Identification of Ga13 as One of the G-proteins That Couple to Human Platelet Thromboxane A2 Receptors*

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Previous studies have shown that ligand or immunoaffinity chromatography can be used to purify the human platelet thromboxane A2 (TXA2) receptor-Gα complex. The same principle of co-elution was used to identify another G-protein associated with platelet TXA2 receptors. It was found that in addition to Gαq, purification of TXA2 receptors by ligand (SQ31,491)-affinity chromatography resulted in the co-purification of a member of the G1 family. Using an antipeptide antibody specific for the human G13 α-subunit, this G-protein was identified as Go13. In separate experiments, it was found that the TXA2 receptor agonist U46619 stimulated [35S]guanosine 5'-O-(3-thiotriphosphate) incorporation into G13 α-subunit. Further evidence for functional coupling of G13 to TXA2 receptors was provided in studies where solubilized platelet membranes were subjected to immunoaffinity chromatography using an antibody raised against native TXA2 receptor protein. It was found that U46619 induced a significant decrease in Gaq and G13 association with the receptor protein. These results indicate that both Goq and Gα13 are functionally coupled to TXA2 receptors and dissociate upon agonist activation. Furthermore, this agonist effect was specifically blocked by pretreatment with the TXA2 receptor antagonist, BM13.505. Taken collectively, these data provide direct evidence that endogenous Gα13 is a TXA2 receptor-coupled G-protein, as 1) its α-subunit can be co-purified with the receptor protein using both ligand and immunoaffinity chromatography, 2) TXA2 receptor activation stimulates GTPγS binding to Gα13, and 3) Gα13 affinity for the TXA2 receptor can be modulated by agonist-receptor activation.

Interaction of the prostaglandin endoperoxide analogue, TXA2 1 (1, 2) with platelet receptors (3–5) has been shown to modulate not only hemostasis but also the development of thromboembolic diseases (6–9). However, despite recent progress, the TXA2-mediated signal transduction pathway is not completely understood. In this regard, previous studies have shown that one mechanism by which TXA2 receptors act is through stimulation of phospholipase C (PLC) leading to inositol 1,4,5-triphosphate (IP3) production, and subsequent intracellular Ca2+ mobilization (10–14). Furthermore, separate studies have linked this stimulation of PLC activity to TXA2 receptor signal transduction through the pertussis toxin-insensitive guanine nucleotide-binding protein (G-protein) Gq (16, 17). On the other hand, experiments conducted in our laboratory provided evidence for the existence of intraplatelet Ca2+ mobilization, which is independent of IP3 production (15). This finding raised the possibility that TXA2 receptors may also couple to a G-protein family separate from Gq. Additional evidence in support of this notion was provided by experiments showing that a C-terminal antibody which recognizes the α-subunit of Gq and G11 was not completely inhibit U46619-stimulated GTPase activity (16). Moreover, ligand and immunoaffinity chromatography purification of the TXA2 receptor-G-protein complex allowed co-purification of G-proteins distinct from Gq (17). Taken together, these results led to the hypothesis that TXA2 receptors might couple to a G-protein(s) to stimulates platelet aggregation independently of the Gq-PLC-IP3 pathway.

Although this putative G-protein has not been identified, recent reports have provided indirect evidence that it may belong to the G12 family (18, 19). In one study, it was shown that activation of platelet TXA2 receptors led to increased incorporation of the photo reactive GTP analogue [α-32P]GTP azidoanilide into both G12 and G13 α-subunits, which may suggest coupling of TXA2 receptors to these α-subunits (20). On the other hand, as all the agonists tested (U46619, thrombin, ADP, and vasopressin) produced [α-32P]GTP azidoanilide incorporation, this labeling could also have been due to activation of a downstream signaling event or to cross-talk between these separate signal transduction pathways. In separate studies, it was shown that the affinity state of TXA2 receptors transfected in COS-7 cells could be influenced by co-expression of Gα13 (21). Although this finding is consistent with the notion that TXA2 receptors have the capacity to couple with G13, it is not clear whether such coupling occurs at physiological concentrations of receptor and/or G-protein. Consequently, two independent reports have provided indirect evidence that TXA2 receptors may couple to a Gα subunit in the G12/13 family. Based on these considerations, in the present study we performed experiments to determine whether this phenomenon occurs in a native platelet preparation using endogenous concentrations of TXA2 receptor and Gα subunits. To this end, affinity purification of

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Copurification of G-proteins with TXA₂ Receptors

The receptor-G-protein complex was employed to measure direct physical association of TXA₂ receptors and Gₐ₁₃/₁₂. This approach has been previously applied in our laboratory to the identification of Gₐ₁₃ as one of the G-proteins associated with the platelet TXA₂ receptors (17). It was found that, in addition to Gₐ₁₃, purification of the TXA₂ receptors resulted in co-elution of Gₐ₁₃. Furthermore, agonist activation of TXA₂ receptors caused an increase in GTPγS binding to G₁₃ α-subunit as well as dissociation of the receptor-G₁₃ complex, providing evidence that G₁₃ is indeed functionally coupled to platelet TXA₂ receptors.

