Identification of \( G_{26} \) as a Pertussis Toxin-Insensitive G Protein in Human Platelets and Megakaryocytes

By Alison Williams Gagnon, David R. Manning, Lucia Catani, Alan Gewirtz, Mortimer Poncz, and Lawrence F. Brass

\( G \) proteins mediate the interaction between cell surface receptors and intracellular effectors. Recent studies have shown that human retina and rat brain contain mRNA encoding a novel 40-Kd G protein \( \alpha \) subunit referred to as \( G_{54} \). Studies with an antiserum selective for the predicted sequence of this protein have suggested that a similar protein is present in human platelets and is phosphorylated during platelet activation. To better understand the structure and function of this protein, the present studies examine its sequence in platelets and compare its abundance in human platelets, megakaryocytes, and two megakaryoblastic cell lines, HEL cells and Dami cells. Three different \( G_{54} \)-selective antisera reacted with a 40-Kd protein in platelet membranes. None of these detected a corresponding protein in HEL or Dami cells, despite the presence in both cell lines of proteins recognized by antisera selective for three members of the \( G \) family. Northern blotting with a \( G_{54} \)-specific probe prepared from retinal \( G_{54} \) showed two hybridizing species in platelet RNA: a major band at 3.5 kb and a minor band at 2.2 kb. Both were detectable in HEL and Dami cells, but at greatly reduced levels compared with platelets. RNA encoding \( G_{54} \) was also detected in individual human megakaryocytes by in situ hybridization. The amount present approached that of \( G_{74} \), the most abundant of the \( G \) species present in platelets. The complete sequence of the platelet homolog to \( G_{54} \) was determined from platelet RNA amplified by the polymerase chain reaction. The encoded protein was the same as those obtained in brain and retina. Thus, based on immunoreactivity and nucleotide sequencing, platelets and megakaryocytes contain substantial quantities of a protein identical to brain and retinal \( G_{54} \). The paucity of \( G_{54} \) protein and RNA in the megakaryoblastic cell lines suggests that either there has been a selective loss of the ability to synthesize \( G_{54} \) from these cells or that \( G_{54} \) appears at a later stage in megakaryocyte development than does \( G_{54} \).

\( G \) proteins that are less widely distributed, such as \( G_{o} \), are present in platelets? These have slightly different biochemical properties, but whether each has a unique role in platelet function remains to be established.

The issue of whether or not a protein identical to brain and retinal \( G_{54} \) is present in platelets is of particular interest for several reasons. First, studies with \( G_{54} \)-selective antibod-
ies suggest that a protein of similar size and antigenicity is prevalent in platelets. Second, because it is not a substrate for pertussis toxin, G\textsubscript{q} is a candidate for involvement in those pathways for signal transduction that are resistant to the effects of the toxin. At least one such pathway, the activation of phospholipase C initiated by the arachidonate metabolite thromboxane A\textsubscript{2} (TXA\textsubscript{2}), has been shown to exist in platelets. Finally, our previous studies show that a platelet protein that can be precipitated with G\textsubscript{q}-selective antisera becomes phosphorylated when platelets are activated by thrombin, phorbol esters, or thromboxane analogs such as U46619. The implications of this phosphorylation for signal transduction and platelet activation are unknown.

Although the sequences reported for G\textsubscript{q} in RNA prepared from rat brain and human retina are essentially identical, experience with other forms of G\textsubscript{q} suggests that additional variants of G\textsubscript{q} are likely to exist. Therefore, as one part of the present studies we have determined the complete coding region sequence of platelet G\textsubscript{q} using RNA isolated from human platelets. In addition we have compared the sequence of G\textsubscript{q} and the relative abundance of immunologically detectable G\textsubscript{q} and mRNA encoding G\textsubscript{q} in platelets and two megakaryoblastic cell lines, HEL cells and Dami cells. The results demonstrate the presence of G\textsubscript{q} in all three types of cells, but also show that there are major differences between platelets, megakaryocytes, and the megakaryoblastic cell lines in the abundance of the protein and mRNA.

