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Specificity of Gα_q and Gα_{11} gene expression in platelets and erythrocytes

Expressions of cellular differentiation and species differences

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Gα_q and Gα_{11}, members of the G_q family of G-proteins, transduce signals from receptors to the β isoenzymes of phosphatidylinositol-specific phospholipase C (PI-PLC). The receptor specificity of these α subunits is unknown. Gα_q and Gα_{11} are ubiquitously expressed in tissues; however, there have been conflicting reports of the presence or absence of Gα_{11} protein in haematopoietic cells. Platelet thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors activate PI-PLC via Gα_q, but the role of Gα_{11} is uncertain. To define their roles in platelet activation we studied Gα_q and Gα_{11} gene expression by immunotransfer blotting and by reverse transcription of mRNA followed by PCR (RT–PCR) and direct sequencing. An antiserum specific for mouse Gα_{11} failed to identify Gα_{11} in dog or human platelets or in dog liver, a tissue known to contain Gα_{11}. RT–PCR performed with gene-specific primers demonstrated Gα_q mRNA, but not Gα_{11} mRNA, in normal human and mouse platelets and in thromboxane-sensitive and thromboxane-insensitive dog platelets. Studies of mouse and dog liver and human retina confirmed that the cDNA, primers and probes used could amplify and recognize Gα_{11} in other tissues. However, species-specific oligonucleotide primers and probes were essential to demonstrate Gα_{11}, but not Gα_q, mRNA. Compared with mouse cDNA, dog and human Gα_{11} cDNA had twice as many nucleotide substitutions (approx. 12% compared with approx. 6%) as Gα_q. Gα_q mRNA was also found in mature erythrocytes but Gα_{11} mRNA was not identified, whereas both Gα_q and Gα_{11} mRNAs were found in bone marrow stem cells. Therefore Gα_{11} gene expression in haematopoietic cells is linked with cellular differentiation. The lack of Gα_{11} indicates that signal transduction from platelet TXA_2/PGH_2 receptors to PI-PLC occurs via Gα_q, and that Gα_{11} deficiency is not responsible for defective activation of PI-PLC in thromboxane-insensitive dog platelets. Despite the high degree of similarity that exists between Gα_q and Gα_{11}, significantly greater species-specific variation in nucleotide sequence is present in Gα_{11} than in Gα_q. Cellular specificity and species specificity are important characteristics of these G_q family G-proteins.

INTRODUCTION

Activation of phosphatidylinositol-specific phospholipase C (PI-PLC) is an important signal transduction mechanism linking cell surface receptors to intracellular effectors [1]. Several membrane receptors are linked to the β isoenzymes of PI-PLC by Gα_q or Gα_{11}, members of the G_q family of G proteins, but the specificity of these α subunits for individual receptor types has not been determined [2–7]. Thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors of human and dog platelets were demonstrated to be linked to PI-PLC by Gα_q/Gα_{11} (Gα_q, Gα_{11} or both, identified by antisera directed toward their common C-terminus) [8–10] and by Gα_q with the use of specific antisera [10]. Platelet TXA_2/PGH_2 receptors were also found to co-purify with proteins of the G_q family [11]. These studies indicated that signal transduction from TXA_2/PGH_2 receptors to PI-PLC involved Gα_q, but they did not define the role of Gα_{11}.

Gα_q and Gα_{11} have a high degree of sequence similarity [12]. Antibodies to the limited number of unique regions of Gα_q and Gα_{11} have differentiated these very similar α subunits in some studies [4,13–15]. Electrophoretic separation and immunoblotting with antisera specific for Gα_q demonstrated the presence of Gα_q protein, but not Gα_{11} protein, in human platelets [16]. These studies were performed without Gα_{11}-specific antisera, so they did not completely exclude the presence of Gα_{11}. Other studies performed with antisera considered specific for Gα_{11} also failed to demonstrate its presence in human platelets [17]. However, the antisera used to distinguish Gα_q from Gα_{11} have been reported to have variable specificity [13,15,18–20], especially in studies performed in different species [19,20]. Antibodies made to peptides synthesized from mouse Gα_{11} oligonucleotide sequences might not recognize Gα_{11} protein in other species because of species-specific amino acid variability. In addition the studies that failed to detect Gα_{11} in platelets also failed to identify Gα_{11} protein in Raji cells [16], despite prior reports of high levels of Gα_{11} mRNA in multiple B-cell lines [21]. Therefore negative immunotransfer blotting studies did not prove that Gα_{11} was absent from platelets. Furthermore other investigators reported that both human platelets and the megakaryocyte cell line MEG-01 contained Gα_{11} protein, the levels of which did not change when differentiation was induced [22].

The uncertainty about the expression of Gα_{11} in platelets has not been clarified by previous molecular studies. Both Gα_q and Gα_{11} mRNA were found to be ubiquitously expressed in murine tissues and many cell lines, including several of haematopoietic origin, although platelets were not evaluated in these studies

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Abbreviations used: DTT, dithiothreitol; Gα_q/Gα_{11}, Gα_q, Gα_{11} or both, identified by antisera directed toward their common C-terminus; TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2; PI-PLC,phosphatidylinositol-specific phospholipase C; RT, reverse transcription of mRNA; TXA_2^+ platelets, TXA_2-sensitive dog platelets; TXA_2^- platelets, TXA_2-insensitive dog platelets.

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The nucleotide sequences reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers L43134 (dog Gα_{11}) and L76256 (human Gα_q).

[12,21]. A recent report of studies of platelet G-protein α subunit mRNAs by reverse transcription of mRNA followed by PCR (RT–PCR) failed to identify $G\alpha_{11}$ mRNA in platelets; however, $G\alpha_q$ mRNA was also not detected [23]. The human leukaemic cell line K562, which has the potential to differentiate into megakaryocytic or erythroid precursors, was found to contain both $G\alpha_q$ and $G\alpha_{11}$ mRNAs [24]. Therefore neither antisera nor molecular studies conclusively established the presence or absence of $G\alpha_{11}$ in platelets.

We previously described impaired activation of PI-PLC in dog platelets that demonstrate minimal aggregation and secretion in response to TXA_2 analogues [10]. These TXA_2-insensitive (TXA_2^-) dog platelets contained $G\alpha_q$ in quantities comparable to those observed in normal human platelets and in TXA_2-sensitive (TXA_2^+) dog platelets; i.e. platelets from other strains of dogs that aggregate and secrete in response to TXA_2 [10,25]. Our studies indicated that signal transduction from TXA_2/PGH_2 receptors to PI-PLC was impaired in TXA_2^- dog platelets [10]. We postulated that the G_q family G-protein involved in this signalling pathway was dysfunctional in dog platelets. Although it was likely that this G-protein was $G\alpha_q$, it was essential that this be established by determining whether or not $G\alpha_{11}$ was present in platelets. Moreover the apparent impairment of function in the G_q family G-protein provided an opportunity to study the functional consequences of expression of these G-protein genes.