**Experimental Procedures**

**Materials**—Outdated platelet concentrates were obtained from Heartland Blood Services (Aurora, IL). SQ intermediate (ethyl-[1S-[1n, 2α-(Z),3α,4α]]-7-[3-aminomethyl] 7-oxa-bicyclo[2.2.1]hept-2-yl]-5-heptenoate) for the synthesis of SQ23,491 was provided by Bristol-Myers Squibb Institute for Medical Research. BM13.177 and BM13.505 were generously supplied by Dr. K. Stegmeier, Roche Molecular Biochemicals (Mannheim, Germany). [³⁵S]GTPγS was purchased from Amersham Pharmacia Biotech. U46619 was purchased from Cayman Chemicals; asolectin was from the American Lectin Co. (Atlanta, GA); CHAPS, protein A-Sepharose CL-4B, GTPγS, α-phorbol ester rabbit prestin, Texas Red-dextran, Gel 102 and 4-chloro-1-naphthol (horseradish peroxidase color development reagent) were from Bio-Rad; and horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L), biotinylated goat anti-rabbit IgG (H+L), and the Vectastain ABC kit were purchased from Vector Laboratories (Burlingame, CA).

**Antibodies**—A 9-amino acid peptide corresponding to residues 40–48 of the human G₁₃ (P₁₃, Table I) (22), with a cysteine added at the N terminus to facilitate coupling to carrier protein was synthesized by Chiron Mimotropes (Raleigh, NC). The peptide was coupled to keyhole limpet hemocyanin using m-maleimidobenzene N-hydroxysuccinimide ester and injected into White New Zealand Pusaurella multico-fiber-rabbits, according to previously described procedures (23). Rabbit polyclonal antibodies against the C-terminal region of G₁₃ (G₁₃-Q, Table I) were produced as described previously (16). Antibodies were purified from rabbit serum by chromatography on protein A-Sepharose CL-4B, and the IgG fractions were labeled with carrier-free Na⁺[³¹P] (Amersham Pharmacia Biotech) using the IODO-BEADS iodo-nation reagent (Pierce). Rabbit polyclonal IgG raised against residues 2–21 (G₁₃-Q) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal IgG directed against the C-terminal region of G₁₃ was a generous gift from J. Sylvia Gutkind (National Institutes of Health, Bethesda, MD) (24). Although this antibody has been suggested to be specific for G₁₃, C-terminal segments of G₁₂ and G₁₃ differ only by 4 amino acids (18, 19). Consequently, this antibody may have limited cross-reactivity with G₁₃ and is named G₁₃. Furthermore, agonist activation of TXA₂ receptors is indeed functionally coupled to platelet TXA₂ receptors.

**Membrane Preparation and Solubilization**—Human platelet membranes were prepared from platelet concentrates and solubilized using 10 mM CHAPS as described previously (5). Typically, this method resulted in a 50%–70% solubilization of platelet membrane proteins, yielding a final protein concentration of 2–3 mg/ml.