### MATERIALS AND METHODS

**Miscellaneous.** Dami cells were provided by Drs Sheryl Greenberg and Robert Handin (Brigham & Women’s Hospital, Boston, MA) and grown in Iscove’s Modified Dulbecco’s Media (IMDM; Gibco, Grand Island, NY) supplemented with 10% equine serum. HEL cells were obtained from the American Type Culture Collection and grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum. Bluescript plasmid containing the full-length human retinal G\textsubscript{q} cDNA was kindly provided by Dr H. Hug (California Institute of Technology, Pasadena).

**Membrane preparation.** HEL and Dami cells were resuspended at greater than 10\textsuperscript{7}/mL in 10 mM Tris-HCl, 1 mM MgCl\textsubscript{2}, 5 mM EDTA, 10 mM benzamidine, 20 \mu g/mL leupeptin, 0.1% (vol/vol) aprotinin, and 0.9 mM phenylmethylsulfonyl fluoride (PMSF) at pH 8.0 and then disrupted by 20 to 40 strokes in a Dounce-style homogenizer at 4°C. Greater than 95% cell disruption was confirmed by microscopic examination. Any cell that remained intact were removed from the lysate by low-speed sedimentation (250g for 5 minutes). A membrane pellet was prepared by centrifugation at 31,400g for 15 minutes at room temperature. The top two thirds of the platelet-rich plasma was carefully removed avoiding the buffy coat layer. The platelets were then collected by centrifugation at 1,000g for 15 minutes. The plasma was immediately removed and RNA was extracted from the platelet pellet using a commercial RNA extraction kit (Pharmacia, Piscataway, NJ). To prepare RNA from HEL and Dami cells, cells growing in culture medium were spun at 1,000 rpm for 5 minutes, the culture medium was removed, and the resultant cell pellet processed as described previously for platelets. HEL cell and Dami cell RNA was quantitated by absorption at 260 nm. RNA prepared in this manner was used in Northern blots or reverse transcription reactions. Northern blotting was performed as described, except that the RNA was transferred to Hybond nylon filters (Amersham, Arlington Heights, IL) and prohybridization performed in 0.5 M sodium phosphate pH 7.5, 7% SDS, and 10% dextran sulfate at 65°C. The filter was then washed twice in 2X SSC (0.3 mol/L sodium chloride, 0.03 mol/L sodium citrate, pH 7.4) at room temperature for 5 minutes, then twice in 2X SSC/1% SDS at 65°C, and finally 0.1X SSC/1% SDS at room temperature. A G\textsubscript{q} specific cDNA probe was made from the terminal 594 bp of the 3’ untranslated region using a unique Pst I restriction site in the human retinal G\textsubscript{q} cDNA.

**Oligonucleotides.** Oligonucleotides for the polymerase chain reactions (PCR) were designed based on the published sequence of G\textsubscript{q} from human retina. Regions of the G\textsubscript{q} subunit with low homology compared with the three known G\textsubscript{q} subtypes, namely G\textsubscript{q14}, G\textsubscript{q16}, and G\textsubscript{q2}, were selected for the synthesis of primer. All of the oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer (Foster City, CA) using standard phosphoramidite chemistry. The sequence and position of each of the primers is shown in Fig 1.

**PCR.** Total RNA from HEL cells, Dami cells, and platelets was prepared as described previously. Approximately 100 ng of HEL cell and Dami cell RNA or platelet RNA made from 50 mL of

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Amino Acid No.</th>
<th>Sequence</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>8730</td>
<td>346-355*</td>
<td>KNLKDQGFL</td>
<td>G\textsubscript{q},G\textsubscript{q},G\textsubscript{q}</td>
</tr>
<tr>
<td>2919</td>
<td>111-125*</td>
<td>TGOAEKGEITPELL</td>
<td>G\textsubscript{q}</td>
</tr>
<tr>
<td>2921</td>
<td>346-355*</td>
<td>QNLKYGLGC</td>
<td>G\textsubscript{q}</td>
</tr>
<tr>
<td>6354</td>
<td>20-291*</td>
<td>RSESSQRRRE</td>
<td>G\textsubscript{q}</td>
</tr>
</tbody>
</table>