Thus the goals of this study were (1) to identify the products of $G\alpha_q$ and $G\alpha_{11}$ gene expression in platelets, (2) to determine the functional significance of this expression in human and TXA_2^+ and TXA_2^- dog platelets, and (3) to compare.the expression of $G\alpha_q$ and $G\alpha_{11}$ in platelets with that in other haematopoietic cells. To accomplish these goals we evaluated $G\alpha_{11}$ protein by immunotransfer blotting, and $G\alpha_q$ and $G\alpha_{11}$ mRNA by RT–PCR, hybridization with sequence-specific oligonucleotide probes and direct sequencing.

MATERIALS AND METHODS

Subjects

Venous blood was obtained from normal human subjects who had not ingested any medications within the previous week, and from unanaesthetized dogs by previously published methods [10]. Studies were performed on platelets from dogs with either TXA_2^+ or TXA_2^- platelets [10]. Mouse platelets were obtained by aortic puncture from NIH Swiss mice anaesthetized with pentobarbital. These studies were approved by the Human Studies and Animal Studies Subcommittees of the Research Committee of the Minneapolis VA Medical Center. Written, informed consent was obtained from human subjects.

Cell and tissue preparation

Platelet-rich plasma was prepared from citrate-anticoagulated blood [10] containing 50 ng/ml PGE_1 by centrifugation at 200 ***g*** for 15 min at 23 °C, and washed twice in buffer containing 96.5 mM sodium chloride, 85.7 mM glucose, 1.1 mM EDTA, 8.5 mM Tris, pH 7.4, and 50 ng/ml PGE_1.

Dog liver tissue was removed from an anaesthetized animal before killing, and kept frozen at −70 °C until the RNA was isolated.

Dog and human erythrocytes were separated from 20 ml of sodium heparin-anticoagulated whole blood by filtration through a column of glass beads [26] to remove more than 95 % of the platelets, followed by a column of α-cellulose/microcrystalline cellulose [27] that removed the leucocytes. These procedures removed all platelets and leucocytes as determined by automated particle counting (Coulter counter model T890) as well as by direct light-microscopic examination of multiple Wright's stained smears.

Human lymphocytes, provided by Dr. Robert Perri (VA Medical Center, Minneapolis, MN, U.S.A.), were separated from whole blood by Ficoll/Hypaque centrifugation followed by monocyte removal by adherence to tissue culture flasks [28].

Human bone marrow stem cells, provided by Dr. Ravi Bhatia (University of Minnesota, Minneapolis, MN, U.S.A.), were obtained from normal human volunteers. Lineage-negative cells were isolated and a $CD34^+$ $HLA\text{-}DR^+$ population was separated by flow cytometry as described [29].

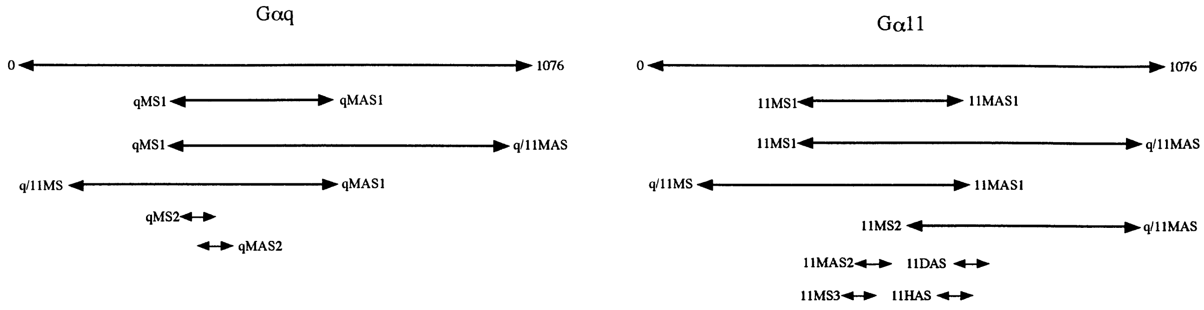
Immunotransfer blotting

The presence of $G\alpha_{11}$ protein was evaluated with SDS/PAGE in the presence of 6 M urea by the method of Milligan et al. [16]. The antiserum used was AS 255 [30], provided by Dr. K. Spicher (Institut für Pharmakologie, Freie Universität Berlin, Berlin, Germany). The immunizing peptide used for AS 255 was (C)VTTFEHQYVNAIK [30]. $G\alpha_q/G\alpha_{11}$ antiserum N345 was generated at HRP (Denver, PA, U.S.A.) by using the KLH-conjugated peptide (C)KDTILQLNLKEYNLV.

RNA isolation, amplification and analysis

Total RNA was isolated from tissues by using a commercial kit (Stratagene, La Jolla, CA, U.S.A.). This method is similar to that of Newman et al. [31]. In brief, washed cells were solubilized (5×10^8 platelets per 0.1 ml, 9.3×10^9 erythrocytes per ml, 10^6 stem cells per ml of denaturing solution), extracted twice and precipitated. Dog liver was thawed, minced and homogenized (1 g of tissue per 10 ml of denaturing solution), extracted twice and precipitated. The amount of RNA was measured by A_{260}/A_{280} comparison. The yield of RNA was 4.4 μg from the platelets (approx. 4×10^9) in 50 ml of blood, 11.4 μg from 2 ml of washed, packed erythrocytes, 3.5 μg from 2×10^6 stem cells and 34.6 μg from 0.38 g of dog liver. Mouse liver QUICK-Clone cDNA, human retinal QUICK-Clone cDNA and mouse liver total RNA were obtained from Clontech Laboratories (Palo Alto, CA, U.S.A.).

