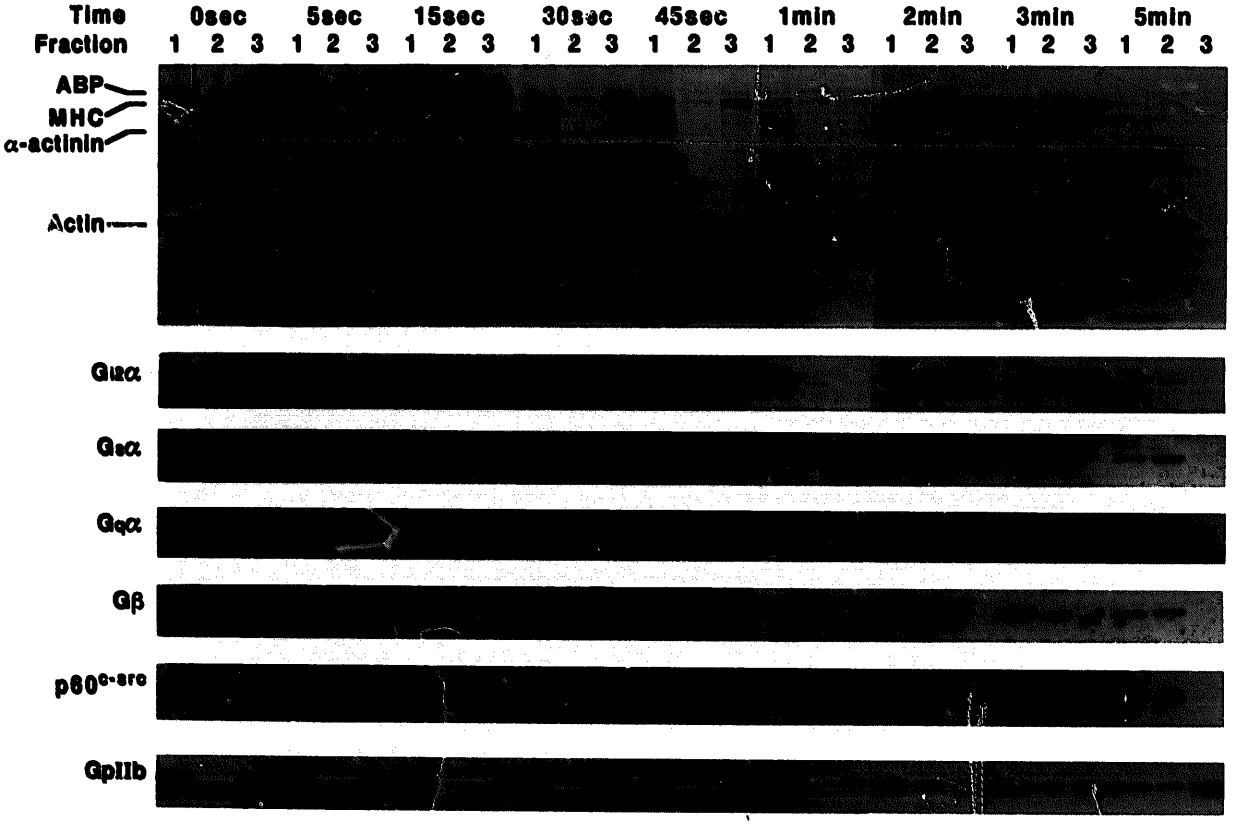

Fig. 2. Time-course of translocation of cytoskeletal proteins, G proteins, $p60^{c-src}$, and GPIIb to the cytoskeleton during platelet activation. Platelets were activated with 40 μM thrombin receptor peptide and aliquots of platelets were lysed with ice-cold lysis buffer at 5 s, 15 s, 30 s, 45 s, 1 min, 2 min, 3 min, and 5 min after activation. The sample designated as 0 s was lysed before addition of TR peptide. The lysate was fractionated into supernatant (lane 1), the low- (lane 2), and the high-speed pellet (lane 3) as described in section 2. Each fraction was separated by 10% SDS-PAGE and stained with Coomassie brilliant blue (upper panel) or electrophoretically transferred to nitrocellulose membranes, followed by immunoblotting with 1:400 dilution of AS/7 (Gi2α), RM/1 (Gsα), QL (Gqα), SW/1 (Gβ), 1:30 dilution of anti-$p60^{v-src}$ antibody ($p60^{c-src}$), or 1:100 dilution of anti-GPIIb-IIIa antibody (GPIIb).