*Data from Jones and Reed.\textsuperscript{28} Data from Fong et al.\textsuperscript{29}
whole blood was used for first-strand synthesis. First-strand synthesis and PCR were performed according to Newman et al.\(^8\) except that 200 ng of the antisense primer was used in the first strand reaction. For PCR, 300 ng of sense primer and 100 ng of antisense primer were used. Reactions with no RNA were routinely performed to exclude possible contamination. A temperature cycling routine in a Perkin Elmer Thermocycler (Perkin Elmer/Cetus, Norwalk, CT) was used that consisted of 35 cycles of denaturation at 94°C for 1.5 minutes, annealing at 60°C for 1.5 minutes, and extension at 72°C for 3 minutes. The amplification reaction was followed by a single incubation at 72°C for 7 minutes to complete the extensions. To boost the yield of the desired PCR product we performed "nested" reactions that consisted of using the same sense primer but substituting the previously used antisense primer with an antisense primer internal to the amplified sequence for the second round of amplification. For these nested reactions, 3 μL of the first PCR reaction was used. Reaction conditions were the same as for the first round of PCR. The desired PCR product was size-fractionated on agarose gel, isolated using an IBI UEA electrophoresis apparatus (New Haven, CT), and quantitated by ethidium bromide staining. PCR resulted in two fragments of the coding region +1 to +684 and +643 to +1075. Both were ligated into pUC18 and sequenced with T7 polymerase by the dideoxy chain termination technique of Sanger et al.\(^9\) (Pharmacia).

Megakaryocytes: Isolation and in situ hybridization. Human megakaryocytes were isolated from normal bone marrow specimens and processed as previously described.\(^1\) In situ hybridization was performed with the Psr 1-derived 3'-untranslated region G\(_m\) cDNA sequence of human retinal G\(_m\) and obtained from amplification of platelet and HEL cell RNA. The sequence of the primers used for amplification is underlined. Above the sequence data is a schematic that shows the location and direction of amplification of platelet and HEL cell RNA. The sequence of the G\(_m\) coding region sequence that was identical to the published sequence of human retinal G\(_m\). Shown is the full-length sequence of G\(_m\) from both platelet RNA and HEL cell RNA and the location and direction of amplification of platelet and HEL cell RNA. The sequence of the primers used for amplification is underlined. Above the sequence data is a schematic that shows the location and direction of amplification of the primers in relation to the full-length G\(_m\) sequence. The primers used in PCR were based on the published sequence of human retinal G\(_m\), and were designed to produce two amplified fragments representing the entire coding region. Three primers were used to obtain a nested reaction product consisting of the first 683 bp of coding region. These were +1, +643 antisense (+643a), and +708. The second reaction product of the final 425 bp was gained by the use of primers +643 sense (+643s), +1036, and +1246. Each amplified fragment was isolated from both platelet RNA and HEL cell RNA and was ligated into pUC18. Sequencing of the two fragments from both platelet RNA and HEL cell RNA resulted in a complete coding region sequence that was identical to the published sequence for human retinal G\(_m\).
probe described previously and a cDNA probe selective for \( G_{\alpha} \) that was described previously. Both probes were oligolabeled with biotin-11-dUTP (Bethesda Research Laboratories, Gaithersburg, MD). cDNA-RNA hybrids were detected using a DNA Detection Kit (Bethesda Research Laboratories) by the sequential addition of streptavidin, biotinylated alkaline phosphatase, and the chromogens nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolylphosphate. Positive reactions consisted of a purple-blue to deep brown-colored precipitate in the cell’s cytoplasm. Biotin-11-dUTP-labeled pBR322 (Promega, Madison, WI) and biotin-11-dUTP-labeled cDNA encoding human \( \beta\)-actin were used as negative and positive controls, respectively.