First-strand cDNA species were synthesized from random hexamers or specific antisense oligonucleotides, derived from published mouse gene sequences (Scheme 1), and recombinant reverse transcriptase (Promega, Madison, WI, U.S.A.) by minor modifications of methods previously described [31,32]. Total RNA (approx. 1 μg) was heated to 68 °C for 10 min and cooled rapidly before adding cDNA buffer [50 mM Tris, pH 8.3, containing 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 0.5 mM dNTP], 1 μM gene-specific antisense primer or random oligonucleotides, 0.25 (manufacturer's) unit of RNase inhibitor, and 10 (manufacturer's) units/μl recombinant reverse transcriptase (MMLV). DNA synthesis was performed at 42 °C for 60 min and stopped by cooling to 4 °C. Amplification of $G\alpha_q$ or $G\alpha_{11}$-specific sequences was performed by PCR [31]. Gene-specific primers (Scheme 1) were added to the PCR mixture (0.5–1.0 μM final concentration) containing 44 mM KCl, 15 mM Tris, 1.5 mM $MgCl_2$, 0.01 % gelatin, 0.025 % Triton X-100, 0.125 mM dNTP and 2.5 mM DTT with 10–25 μl of cDNA, and heated to 94 °C for 5 min. *Taq* polymerase (Promega, Madison, WI, U.S.A.) (5 units; 1 unit incorporates 10 nmol of dNTPs into acid-insoluble material in 30 min at 74 °C) was added, and the samples were overlaid with mineral oil. Amplification was performed using the 'step–cycle' programme on a Thermal Cycler (Perkin-Elmer–Cetus, Norwalk, CT, U.S.A.) set to denature at 94 °C for 1 min, anneal at 50 °C for 0.5 min and extend



Gαq Specific Oligonucleotides

Mouse

qMS1 (359-384)
qMS2 (408-426)

qMAS1 (683-702)*
qMAS2 (535-557)

Gαq/Gα11 Common Oligonucleotides

Mouse

q/11MS (114-130)
q/11MAS (1044-1067)

Gα11 Specific Oligonucleotides

Mouse

11MS1 (362-387)
11MS2 (644-670)
11MS3 (486-504)

11MAS1 (686-701)‡
11MAS2 (585-602)

Dog

11DAS (699-722)§

Human

11HAS (686-701)

S= sense; AS= antisense

* Designated GQ4 in Ref. 21
‡ Designated CT106 in Ref. 21
§ Derived from dog liver sequence

Scheme 1 Primers and probes used to amplify and identify Gα$_q$ and Gα$_{11}$

at 72 °C for 1.5 min for 35 cycles, with final extension at 72 °C for 10 min. Minor variations on this basic protocol were employed in some experiments. PCR products were analysed on 2 % (w/v) agarose gels, stained with ethidium bromide and then transblotted onto Zeta-probe GT membranes (Bio-Rad, Hercules, CA, U.S.A.). After they had been fixed with 0.4 M NaOH, the blots were cross-linked with a UV Stratalinker 1800 (Stratagene). Blots were hybridized with radiolabelled probes for Gα$_{11}$ or Gα$_q$ in QuikHyb (Stratagene) at the probes' $T_m - 5$ °C for more than 1 h. Blots were washed twice in 2 × SSC/0.1 % SDS (where SSC is 0.15 M NaCl/0.015 M sodium citrate) for 15 min at 22 °C and in 0.1 × SSC/0.1 % SDS for 30 min at $T_m - 5$ °C. Films were exposed with intensifying screens at −70 °C.

Oligonucleotides (Scheme 1) were synthesized by the University of Minnesota MicroChemical Facility with an Applied Biosystems Inc. (Foster City, CA, U.S.A.) 394 DNA synthesizer and purified by HPLC. They were evaluated by the computer primer programs OLIGO (National Biosciences, Plymouth, MN, U.S.A.) and/or PRIMER (Scientific and Educational Software, State Line, PA, U.S.A.). Primers for RT–PCR of leucocyte HLA-DQB RNA [23] were provided by Dr. G. van Willigen (Department of Haematology, University Hospital, Utrecht, The Netherlands).

Probes were radiolabelled by using 3′-end labelling of the specified oligonucleotide in reaction with [α-^{32}P]dCTP and terminal deoxynucleotidyl transferase (Promega, Madison, WI, U.S.A.) in 100 mM cacodylate buffer (pH 6.8) containing 1 mM $CoCl_2$ and 0.1 mM DTT for 30 min at 37 °C. Unreacted [α-^{32}P]dCTP was removed from the radiolabelled probe by use of a CHROMA SPIN-10 column (Clontech, Palo Alto, CA, U.S.A.).

Sequencing

PCR samples with sequence primers to yield sense and anti-sense sequence were reacted by using the PRISM® Ready Reaction DyeDeoxy® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.). Sequencing gels were run by the University of Minnesota MicroChemical Facility with an Applied Biosystems 373A DNA Sequencing System.

RESULTS

Urea gel electrophoresis of proteins obtained from rat brain and dog liver revealed two bands that were identified as Gα$_q$/Gα$_{11}$ by immunotransfer blotting with an antiserum (N345) directed against the common C-terminus of Gα$_q$ and Gα$_{11}$ (Figure 1). The

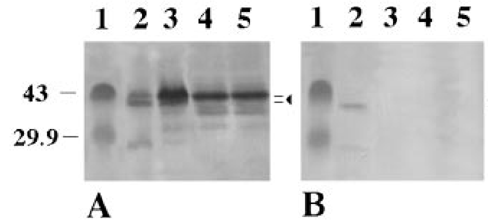


Figure 1 Identification of Gα$_q$ and Gα$_{11}$ by immunotransfer blotting

Urea/SDS/PAGE gel (6 M urea, 11% gel) electrophoresis of proteins obtained from the designated tissues followed by incubation with (**A**) Gα$_q$/Gα$_{11}$ antiserum N345 or (**B**) Gα$_{11}$ antiserum AS 255, and identification by HRP-DAB (Bio-Rad) development. Gα$_q$ is the upper band indicated by the arrowhead and Gα$_{11}$ is the lower band. (**A**) Lane 1, molecular mass standards (shown at the left in kDa); lane 2, dog liver; lane 3, rat brain; lane 4, human platelets; lane 5, TXA_2^- dog platelets. (**B**) Lane 1, molecular mass standards; lane 2, rat brain; lane 3, dog liver; lane 4, human platelets; lane 5, TXA_2^- dog platelets. Note that the lane orders in (**A**) and (**B**) are different. Protein concentrations on both blots were: human platelets, 104 μg of cellular protein; TXA_2 dog platelets, 87 μg of cellular protein; dog liver, 150 μg of cellular protein; rat brain, 37 μg of membrane protein.