**Ligand Affinity Chromatography Purification of the Thromboxane A₂ Receptor-G-protein Complex**—TXA₂ receptors were purified as described previously (5). Briefly, the TXA₂ receptor antagonist SQ23,491 was immobilized to Affi-Gel 102, and CHAPS-solubilized membranes (4 mg of protein) in buffer A (20% glycerol, 500 mM KCl, 0.2 mM EGTA, and 0.5 mM/mg asolectin) were incubated with the matrix overnight. Bound proteins were eluted as flow-through, and the column was washed with buffer D (20 mM Tris-HCl, 10 mM CHAPS, 20% glycerol, 500 mM KCl, 0.2 mM EGTA, 0.5 mg/ml asolectin, pH 7.4). TXA₂ receptors and receptor-associated proteins were then eluted with buffer D containing 50 mM TXA₂ receptor antagonist, BM13.177 (25, 26). After elution of the first 1-ml fraction, the flow was stopped for 30 min and restarted to elute the subsequent 1-ml fractions. TXA₂ receptor binding activity as well as G₁₃ immunoreactivity were found to be concentrated in the first fraction following the 30-min incubation (17). A modification of this method was used in order to allow further identification of the TXA₂ receptor-associated G-proteins (27). Specifically, after unbound proteins were washed with buffer D, 5 μg/ml [³¹P]G₂-QL IgG, [³¹P]G₁₂ IgG, or [³¹P]G₁₃ IgG (or the same protein concentration of [³¹P]labeled preimmune IgG (PI IgG) was added), and the reaction mixture was allowed to incubate for 1 h at 20°C. Unbound antibodies were washed with buffer D, and elution of TXA₂ receptors and receptor-associated proteins was performed as described above. The elution fractions were counted for [³¹P] activity and specific binding attributable to G₁₃, G₁₂, and G₁₃ was defined as the difference between the counts eluted from the PI antibody columns minus the counts eluted from the [³¹P]-IgG column.

**Immunaffinity Chromatography Purification of the Thromboxane A₂ Receptor-G-protein Complex**—Solubilized platelet membranes were prepared as described (5), and the CHAPS concentration was adjusted to 2 mM. The preparation (4 mg of protein) was then incubated with an immunosorbent matrix coupled to an anti-TXA₂ receptor antibody (Texas Red-dextran-Texas Red-dextran) for 1 h at 20°C (28). [³¹P]-Labeled G₂-QL IgG, G₁₃ IgG, or PI IgG was added (final concentration 150 μg/ml), and the reaction mixture was allowed to incubate for 5 min. The preparation was then incubated with vehicle or the TXA₂ agonist U46619 (100 nM) (29) for an additional 5 min. The matrix was loaded on a column and washed with buffer D to elute unbound proteins. The column was eluted with 100 mM glycine (pH 2.5), and the 3-ml elution fraction was counted for [³¹P] activity. Specific binding attributable to G₁₃ or G₁₂ was defined as the difference between the counts eluted from the [³¹P]-G₂-QL or [³¹P]-G₁₂ column, respectively, minus the counts eluted from the [³¹P]-IgG column. Eluted counts were normalized to the amount of purified TXA₂ receptor protein, as measured by densitometric analysis of the immunosorbent column elution fractions immunoblotted with TXA₂ antibody. In order to purify TXA₂ receptor-associated G-proteins, as described previously (5), and with 10 mM G₁₂-3 peptide for 1 h at room temperature to specifically elute G₁₂ subunits. Eluted fractions were then concentrated to 1 ml of Ecosafe (Research Product International, IL) and analyzed by scintillation spectrometry. Eluted counts were normalized to the amount of immunoprecipitated G₁₂ α-subunit, as measured by densitometric analysis of the elution fractions immunoblotted with G₁₃-13 IGG.

**ELISA**—Immunol 2 microtiter plates were coated with either 12.5 μg of synthetic peptide or 125 μg of solubilized platelet membranes. Following incubation for 1 h at room temperature, the plates were washed three times with modified Tyrode’s buffer containing 0.1% bovine serum albumin, 5 mM dextrose, 1 mM CaCl₂, 5 mM HEPES, pH 7.4, and then blocked by incubation for 1 h with 5% bovine serum albumin in the same buffer. Serial dilutions of antisera were applied to the wells and incubated for an additional 1 h at room temperature. The wells were washed three times with the modified Tyrode’s buffer, and bound antibodies were detected by incubation for 1 h with goat anti-rabbit IgG (H+L) conjugated to horseradish peroxidase. After extensive washing, the color reaction was developed by addition of 50 μl of 0.4 mg/ml o-phenylenediamine, 0.012% H₂O₂ in 80 mM citrate phosphate, pH 5. An equal volume of 2 × H₂SO₄ was then added, and the presence of specific antibodies was measured by absorbance at 490 nm.

**Polyacrylamide Gel Electrophoresis and Immunoblot Assay**—The affinity column eluates were first concentrated using Millipore Ultrafree-MC filters. 20–40 μl of sample (30–40 μg of protein) was then subjected to SDS-PAGE according to the method of Laemmli (31) using 10% minigels, under nonreducing conditions, and the proteins were electrophoretically transferred onto nitrocellulose membranes according to the method of Towbin et al. (32). After transfer, the nitrocellulose membranes were blocked with 5% gelatin in Tris-buffered saline and incubated overnight at room temperature with the indicated dilution of G₂-QL, G₁₂-13, or G₁₃ common IgG. The blots were washed and treated with biotinylated goat anti-rabbit IgG (H+L) as the secondary antibody. The immunoreactive proteins were detected with avidin and horseradish peroxidase, followed by 0.5 mg/ml 4-chloro-1-naphthol.