RESULTS

**Northern analysis.** Figure 2 shows a Northern analysis of platelet RNA probed with a 594-bp fragment from the 3’ untranslated region of human retinal \( G_{\alpha} \), a region selected because of its low homology with other forms of \( G_{\alpha} \). By autoradiography two hybridizing species are present: a major band at 3.5 kb and a minor band at 2.2 kb. These two species are comparable in size to the two species detected in brain and retinal RNA, and are thought to arise from alternative splicing of a single gene product. Figure 2 also shows the results that were obtained with RNA from HEL cells and Dami cells: two human leukemic cell lines that are known to synthesize proteins characteristic of the megakaryocyte/platelet lineage, including several membrane glycoproteins and secretory proteins, and to possess functional receptors for platelet agonists such as thrombin, epinephrine, and TXA2. Northern analysis of total RNA isolated from both cell lines shows the same two hybridizing species that are present in total platelet RNA, but the extent of reactivity with similar quantities of RNA (determined by ethidium bromide staining) gel is far less. This finding suggests that although RNA encoding \( G_{\alpha} \) is detectable in the cell lines, the amount is much lower than in platelets.

**In situ hybridization with human megakaryocytes.** Because Northern analysis demonstrated the presence of \( G_{\alpha} \) message in human platelets, in situ hybridization studies were used to examine the expression of \( G_{\alpha} \) in normal human megakaryocytes. Two different probes were used. One, which was specific for \( G_{\alpha} \), was comprised of the same fragment from the 3’ untranslated end of the retinal \( G_{\alpha} \) cDNA that was used for Northern analysis. The second probe consisted of the corresponding 3’ untranslated region of \( G_{\alpha} \), the most abundant of the three forms of \( G_{\alpha} \) present in megakaryocytes and platelets. Both probes were biotinylated to permit detection by a strepavidin-biotin-alkaline phosphatase coupled reaction. A probe for pBR322 was used as a negative control; one for actin was used as a positive control. The results are shown in Fig 3. Panel A shows the minimal signal obtained with the pBR322 probe. Panel B demonstrates reactivity of the megakaryocytes with the \( \beta\)-actin probe. The reactions with the \( G_{\alpha} \) and \( G_{\alpha2} \) probes are shown in panels C and D. The extent of the reaction with the \( G_{\alpha} \)-specific probe approached that of the \( G_{\alpha2} \), indicating that human megakaryocytes contain mRNA for both in roughly similar amounts. Under the same conditions, HEL cells also gave a positive reaction with the \( G_{\alpha2} \) probe, but \( G_{\alpha} \) reactivity was not detectable (not shown).

**Immunotransfer blotting of a \( G_{\alpha2} \)-like species in human platelets.** A set of rabbit antisera were prepared against peptides whose sequence was specific for \( G_{\alpha} \) amongst the known forms of \( G_{\alpha} \). The sequences of the peptides that were used to prepare each of these antisera are shown in Table 1. The selectivity of each of the antibodies was confirmed with recombinant \( G_{\alpha1}, G_{\alpha1}, G_{\alpha3}, \) and \( G_{\alpha3} \). Three of these antisera, referred to as 2919, 2921, and 6354, were prepared against peptide sequences from three widely separated regions of \( G_{\alpha1} \). Based on immunotransfer (Western) blotting, these antisera react with recombinant \( G_{\alpha1} \) but not with any of the recombinant forms of \( G_{\alpha} \) (not shown). An additional antiserum, referred to as 8730, was raised against a peptide sequence that is similar in all three forms of \( G_{\alpha} \), but not present in \( G_{\alpha1} \). This antibody recognizes recombinant \( G_{\alpha1} \) and \( G_{\alpha2} \), and, to a lesser extent, recombinant \( G_{\alpha3} \). It does not recognize recombinant \( G_{\alpha1} \).

Figure 4 shows the results of immunotransfer blotting...
Fig 3. In situ hybridization of isolated human megakaryocytes. Representative photomicrographs are shown of normal human megakaryocytes that were probed for the expression of G$_a$ and G$_m$ mRNA by hybridization with biotin-11-dUTP-labeled cDNA probes. The large cells are megakaryocytes. The smaller cells are from other hematopoietic cell lines. A positive signal is indicated by a purple-brown precipitate in the cytoplasm of the cell. Panel A shows the minimal signal obtained with a probe for pBR322. Panel B shows the signal obtained with a probe for $\beta$-actin, used as a positive control. Panel C and D show the results obtained with the probes for G$_a$ and G$_m$, respectively.