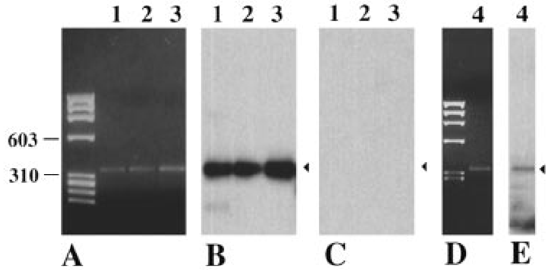


Figure 2 Identification of $G\alpha_q$ but not $G\alpha_{11}$ in platelet RNA

(**A**) Ethidium bromide-stained 2% agarose gel of PCR products derived from qMAS1 cDNA and oligonucleotide primers qMS1 and qMAS1. RNA derived from: lane 1, human platelets; lane 2, TXA_2^+ dog platelets; lane 3, TXA_2^- dog platelets. The positions of 603 and 310 bp fragments are shown at the left. (**B**) PCR products in (**A**) hybridized with radiolabelled probe qMAS2. (**C**) PCR performed with 11MAS1 cDNA and oligonucleotide primers 11MAS1 and 11MS1, and probed with radiolabelled oligonucleotide 11MAS1. RNA derived from: lane 1, human platelets; lane 2, TXA_2^+ dog platelets; lane 3, TXA_2^- dog platelets. (**D**) Ethidium bromide-stained 2% agarose gel of PCR product derived from commercial mouse liver cDNA and oligonucleotide primers 11MS1 and 11MAS1 (lane 4). (**E**) PCR product in (**D**) hybridized with radiolabelled probe 11MAS1.

studies of Milligan et al. [14,16] indicated that the two proteins observed were $G\alpha_q$ and $G\alpha_{11}$. Immunotransfer blotting with an antiserum (AS 255), reported to recognize $G\alpha_{11}$ [30], successfully identified $G\alpha_{11}$ in rat brain (Figure 1). Antiserum AS 255 failed to identify $G\alpha_{11}$ protein in human and dog platelets; however, it also failed to identify $G\alpha_{11}$ in dog liver (Figure 1). Because liver contains $G\alpha_{11}$ [12,14,20], this result indicated that the previously reported absence of $G\alpha_{11}$ from platelets required confirmation by alternative methods. We then evaluated platelets for the presence of $G\alpha_{11}$ and $G\alpha_q$ mRNA.

The PCR products obtained from dog TXA_2^+, TXA_2^- and human platelet RNA by using mouse $G\alpha_q$-specific cDNA (qMAS1 cDNA) and $G\alpha_q$-specific primers qMS1 and qMAS1 (Scheme 1) were of the appropriate size (approx. 343 bp) on ethidium bromide-stained agarose gels (Figure 2A). When these products were transblotted and probed with the radiolabelled $G\alpha_q$-specific oligonucleotide qMAS2, hybridization occurred (Figure 2B). When an analogous experiment was conducted with mouse $G\alpha_{11}$-specific cDNA (11MAS1 cDNA) and $G\alpha_{11}$-specific primers 11MS1 and 11MAS1 (Scheme 1), no appropriately sized PCR product was observed and hybridization with radiolabelled 11MAS1 did not occur (Figure 2C). Mouse liver cDNA [oligo(dT) primed] yielded a product of appropriate size (Figure 2D) that hybridized with 11MAS1 (Figure 2E). Similar results were obtained when cDNA was generated from random hexamers (results not shown). $G\alpha_{11}$ message was not identified in human platelets, TXA_2^+ dog platelets or TXA_2^- dog platelets.

As an alternative approach we generated first-strand cDNA with an oligonucleotide (q/11MAS) common to both $G\alpha_{11}$ and $G\alpha_q$. PCR performed on human and dog platelet q/11MAS cDNA with two sets of $G\alpha_{11}$-specific primers failed to generate products of appropriate size, and no hybridization was observed with the $G\alpha_{11}$-specific probes 11MAS1 (Figure 3B) and 11MS1 (Figure 4B). However, when PCR was performed concurrently with the same preparation of q/11MAS cDNA and $G\alpha_q$-specific primers, PCR products of appropriate size that hybridized with $G\alpha_q$-specific probes were observed (Figures 3A and 4A). Random-hexamer cDNA generated from platelet RNA produced similar results (not shown).

Control experiments were performed with mouse liver cDNA

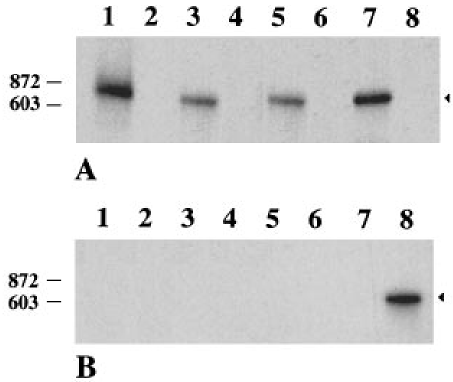


Figure 3 Identification of $G\alpha_q$ in platelet RNA and $G\alpha_q$ and $G\alpha_{11}$ in mouse liver cDNA

(**A**, **B**) PCR performed with q/11MAS cDNA (platelets) or oligo(dT)-primed cDNA (mouse liver) and either primer q/11MAS plus qMS1 (lanes 1, 3, 5 and 7) or 11MS1 (lanes 2, 4, 6 and 8): (**A**) probed with radiolabelled oligonucleotide qMS2; (**B**) probed with oligonucleotide 11MAS1. Lanes 1 and 2, human platelet RNA; lanes 3 and 4, TXA_2^+ dog platelet RNA; lanes 5 and 6, TXA_2^- dog platelet RNA; lanes 7 and 8, mouse liver cDNA. The positions of 872 and 603 bp fragments are shown at the left.

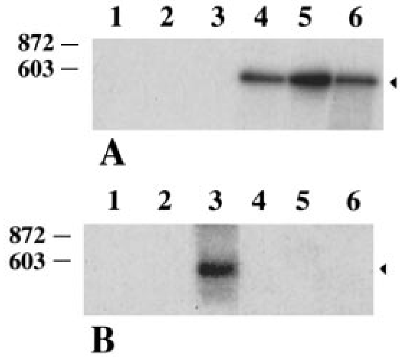
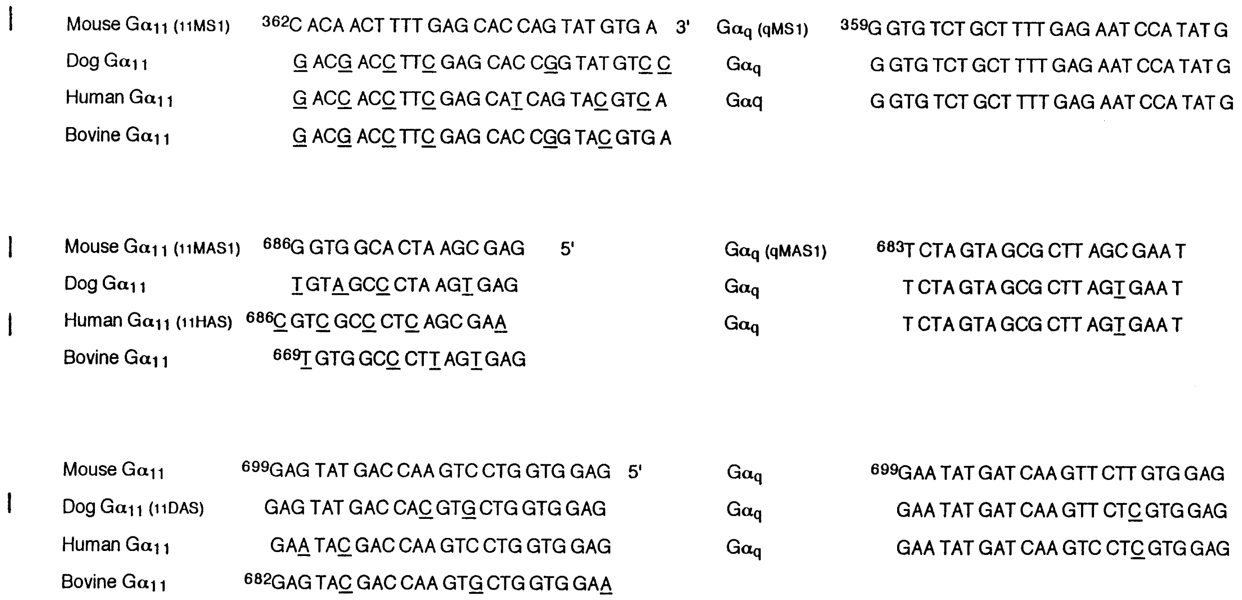