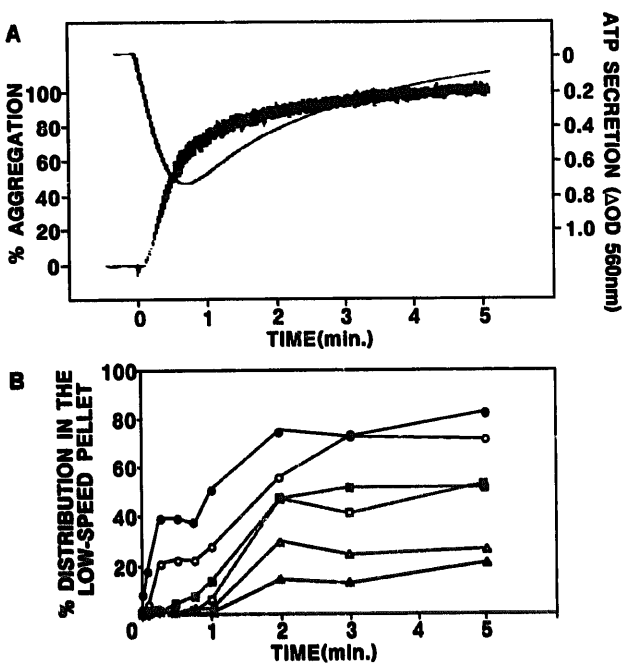

Fig. 3. Comparison of the kinetics of platelet aggregation, ATP secretion, and translocation of G proteins, $p60^{c-src}$, GpIIb. (A) Platelet aggregation and ATP secretion was monitored in the same experiment. 100% aggregation was defined as the light transmission at 5 min. ATP secretion was monitored as described in section 2.3. (B) The percentage of Gi2α (○—○), Gsα (●—●), Gqα (△—△), Gβ (■—■), $p60^{c-src}$ (□—□), and GPIIb (▲—▲) in the low-speed pellet was determined by densitmetric scanning of immunoblots. The data presented are a representative result of at least 6 separate experiments.

It has been shown that thrombin receptor is activated by a novel mechanism involving proteolytic cleavage and tethered ligand formation [17]. A synthetic peptide with the sequence SFLLRN has been shown to be a potent stimulant of the receptor, and causes platelet activation [18]. In our preliminary experiments, 40 μM TR peptide was roughly equivalent to 0.1 U/ml thrombin with respect to induce platelet aggregation (data not shown). We used the peptide as an agonist and examined the subcellular distribution of G proteins during platelet activation.

The upper panel of Fig. 2 shows the subcellular distribution of platelet proteins during activation. In resting platelets (0 s), only a trace amount of actin was found in the low-speed pellet. At 5 s after activation, a significant amount of actin and α-actinin translocated to the low-speed pellet. At 15 s, actin-binding protein (ABP or filamin) and myosin heavy chain were detectable in the low-speed pellet. Thereafter, the contents of actin, myosin, ABP, and α-actinin in the low-speed pellet gradually increased until 2 min. In the low-speed pellet, these cytoskeletal proteins were the most abundant. The subcellular distribution of these proteins seems to remain unchanged subsequently.

All G proteins examined also translocated to the low-speed pellet (Fig. 2 bottom panels); however, they were categorized into two groups with respect to the pattern of translocation. The first group consists of Gi2α and Gsα; the second Gqα and Gβ. In resting platelets, Gi2α and Gsα were recovered in both the supernatant and the high-speed pellet. At 5 s after activation, small amounts of both Gi2α and Gsα could be detected in the low-speed pellet. The amounts of Gi2α and Gsα in the low-speed pellet increased between 45 s and 2 min, at which time Gi2α and Gsα were predominant. On the other hand, Gqα and Gβ distributed mainly in the supernatant of resting platelets. Both G protein subunits were not detected in the low-speed pellet until 45 s after activation. Significant amounts of both Gqα and Gβ were detected in the low-speed