with these antisera using membranes prepared from platelets, HEL cells, and Dami cells. All three contained a 41-Kd protein that was immunoreactive with the G$_m$-selective antiserum, 8730. Platelets also contained a 40-Kd protein that was immunoreactive with the G$_m$-selective antiserum, 2921 and 6354 (Fig 4), as well as a third G$_m$-selective antiserum, 2919, that isn’t shown. Preincubation of antisera 2921 and 6354 with their corresponding peptides, but not with an unrelated peptide, caused the 40-Kd band of immunoreactive protein present in platelets to disappear. The additional bands of immunoreactivity seen in Fig 4, many of which were also present with the preimmune antisera, were unaffected (not shown). Therefore, these are unlikely to represent other, previously unsuspected forms of G$_m$.

These results confirm the presence of immunologically recognizable G$_m$ in platelets and suggest that the platelet protein is sufficiently similar to retinal and brain G$_m$ to share three widely spaced epitopes. A different result was obtained with the HEL cells and Dami cells. Although both cell lines contained readily detectable amounts of G$_m$, neither contained protein that was specifically immunoreactive with the G$_m$-selective antisera (Fig 4), even when the amount of membrane protein used in the analysis was increased by 50%. This finding suggests that HEL and Dami cells express insufficient amounts of G$_m$ to be detected in this fashion, despite the fact that they contain small amounts of RNA encoding G$_m$. In a separate experiment, HEL cells were incubated for 48 hours with 1 nmol/L, 10 nmol/L, and 100 nmol/L phorbol 12-myristate 13-acetate (PMA) to determine whether differentiation would induce the expression of G$_m$. The treated HEL cells displayed clear morphologic changes in comparison to cells treated with vehicle. However, incubation with PMA failed to induce the expression of G$_m$ at any of these concentrations as determined by Western blotting (not shown). The level of immunoreactive G$_m$ detected with antisera 8730 was unaffected.

Sequence of G$_m$ in platelets and HEL cells. The complete sequence of platelet and HEL cell G$_m$ was obtained after amplifying total RNA from these sources by PCR. The oligonucleotide primers that were used were based on the human retinal G$_m$ sequence (Fig 1). Details of the sequencing strategy are given in the figure legend. Within the coding region, the sequence obtained from platelets and HEL cells is identical to human retinal G$_m$. 
Table 1. Preincubation of antisera 2921 and 6354 with their corresponding cells were subjected to SDS-PAGE followed by immunotransfer blotting with peptide-directed antisera of the peptide sequences used to prepare the antisera are shown in G, (2921,6354), or the respective preimmune sera (PI). The locations showing that the effects of agonists on phospholipase C and adenylyl cyclase can be inhibited by preincubating the membranes. Membranes (100 µg) from platelets, HEL cells, and Dami cells were subjected to SDS-PAGE followed by immunotransfer blotting with peptide-directed antisera (I) selective for Gα (8730) and Gα (2921,6354), or the respective preimmune sera (PI). The locations of the peptide sequences used to prepare the antisera are shown in Table 1. Preincubation of antisera 2921 and 6354 with their corresponding peptides, but not with an unrelated peptide, caused the 40-Kd band of immunoreactivity to disappear. The additional bands of immunoreactivity were unaffected.

Fig 4. Immunotransfer blotting of platelet, HEL, and Dami cell membranes. Membranes (100 µg) from platelets, HEL cells, and Dami cells were subjected to SDS-PAGE followed by immunotransfer blotting with peptide-directed antisera (I) selective for Gα (8730) and Gα (2921,6354), or the respective preimmune sera (PI). The locations of the peptide sequences used to prepare the antisera are shown in Table 1. Preincubation of antisera 2921 and 6354 with their corresponding peptides, but not with an unrelated peptide, caused the 40-Kd band of immunoreactivity to disappear. The additional bands of immunoreactivity were unaffected.