Figure 4 Identification of $G\alpha_q$ in platelet RNA and $G\alpha_q$ and $G\alpha_{11}$ in reverse-transcribed mouse liver RNA

(**A**, **B**) PCR performed with q/11MAS cDNA, and primer q/11MS plus either primer 11MAS1 (lanes 1, 2 and 3) or primer qMAS1 (lanes 4, 5 and 6): (**A**) probed with radiolabelled oligonucleotide qMS1; (**B**) probed with radiolabelled oligonucleotide 11MS1. RNA derived from: lanes 1 and 4, human platelets; lanes 2 and 5, TXA_2^+ dog platelets; lanes 3 and 6, mouse liver. The positions of 872 and 603 bp fragments are shown at the left.

and reverse-transcribed mouse liver RNA. PCR performed with mouse liver cDNA and oligonucleotides q/11MAS, and either qMS1 or 11MS1, yielded products that hybridized with the $G\alpha_q$-specific probe qMS2 (Figure 3A, lane 7) and the $G\alpha_{11}$-specific probe 11MAS1 (Figure 3B, lane 8) respectively. Similarly, mouse liver q/11MAS cDNA combined with the $G\alpha_q/G\alpha_{11}$ common primer q/11MS and either qMAS1 or 11MAS1 yielded PCR products that hybridized with the $G\alpha_q$-specific probe qMS1 (Figure 4A, lane 6) and the $G\alpha_{11}$-specific probe 11MS1 (Figure 4B, lane 3) respectively. Therefore the mouse $G\alpha_{11}$-specific primers and probes used identified $G\alpha_{11}$ message in liver but not in platelets.

These results indicated that $G\alpha_q$ message could be identified across species lines by the use of oligonucleotides based on mouse sequences, and that mouse $G\alpha_{11}$ message could be identified by the use of the mouse $G\alpha_{11}$ primers and probes



Scheme 2 Species-related sequence specificity of $G\alpha_{11}$ and $G\alpha_q$ oligonucleotides

employed. To provide species-specific controls for $G\alpha_{11}$ mRNA, commercial human retinal cDNA and reverse-transcribed dog liver RNA were studied. PCR performed with mouse-specific primers (q/11MAS and 11MS1) with human retinal $G\alpha_{11}$ cDNA yielded no specific PCR product when probed with oligonuclotide 11MAS1. Therefore PCR was performed with a human $G\alpha_{11}$-specific primer, 11HAS, and q/11MS (identical mouse and human sequences). A new $G\alpha_{11}$ oligonucleotide (11MAS2) (Scheme 1) with human and mouse identity was synthesized. Oligonucleotide 11MAS2 hybridized with a PCR product derived from human retinal cDNA, and with a PCR product derived from primers 11MAS1 and q/11MS by using mouse liver cDNA. However, hybridization with 11MAS2 was not observed when PCR was performed by using either of the above primer sets with dog liver random-hexamer cDNA.

Apparently the nucleotide sequence of dog $G\alpha_{11}$ differed sufficiently from that of mouse and human $G\alpha_{11}$ that a species-specific oligonucleotide was required for successful PCR. To provide that oligonucleotide, a PCR product was generated from dog liver random-hexamer cDNA and oligonucleotide primers q/11MAS (highly similar in human and mouse, and identical in $G\alpha_q$ and $G\alpha_{11}$) and 11MS2 (highly similar in human and mouse, and unique to $G\alpha_{11}$). This PCR reaction made a product of appropriate size (approx. 400 bp) that was directly sequenced by using oligonucleotide 11MS2. A fragment of this PCR product was homologous with mouse and human $G\alpha_{11}$. On the basis of the sequence of this fragment, a dog $G\alpha_{11}$-specific probe (11DAS) was synthesized (Scheme 2). PCR performed with dog liver random-hexamer cDNA and primers q/11MAS and 11MS2 yielded a product that hybridized with oligonucleotide 11DAS (Figure 5, lane 1). Similarly, species-specific probes also identified $G\alpha_{11}$ in mouse liver and human retina (Figure 5, lanes 4 and 6). RT–PCR of both dog and human platelet mRNA failed to yield evidence of $G\alpha_{11}$ with the species-specific probes (Figure 5, lanes 2, 3 and 7). Similarly, RT–PCR of mouse platelet RNA probed with oligonucleotide 11MAS1 also revealed no evidence of $G\alpha_{11}$ message in mouse platelets (Figure 5, lane 5). Therefore $G\alpha_{11}$ mRNA was not detected in human, mouse or either TXA_2^- or TXA_2^+ dog platelets, but $G\alpha_q$ mRNA was readily demonstrable in all four types of platelet. No differences in the quantity of $G\alpha_q$ message were apparent between TXA_2^- and TXA_2^+ dog platelets.

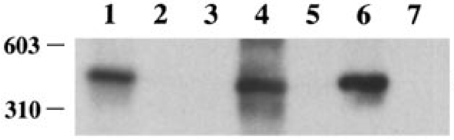


Figure 5 Identification of $G\alpha_{11}$ in dog liver RNA, mouse liver RNA and human retinal cDNA

PCR performed with random hexamer cDNA and primer 11MS2 and q/11MAS. All lanes were probed with radiolabelled, $G\alpha_{11}$-specific oligonucleotides (11DAS, 11MAS1, 11HAS). Lane 1, dog liver RNA; lane 2, TXA_2^+ dog platelet RNA; lane 3, TXA_2^- dog platelet RNA; lane 4, mouse liver RNA; lane 5, mouse platelet RNA; lane 6, human retinal cDNA; lane 7, human platelet RNA. The positions of 603 and 310 bp fragments are shown at the left.