**Statistical Analysis**—Data were analyzed according to Student’s t test (*, p < 0.05; **, p < 0.005).

**Results**

In order to purify TXA₂ receptor-associated G-proteins, solubilized platelet membranes were subjected to ligand affinity
standard procedures. In the next experiments, solubilized platelet membranes were incubated with the ligand affinity matrix (5). The column was washed with buffer and then equilibrated with 125I-G-13 IgG. The TXA2 receptor-G-protein complex was next eluted by BM13.177 (25, 26), and the elution fractions were quantitated for 125I. As Gaq is known to couple to TXA2 receptors (16, 17), a positive control experiment was conducted using 125I-G-QL IgG. Control experiments were also performed using 125I-labeled preimmune IgG to determine nonspecific binding of both G-QL and G-13 IgG. Reported specific binding represents the difference between the counts eluted from the 125I-anti-α-subunit IgG column and the counts eluted from the 125I-labeled preimmune IgG column. Using this procedure, it was found that TXA2 receptors co-purified not only with Gaq but also with Ga13. Thus, Fig. 4 illustrates that the affinity column eluate contained 53 ± 5% and 24 ± 5% specific binding for Gaq (solid bar) and Ga13 (open bar), respectively. These findings, therefore, provide evidence that both Gaq and Ga13 are in direct physical association with endogenous platelet TXA2 receptors.

The next series of experiments was performed to determine whether Ga13 also copurified as part of the TXA2 receptor-G-protein complex. These studies employed a specific antibody directed at a unique N-terminal segment of Ga12 (G-12, Table 1), which was also iodinated for quantitative purposes. Again, the TXA2 receptor-G-protein complex was purified by ligand affinity chromatography and the amount of Ga12 present in the eluate was determined using 125I-G-12 IgG. As above, specific binding was determined by parallel experiments using 125I-labeled preimmune IgG. It was found that, in contrast to Gaq, Ga13 did not appear to co-purify with TXA2 receptors (Fig. 4, hatched bar). In these experiments, it can be seen that the counts attributable to Ga13 are less than the counts representing nonspecific binding by preimmune IgG. Although this decrease is not significant, it can be explained on the basis that G-12 IgG is enriched in immunoglobulins against Ga12 and consequently contains a lesser percentage of nonspecific proteins than preimmune IgG. As nonspecific protein is presumably responsible for nonspecific binding observed with preimmune IgG, the difference between 125I-G-12 IgG counts and 125I-labeled preimmune IgG counts yields a negative number. The same phenomenon would also suggest that the specific binding observed for both G-QL and G-13 IgG (Fig. 4) is probably underestimated, as each of these IgG fractions contain less nonspecific proteins than their preimmune IgG controls. Furthermore, this consideration would indicate that the relative percentage of specific binding with G-QL and G-13 IgG may not necessarily represent the actual distribution of Gaq and Ga13 within the TXA2 receptor-G-protein complex. Taken together, the above results provide evidence that in addition to Gaq, platelet TXA2 receptors are coupled to endogenous Ga13.

In the next series of experiments, the antigen U46619 was used to determine whether Ga13 is functionally coupled to TXA2 receptors. In these studies, solubilized platelet membranes were incubated with [35S]GTPγS in the presence and absence of U46619 (10 nM) and subjected to immunoprecipitation with G-13 IgG. Immunoprecipitated Ga13 was eluted using 1 mM G-13 peptide, the elution fractions were counted for [35S]GTPγS.
The present study employed ligand affinity (5) and immunoaffinity chromatography techniques (28) to purify and identify G-proteins associated with human platelet TXA2 receptors. These techniques have previously been used for the purification of TXA2 receptor-G-protein complexes from solubilized platelet membranes (17). Using both ligand and immunoaffinity chromatography, it was found that in addition to Gαq, a member of the Go13 family of G-proteins co-purifies with platelet TXA2 receptors. This G-protein was identified as Gα13 using an antibody raised against a unique internal sequence of human G13-α-subunit (22). Additional studies demonstrated that this Gα13-α-subunit was functionally coupled to TXA2 receptors.