DISCUSSION

The identification of Gα in platelets began with studies showing that the effects of agonists on phospholipase C and adenylyl cyclase can be inhibited by preincubating the platelets with phorbol esters. Initially, this inhibition was attributed to the phosphorylation of Gα by protein kinase C. However, subsequent studies from our laboratories using Gα-selective antisera suggested that the G protein which is phosphorylated in platelets by protein kinase C is not Gα, but a protein immunologically related to Gα. Therefore, the first goal of the present study was to determine whether the Gα-like protein in platelets, “Gα(plt)”, was identical to retinal and brain Gα, or represented a new member of the Gα family. To this end, additional antisera were prepared against peptide sequences unique to Gα among the known forms of Gα. The domains selected were distributed across the length of Gα. Western blots with each of these shows a 40- to 41-Kd immunoreactive protein in platelet membranes. Northern blots of platelet RNA using a portion of the 3' untranslated end of retinal Gα show two reactive species that were comparable in size with those reported in other tissues. The same probe also bound to megakaryocytes in an in situ hybridization assay.

These results suggest that Gα(plt) must be, at the very least, closely related to Gα. Sequence analysis of the amplified product of platelet RNA by PCR using primers derived from retinal Gα was identical to that published for Gα, including the region of the molecule near the N-terminus containing the serine residue that is thought to be the site for phosphorylation by protein kinase C. This does not, of course, exclude the presence in megakaryocytes and platelets of additional, still unidentified members of the Gα family. However, so far analysis of genomic DNA suggests that there is only one copy of the Gα gene per haploid genome and that a splice variant probably accounts for the presence of the two mRNA species seen on Northern blots.

Expression of Gα. Having established that platelets contain a form of Gα identical to that present in retina and brain, our second goal was to compare the expression of Gα in platelets, mature megakaryocytes, and two leukemic cell lines, HEL cells and Dami cells. Both of these cell lines have been shown to produce megakaryocyte-specific proteins, including several characteristic membrane glycoproteins and secretory proteins. In addition, HEL cells have been shown to respond to platelet agonists and have proven to be a useful model system for the study of the structure and function of platelet proteins. When tested with a Gα probe, RNA from both cell lines contained the same two hybridizing species present in platelets. However, the reactivity of HEL cells and Dami cell RNA was considerably less than that obtained with a similar amount of platelet RNA and neither cell line displayed detectable immunoreactivity with the Gα-selective antisera. These results suggest that relatively small amounts of mRNA encoding Gα are present in HEL cells and Dami cells and that little, if any, protein is produced from it. This finding contrasts with the results of the present and previous studies, which show that HEL cells contain readily detectable amounts of Gα. PCR was used to amplify HEL cell RNA using Gα-directed primers. As in platelets, the sequence obtained was identical to that for retinal and brain Gα.

The differences between the results obtained with platelets, HEL cells, and Dami cells may reflect differences in the biology of Gα and the Gα family members in megakaryocytes. Insufficient numbers of purified human megakaryocytes were available to permit direct comparisons of the amount of Gα RNA and protein present in megakaryocytes and the two cell lines. However, when HEL cells and mature megakaryocytes were compared by in situ hybridization, RNA encoding Gα was readily detectable in megakaryocytes, but not in HEL cells. Gα was readily detectable in both.

There are several possible explanations for this difference between mature megakaryocytes and HEL and Dami cells. One is that Gα is produced at a later stage in megakaryocyte development than the Gα family members. If so, then the megakaryoblastic cell lines may reflect a
stage of development in which $G_a$ but not $G_{ar}$ is produced. Incubation with phorbol ester, which has been reported to alter the level of expression of other proteins in HEL cells, had no effect on the expression of $G_a$. For now, the function of $G_{ar}$ remains unknown, as does the effect of phosphorylation. The fact that $G_{ar}$ which has a generally limited tissue distribution, is abundant in platelets suggests that it performs an important role. This may prove to be a role in the regulation of signal transduction during platelet activation. If so, then the process regulated should be resistant to the effects of pertussis toxin because $G_{ar}$ lacks the consensus sequence for ADP-ribosylation by the toxin. A candidate pathway may be the activation of phospholipase C by Tx$\alpha$, a process known to be pertussis toxin resistant. However, for now, this remains speculative.

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REFERENCES


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