Additional evidence of $G\alpha_{11}$ species specificity was sought by comparison of the sequence of dog $G\alpha_{11}$ with those of other species. PCR was performed with random-hexamer dog liver cDNA and primers q/11MS and 11DAS. The 594 bp products were directly sequenced by using oligonucleotides 11MAS2 or 11MS3 (Scheme 1). The dog $G\alpha_{11}$ sequence (Figure 6) was similar to those of mouse, human and bovine $G\alpha_{11}$ [12,33,34], but significant variations were observed in three oligonucleotides used to study $G\alpha_{11}$ (Scheme 2), two of which coincide with unique areas of $G\alpha_{11}$. The sequence variability seemed to account for the failure of these oligonucleotides to function interchangeably in PCR. Mouse oligonucleotide 11MS1 differed from dog by 7 of 26 nucleotides. In contrast, no significant species-specific sequence variability was found in comparable $G\alpha_q$ oligonucleotides (Scheme 2). The contrast in species-specific variability in $G\alpha_{11}$ in comparison with $G\alpha_q$ was further illustrated

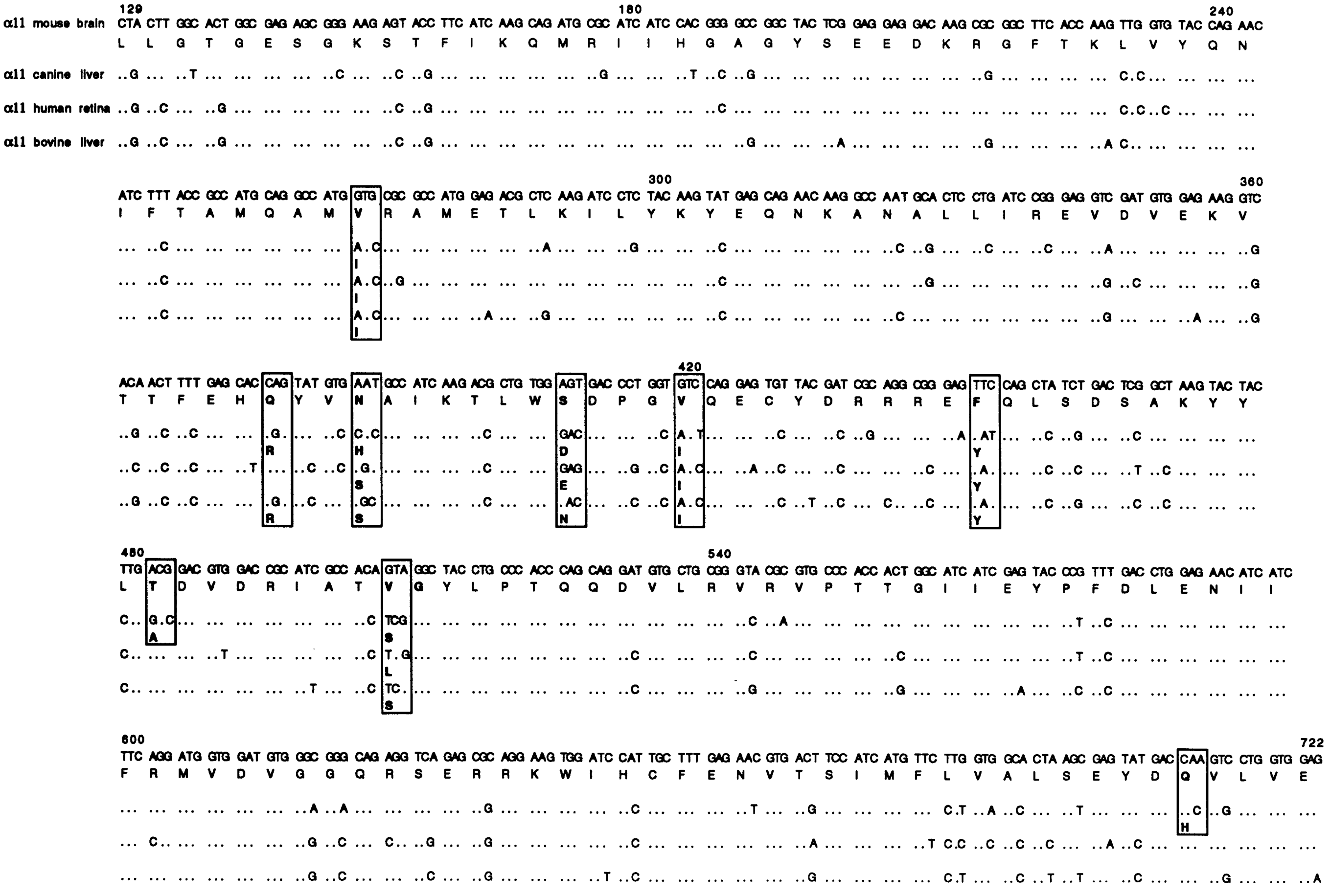


Figure 6 Sequence of mouse brain Gα_{11} fragment, bp 129–722, compared with dog liver, human retina and bovine liver sequences

The sequences of mouse, human and bovine Gα_{11} are derived from [12,33,34]. Identical nucleotides are designated by dots. Boxes denote amino acid differences. The primers used for sequencing dog Gα_{11} were 11MS3 and 11MAS2.

by comparing the nucleotide similarities of bp 129–722 of dog and mouse cDNA (Figure 6). Most of the amino acid differences that distinguish Gα_{11} from Gα_q are located in this region. Dog Gα_{11} differed from mouse Gα_{11} by 70 of 594 (11.8 %) nucleotides, including nucleotides that code for nine amino acid differences (Figure 6). Bovine and human Gα_{11} nucleotides differed from those of mouse Gα_{11} by 11.3 % and 10.9 % respectively. In contrast, only 6.9 % of Gα_q nucleotides 129–722 differed between dog or human and mouse sequences, and these differences coded for only one amino acid difference. The full-length nucleotide sequence of human Gα_q varied from the mouse sequence by 6 % (Figure 7).