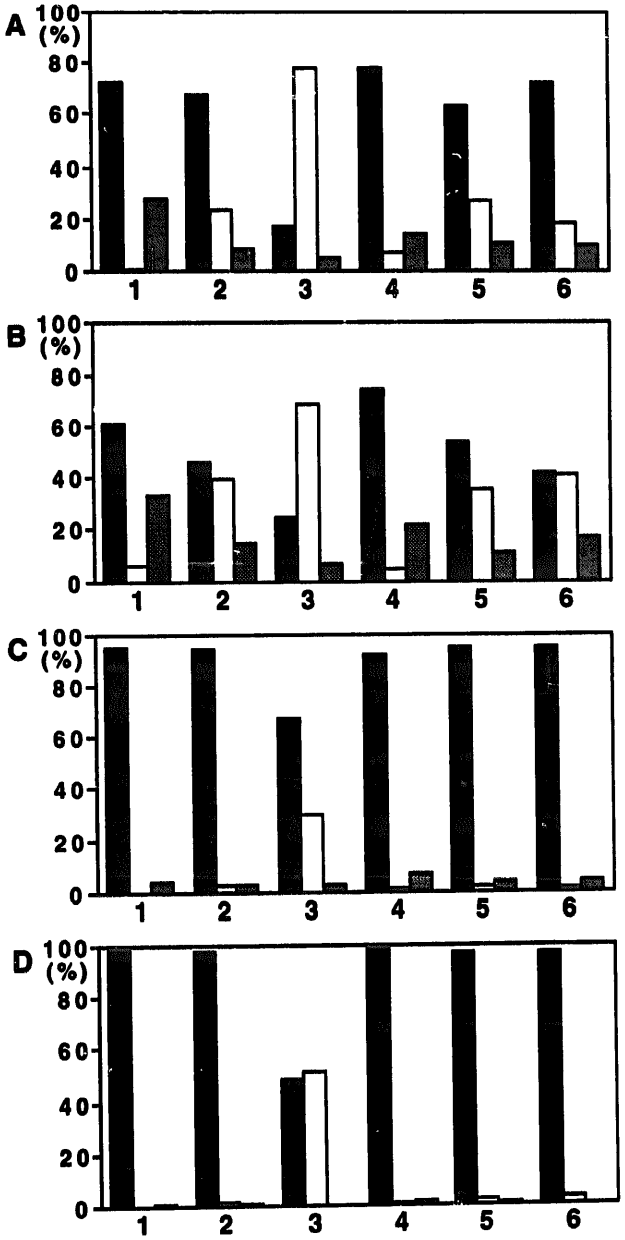

Fig. 4. Effect of RGDS peptide or cytochalasin D on the translocation of G proteins during platelet activation. Platelets were preincubated with 0.5 mM RGDS peptide (5) or 10 μg/ml cytochalasin D (4) and activated with 40 μM TR peptide with (1–5) or without (6) stirring. At 0 s (1), 15 s (2), or 3 min (3–6), platelets were lysed and fractionated into supernatant (black column), the low-speed pellet (open column), and the high-speed pellet (shaded column). The contents of Gi2α (A), Gsα (B), Gqα (C), and Gβ (D) in each fraction were determined by immunoblotting followed by densitometric scanning. The vertical axis indicates percent distribution of individual subunits. The data presented are a typical result of at least 6 separate experiments.

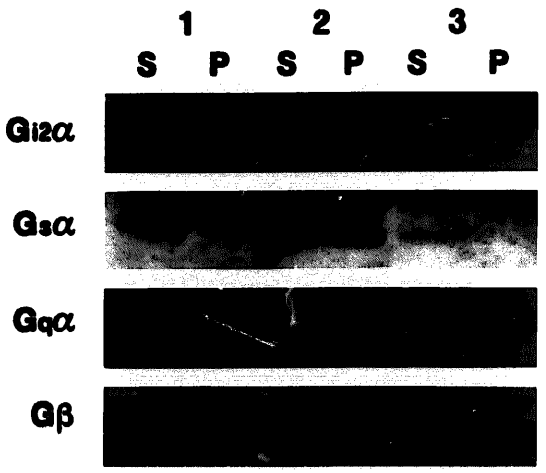

Fig. 5. Actin-binding assay of hypotonic extract from the low-speed pellet. Hypotonic extract of the low-speed pellet prepared as described in section 2 was incubated with (2,3) or without (1) 0.3 mg/ml of actin in the presence (3) or absence (1,2) of 0.4 mg/ml DNase I, and separated into 100 000×g supernatant (S) and pellet (P). Immunoblotting was performed with 1:400 dilution of AS/7 (Gi2α), RM/1 (Gsα), QL (Gqα), or SW/1 (Gβ).