The Go12 family of G-proteins defines the fourth and the
most recently discovered class of α-subunits (18, 19). The members of this family share high sequence homology and are ubiquitous and immunodetectable in most membranes of various mammalian cells and tissues (22, 34, 35). However, despite intensive research in the past years, no definitive effector(s) has been assigned to either G\textsubscript{12} or G\textsubscript{13} (36, 37). Both G\textsubscript{12} and G\textsubscript{13} are oncogenic, and expression of their mutationally activated forms stimulates cell proliferation and induces neoplastic transformation in NIH3T3 and Rat1 cells (24, 38–40). Furthermore, GTPase-deficient mutants of G\textsubscript{12} and G\textsubscript{13} have been shown to stimulate Jun kinase/stress-activated protein kinase (JNK/SAPK) in NIH3T3, HEK293, and COS-1 cells (41, 42). In addition, both activated α-subunits have been shown to stimulate stress fiber formation/focal adhesion assembly in Swiss 3T3 cells (43) and induce apoptosis when transfected in Chinese hamster ovary or COS-7 cells (44, 45). Finally, signal transduction through G\textsubscript{12} and G\textsubscript{13} appears to involve small molecular weight GTP-binding proteins such as RhoA, cdc42, and Ras (46–48). However, even though there is similarity between G\textsubscript{12} and G\textsubscript{13}-associated pathways, evidence has been provided that both subunits seem to fulfill distinct cellular and biological functions. Specifically, G\textsubscript{12} has been shown to be involved in the transcriptional activation of the serum response element (47). On the other hand, G\textsubscript{13} but not G\textsubscript{12} is involved in the induction of inducible nitric-oxide synthase in MCT cells (49) and in lysosphatidic acid-induced activation of Rho (50). Other studies showed that, whereas the guanine nucleotide exchange factor (GEF) for Rho, p155RhoGEF, was able to act as a GTPase-activating protein toward both G\textsubscript{12} and G\textsubscript{13}, only G\textsubscript{13} bound to p155RhoGEF and stimulated its capacity to catalyze nucleotide exchange on Rho (51, 52). In addition, disruption of the gene encoding G\textsubscript{13} α-subunit in mice impaired the ability of endothelial cells to develop into organized vascular-system, resulting in intrauterine death and demonstrating a role for G\textsubscript{13} in the regulation of cell movement and developmental angiogenesis (53).

Two potential effectors for G\textsubscript{13} have been proposed that would be of interest in the signal transduction pathways associated with TXA\textsubscript{2} receptors in platelets. In this connection, G\textsubscript{13} has been shown to stimulate the ubiquitously distributed Na/H exchanger isofrom, NHE1 (46, 53–55). Moreover, substitution of C-terminal residues from α\textsubscript{c} conferred on α\textsubscript{13} the ability to respond to stimulation by the D\textsubscript{2}-dopamine receptor and to activate NHE1 in an agonist-dependent manner (54).

In platelets, regulation of Na/H exchange has been shown to modulate receptor-mediated phospholipase A\textsubscript{2} and phospholipase C activation as well as intracellular Ca\textsuperscript{2+} mobilization (57, 58). In regard to platelet TXA\textsubscript{2} receptors, it was found that U46619 caused an increase in intracellular pH, which was required for full U46619-induced Ca\textsuperscript{2+} mobilization (59). Thus, coupling of TXA\textsubscript{2} receptors to NHE1 activity stimulation could be a possible mechanism by which G\textsubscript{13} is involved in TXA\textsubscript{2}-mediated signal transduction in platelets.

In addition to its indirect effects on intracellular Ca\textsuperscript{2+} via Na/H exchange activity stimulation, G\textsubscript{13} has also been implicated in the activation of L-type Ca\textsuperscript{2+} channels (60, 61). Specifically, in rat portal vein myocytes, the heterotrimer α\textsubscript{13}β1γ2 couples to the angiotensin AT\textsubscript{1A} receptors to increase cytoplasmic Ca\textsuperscript{2+} concentration (60). Furthermore, it was found that the βγ dimer released from α\textsubscript{13} upon angiotensin AT\textsubscript{1A} receptor activation was responsible for the activation of L-type Ca\textsuperscript{2+} channels (61). Although extracellular Ca\textsuperscript{2+} influx through L-type and non-L-type Ca\textsuperscript{2+} channels has been associated with TXA\textsubscript{2} receptor-mediated contraction in rat aorta (62), no such channels have been identified on the platelet surface thus far.

In summary, the present data demonstrate that platelet TXA\textsubscript{2} receptors are functionally coupled to G\textsubscript{13}. The physiological significance of the signal transduction pathway associated with such coupling requires further investigation.

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