To investigate further the selectivity of Gα_{11} mRNA expression in haematopoietic cells, we performed RT–PCR on human and dog erythrocyte RNA species by using random-hexamer cDNA and the primers used for the studies of platelets. Human erythrocytes, like platelets, contained Gα_q mRNA, but no Gα_{11} mRNA was detected (Figure 8). Similar results were obtained with dog erythrocytes (results not shown). The quantity of Gα_q mRNA in erythrocytes was less than in platelets (Figure 8B) but it was readily detected. In contrast with platelets and erythrocytes, their precursors, CD34$^+$ HLA-DR$^+$ haematopoietic stem cells, were found to contain mRNA species for both Gα_q and Gα_{11} (Figure 8). Consistent with the results of prior studies of lymphoid cell lines [21], both Gα_q and Gα_{11} mRNAs were detected in a human lymphocyte (T and B cells) preparation (results not shown). The quantity of Gα_q in this lymphocyte preparation exceeded that of Gα_{11} approx. 10-fold. The platelet preparations used for the studies reported above contained rare leucocytes. Evidence of minimal contamination with lymphocytes was found in some platelet preparations by the presence of a PCR product of appropriate size generated by HLA-DQB-specific primers [23]. Despite this contamination, however, multiple PCR runs performed, under the conditions previously specified, on more than 20 separate platelet RNA preparations failed to demonstrate evidence of Gα_{11} mRNA with the use of any of the Gα_{11}-specific probes reported above. On occasion, when larger quantities of RNA were used in PCR to maximize the reaction products, or when more stringent conditions were imposed, a very faint band was observed on probing with Gα_{11}-specific oligonucleotides. This band was considered to represent minute amounts of Gα_{11} message derived from contaminating lymphocytes. The erythrocyte preparations were free of identifiable platelets and lymphocytes, and RT–PCR performed with HLA-DQB primers revealed no evidence of lymphocyte contamination.

DISCUSSION

Previous studies demonstrated that platelet TXA$_2$/PGH$_2$ receptors are associated with the G$_q$ family of G-proteins [11]

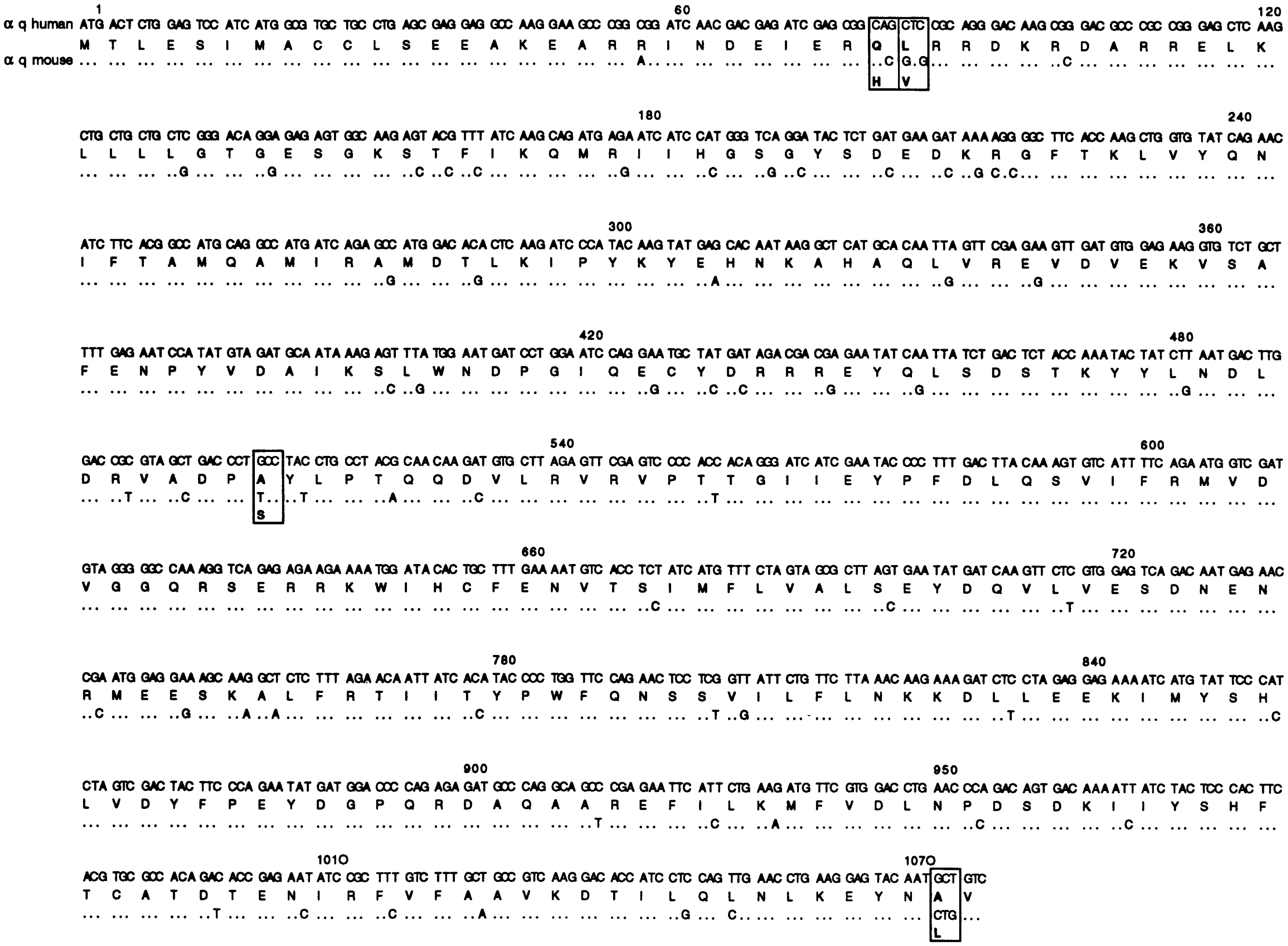


Figure 7 The sequence of human Gα$_q$ cDNA compared with mouse Gα$_q$

The sequence of mouse Gα$_q$ is from [12].

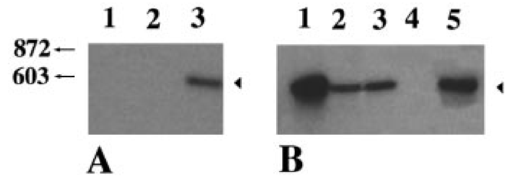


Figure 8 Identification of Gα$_q$ in human erythrocyte RNA, and of Gα$_q$ and Gα$_{11}$ in human bone marrow stem cell RNA

(**A**) PCR performed with random primed cDNA and primer 11HAS plus primer q/11MS and probed with radioactive oligonucleotide 11MAS2. Lane 1, human platelet RNA; lane 2, human erythrocyte RNA; lane 3, human stem cell RNA. The positions of 872 and 603 bp fragments are shown at the left. (**B**) PCR performed with random primed cDNA and primer qMAS1 plus q/11MS and probed with radioactive oligonucleotide qMS2. Lane 1, human platelet RNA; lane 2, human platelet RNA diluted 1:50; lane 3, human erythrocyte RNA; lane 4, human erythrocyte RNA diluted 1:50; lane 5, human stem cell RNA.

and linked to PI-PLC by Gα$_q$ and questionably by Gα$_{11}$[8–10]. To determine whether dog platelets contained Gα$_{11}$ we immunoblotted membranes with an antiserum previously reported to have Gα$_{11}$ specificity [30]. Gα$_{11}$ was not identified in dog or human platelets, or in dog liver, a tissue known to contain both Gα$_{11}$ message and protein [12,16,20,21]. The limitations of negative immunoblotting studies were illustrated by this experiment as well as reports of a lack of specificity of Gα$_q$ and Gα$_{11}$ antisera [13,15,19,20]. Therefore we chose to pursue our studies by using molecular methodology.