pellet after 2 min. $p60^{c-src}$ and GPIIbIIIa showed a translocation pattern similar to that of Gqα and Gβ. The present results indicate that Gi2α and Gsα translocate to the low-speed pellet at an early stage, followed by further translocation in the late stage, while Gqα and Gsα translocated to the low-speed pellet only in the late stage of activation.

To assess the significance of G protein translocation to the cytoskeleton in platelet function, we quantified the relative contents of G proteins, $p60^{c-src}$, and GpIIb in the low-speed pellet by scanning the blots of Fig. 2 (Fig. 3B). The percent distributions of Gi2α and Gsα in the low-speed pellet reached a plateau within 1 min after activation, while Gqα, Gβ, $p60^{c-src}$, and GpIIb were gradually increased during this period. All proteins showed marked increases after 1 min, reaching a second plateau at about 2 or 3 min. The first peak of Gi2α and Gsα corresponded to maximal ATP secretion (Fig. 3A). Platelets also changed shape within seconds after activation. These results suggest that rapid translocation of Gi2α and Gsα to the cytoskeleton is associated with platelet shape change and/or granule secretion. The second peak of all proteins coincided with platelet aggregation, suggesting an association between translocation in the second peak and platelet aggregation. It is noted that the extent of translocation differed between proteins. About 70–80% of Gi2α and Gsα was in the low-speed pellet at 5 min, compared to approximately 50% of Gβ and $p60^{c-src}$ and about 20% of Gqα and GpIIb. These differences were reproducible with 4 different experiments, and suggest that translocation of G proteins to the low-speed pellet is mediated by some specific interaction with their targets, not an artificial phenomenon such as trapping by fibrin clots.

We then characterized the translocation of G proteins. GPIIbIIIa (αIIbβ3 integrin) is essential for platelet aggregation. When GPIIbIIIa ligation and subsequent platelet aggregation were inhibited by preincubation with RGDS peptide (Fig. 4, column 6) or activation without stirring (Fig. 4, column 5), the second phase translocation of G proteins to the low-speed pellet was suppressed compared with aggregated platelets (Fig. 4, column 3). The subcellular distribution of G proteins under these conditions was comparable to that normally seen at 15 s in aggregated platelets (Fig. 4, column 2). The result indicates that translocation of G proteins to the low-speed pellet shows two phases which are clearly distinguished by GPIIbIIIa dependency. Here, we defined these two phases of translocation: the first phase translocation occurs within 15 s after activation, independent of GPIIbIIIa engagement; whereas the second phase translocation begins at around 1 min and is strictly dependent on GPIIbIIIa. Cytochalasin D, which inhibits actin polymerization, suppressed the first phase translocation of Gi2α and Gsα (Fig. 4 column 4) as well as actin (data not shown). Thus, the first phase translocation is dependent on actin polymerization or exposure of barbed ends of actin filaments, suggesting the association of G proteins with the actin-based cytoskeleton.

In order to confirm the interaction of G proteins with the actin-based cytoskeleton, a reconstitution assay was performed. Immunoblotting and SDS-PAGE revealed that the hypotonic extract of the low-speed pellet at 3 min after activation contains G proteins as well as cytoskeletal proteins such as actin, ABP, and α-actinin (data not shown). G proteins in the extract were co-precipitated with exogenously added actin (Fig. 5, lane 2). Preincubation with DNase I, which induces depolymerization of actin filaments, completely inhibited sedimentation of G proteins (Fig. 5, lane 3). In the absence of exogenous actin, neither G proteins nor actin was precipitated, probably because the concentration of actin in the final assay mixture was lower than the critical concentration (Fig. 5, lane 1). This result suggests that G proteins in the low-speed pellet are associated with the actin-based cytoskeleton.