By RT–PCR we identified Gα$_q$ mRNA but not Gα$_{11}$ mRNA in platelets and erythrocytes. In contrast, Gα$_{11}$ message was readily detected in mouse liver. However, these mouse-specific primers and probes failed to identify Gα$_{11}$ in human retina or dog liver. Despite the high degree of conservation of G-protein α subunits, Gα$_{11}$ cDNA sequences have greater (approx. 3 %) species-specific amino acid variation and less extensive oligonucleotide identity (approx. 90 %) than those of Gα$_s$ (less than 0.1 % and more than 95 % respectively) [12,33,34]. These results suggested that dog Gα$_{11}$ contained species-specific sequence variations that could have precluded detection with mouse-derived oligonucleotides or antisera. Our observation that an antiserum based on mouse sequences failed to identify Gα$_{11}$ in dog liver supported this assumption. Gα$_q$, in contrast, showed conservation between mammalian species as indicated by the utility of mouse gene-specific primers and probes in the recognition of Gα$_q$ message in dog and human tissues, and of mouse-specific antisera in the identification of protein [10]. RT–PCR of dog, human and mouse RNA performed with species-specific primers and probes demonstrated the oligonucleotide sequence specificity of Gα_{11}. The nucleotide differences between Gα_q and Gα_{11} demonstrated in the present study were previously unknown because only mouse data were available for this comparison [12].

A technical aspect of our studies that requires comment is the susceptibility of cell-specific PCR studies to contamination by RNA from other cell types. We confirmed in some platelet RNA preparations the presence of message for the HLA Class II subregion antigen, DQB, resulting from a low level of contaminating lymphocyte RNA. Leucocyte contamination can be minimized by filtration [23]. Although this was not essential to obtain PCR products lacking Gα_{11}, perhaps because Gα_q message is much more abundant than Gα_{11} message in lymphocytes, it is important to consider the potential implications of even low-level contamination from lymphocytes. Similarly, it was important to exclude leucocyte and platelet contamination from erythrocyte preparations, as was done in our studies, to avoid detection of Gα_q or Gα_{11} derived from other cells.

The cellular specificity of Gα_q and Gα_{11} gene expression in platelets and erythrocytes seems to be related to cellular differentiation. Independent Gα_q and Gα_{11} mRNA expression was also reported to occur in mouse embryos [35] and C6 cells in culture [13]. Expression of Gα_{16}, a member of the Gq family restricted to haematopoietic cells, is linked to cellular developmental change [36], and Gα_{14} is predominantly expressed in early haematopoietic lineages [21]. Because platelets result from terminal differentiation of megakaryocytes, the absence of Gα_{11} message in platelets might be analogous to the down-regulation of Gα_{16} observed in HL-60 cells induced to differentiate [36]. K562 stem cells contain Gα_{11} mRNA [24]; therefore our observations suggest that Gα_{11} gene down-regulation occurs during megakaryocyte maturation. Similarly, Gα_{11} mRNA [21] and Gα_q/Gα_{11} protein [37] occur in mouse erythroleukaemia cells, so Gα_{11} down-regulation probably also takes place during maturation of erythroid precursors. Therefore the expression of Gα_q and Gα_{11} genes varies in individual cell types and at different stages of maturation. The concept that they are ubiquitously expressed [12,21] requires revision.

It can be concluded from the current study that Gα_{11} gene products are not expressed in platelets or erythrocytes, in contrast with other tissues and cell lines previously studied. The failure to detect Gα_{11} mRNA in platelets provides independent confirmation of the observations of Milligan et al. [16] and Ushikubi et al. [17] about the lack of Gα_{11} protein in platelets. Therefore platelet TXA_2/PGH_2 receptors are linked to PI-PLC via Gα_q, but not via Gα_{11}. Because they lack Gα_{11}, platelets provide a cell for the study of the function of Gα_q independently of Gα_{11} that may have advantages over transfection studies of G-protein-mediated signal transduction.

A second conclusion that can be drawn from this work is that Gα_{11} has no role in signal transduction from platelet TXA_2/PGH_2 receptors to the β isoenzymes of PI-PLC. Thus the impaired activation of PI-PLC in TXA_2^- dog platelets cannot be explained by the absence of Gα_{11}. Additional studies will be necessary to determine the functional roles of Gα_q and Gα_{11} in individual cells and to define the mechanism responsible for TXA_2^- dog platelets.

Finally it can be concluded that species specificity is of considerable significance in studies of G_q family proteins. The present study emphasizes the species specificity of Gα_{11}, but precedent exists for species variability within the G_q family. Gα_{15} and Gα_{16} are mouse and human homologues of the same α subunit [21]. The consequences of species variability have often been overlooked. An example is the identification of α subunits in other species by using antisera formed in response to mouse sequence peptides. In addition to our experience with antibody AS 255, a report that human platelets contain Gα_{11} protein [22] is a case in point. We found that the mouse peptide antiserum used identified human Gα_q as Gα_{11} (P. C. Dunlop, unpublished work). This antiserum cross-reactivity most probably occurred because the antigenic terminal QL sequence present in mouse Gα_{11}, but absent from mouse Gα_q, is also present in human Gα_q (Figure 7). Species-specific sequence variability has obvious significance for studies with RT–PCR and antisera, but anti-sense methodology is also vulnerable. Anti-sense oligonucleotides synthesized from mouse Gα_q sequences did not inhibit PI-PLC activation in *Xenopus* oocytes, whereas Gα_s and Gα_{common} anti-sense oligonucleotides were inhibitory [38]. Therefore it was concluded that PI-PLC activation was mediated by G_s. However, the substantial nucleotide sequence differences between *Xenopus* Gα_q and mouse Gα_q [12,39] resulted in significant 3′ sequence mismatches that were likely to render the anti-sense oligonucleotides inactive. Thus the results of this study would probably have been different if *Xenopus*-specific anti-sense oligonucleotides had been used. Future studies of the G_q family of G-proteins should carefully consider the potential effects of species-specific sequence differences.

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