4. Discussion

Differential centrifugation analysis of Triton X-100-treated platelet lysates is a useful and well-established method to examine and characterize the reorganization of the platelet cytoskeleton [5,13]. The low-speed pellet is thought to be composed of a network of actin filaments and their associated proteins. The high-speed pellet is proposed to represent the membrane cytoskeleton including filamentous actin and associated proteins. In resting platelets, cytoplasmic actin filaments are not sufficiently cross-linked and recovered in the high-speed pellet, contrary to the low-speed pellet [5] (Fig. 2). After activation, actin filaments are rapidly cross-linked resulting in an increased sedimentation in the low-speed pellet. α-Actinin, ABP, and tropomyosin are also increased in the low-speed pellet. The aggregation-independent first phase translocation of Gi2α and Gsα is quite similar to translocation of those cytoskeletal proteins, suggesting that this pool of Gi2α and Gsα is associated with cytoplasmic actin filaments. It is possible that Gi2α and Gsα are localized at the site where the cytoplasmic actin filaments terminate at the plasma membrane, and are associated with the reorganization of the cytoskeleton in an early phase of platelet activation.

The second phase translocation of G proteins is associated with platelet aggregation, which is a typical example of integrin-mediated cell adhesion. It is possible to consider that a multimolecular cell adhesion apparatus which links GPIIbIIIa to the actin-cytoskeleton is formed in aggregated platelets as seen in focal contacts of cultured cells [19]. Proteins which are

recovered in the low-speed pellet in the second phase, including G proteins and $p60^{c-src}$, may be involved in the formation or maintenance of the cell adhesion apparatus. In this regard, it is of interest that γ5 subunit has been shown to be localized with vinculin at the focal adhesion of cardiac fibroblasts [20]. It is possible that G proteins are generally involved in the integrin-mediated cell adhesion in association with the actin-based cytoskeleton.

G proteins transduce signals from receptors to the appropriate effectors. In platelets, Giα has been shown to inhibit adenylyl cyclase activity during platelet activation. Gs stimulates adenylyl cyclase activity; however, the significance of Gsα in platelet activation remains unclear. Gq, a member of another family of G proteins (Gq/G11 family), has been shown to activate phospholipase Cβ [21]. Thrombin receptor functionally couples with Gi. Further, Gq/11, G12, and G13 are also activated via thrombin receptor [22]. Recent understanding of G protein function indicates that both α and βγ subunits which are dissociated upon receptor activation regulate specific effectors [23]. At present it is unclear whether cytoskeleton-associated α and β subunits bind to the same cytoskeletal protein in the trimeric state or bind to different proteins, or whether cytoskeletal association of G proteins reflects the activation of individual G proteins. Translocation of Gi2α and Gsα, but not Gβ, in the first phase (Figs. 2 and 3) suggests that α subunits of Gi2 and Gs dissociated from βγ subunits independently translocate to the low-speed pellet. Further examination of this problem is required.

Association of G proteins with the cytoskeleton has also been reported in neutrophils, Sf49 mouse lymphoma cells, and cardiac fibroblasts [20,24,25]. Gq/G11 α subunits have been recently shown to associate with actin filaments in WRK1 cells [26]. As G protein does not directly bind F-actin, it is likely that the cytoskeletal association of G proteins is mediated by certain cytoskeletal proteins. Recently, a number of signal transducing molecules have been shown to translocate to the cytoskeleton during platelet activation. These include $p60^{c-src}$, $p62^{c-yes}$, phosphatidylinositol-3 kinase, phospholipase A2, phospholipase C, GTPase activating protein, protein kinase C, $p125^{FAK}$, rap1B, rap2B, rho, rac, and cdc42Hs. Stimulation of G protein-coupled receptor activates multiple signalling cascades, including tyrosine kinases, phospholipase C, and Ras pathway [27]. The cytoskeleton may provide the site where these signal transducing molecules, including G proteins, assemble and interact with each other, resulting in cross-talk of signalling pathways. Alternatively, the cytoskeleton may be directly involved in the G protein-mediated signal transducing events. It has been reported that calspectin (fodrin or non-erythroid spectrin) modulates the activity of CD45, a transmembrane protein tyrosine phosphatase [28]. Similar regulation may be present in G protein-mediated signalling. In order to examine these possibilities, it is important to examine the cytoskeletal target of G proteins and how the interaction is regulated. Investigation is currently underway to identify G protein-binding protein(s) in the activated platelet cytoskeleton.

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