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IDENTIFICATION OF α-SUBUNITS OF TRIMERIC GTP-BINDING PROTEINS IN HUMAN PLATELETS BY RT-PCR

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In a search for new α-subunits of trimeric GTP-binding proteins in human platelets, we prepared leucocyte-free platelet concentrates and analyzed total RNA for areas homologous to known α-subunits. RT-PCR based on two degenerate primers revealed the expected band of 495 base pairs and an additional band of 540 base pairs reflecting the alternative splice product of $G_i\alpha$. Following subcloning in pGEM-T vector and sequencing, we identified the α-subunits $G_i\alpha$-2 and $G_i\alpha$-S of the regulating GTP-binding proteins of adenyl cyclase as well as $G_z\alpha$ whose function is unknown, confirming earlier immunological identification. In addition, we identified $G_i\alpha$-L (differing from $G_i\alpha$-S by an insertion of 45 base pairs), $G_{16}\alpha$, (a member of the pertussis toxin insensitive G_q-family), and two new variants of both $G_i\alpha$-S and $G_i\alpha$-L each containing a C-A-G triplet. With G_{16} we have identified another candidate for pertussis-toxin insensitive signal transduction in platelets. The C-A-G containing sequences of $G_i\alpha$ lead to an insertion of a Ser-residue, which results in the consensus sequence of a phosphorylation site for protein kinase C (Ser-X-Lys), making these variants candidates for protein kinase C-sensitive cyclic AMP formation.

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Seven transmembrane receptors transduce signals to intracellular effector enzymes via heterotrimeric GTP-binding proteins that consist of α-, β- and γ-subunits. The α-subunit contains the GTP-binding site and mediates signal transduction from the receptor to the effector enzymes. Based on toxin sensitivity, α-subunits have been grouped as G_i-proteins (inhibited by pertussis toxin), G_s-proteins (activated by cholera toxin) and a third, toxin-insensitive group consisting of the G_q- and $G_{12/13}$ families and G_z. The β- and γ-subunits form a isoprenylated heterodimer which anchors the protein to the plasma membrane and in some cases activate effector enzymes independent of the α-subunit (for reviews on these subjects see (1-6)). Western blots and toxin-sensitive GTP hydrolysis have been applied for identification of trimeric GTP-binding proteins in platelets. So far, three subtypes of the G_i-family, known to inhibit adenyl cyclase and to activate phospholipase C-β through their $\beta\gamma$ complexes (7,8) have been identified ($G_i\alpha$-1,2,3) (9). In addition, $G_s\alpha$-S which stimulates adenyl cyclase (10), has been detected as well as a member of the and G_q-family which couples the thromboxane receptor to phospholipase C-β (11,12). GTP-binding proteins with unknown function include the subunits $G_{12}\alpha/G_{13}\alpha$ (13,14) and $G_z\alpha$ which is phosphorylated in activated platelets (15,16).

In the present study we searched for new α-subunits in platelets using RT-PCR on platelet RNA. Apart from the notable absence of certain α-subunits earlier detected by Western blotting, we identified $G_{16}\alpha$ and two variants of G_s that are new for platelets and might serve in protein kinase C-sensitive control of cyclic AMP production.

Materials and Methods

The synthetic oligonucleotide primers were synthesised on an Applied Biosystems synthesizer model 381A (Applied Biosystems Inc., Foster City, CA, USA). Cesium chloride, deoxynucleotides and T7-DNA polymerase were purchased from Pharmacia Biotech (Brussels, Belgium) and RQ1 DNase, Taq-polymerase and the pGEM-T cloning system were from Promega Corporation (Madison, WI, USA). The dye primer sequencing kit was derived from Applied Biosystems Inc. (Foster city, CA, USA). AmpliTaq and the GeneAMP RNA PCR Kit were derived from Perkin Elmer(Norwalk, CT, USA) and the oligo-dT primer was from Invitrogen (Leek, the Netherlands). The Prep-A-Gene kit was obtained from Biorad (Hercules, CA, USA) and Superscript II reverse transcriptase was from Gibco BRL (Gaithersburg, MA, USA). Guanine isothiocyanate and β-mercaptoethanol were purchased from Sigma chemicals (StLouis, MO, USA) and sarkosyl from BDH chemicals LTD (Poole, England). The di-deoxynucleotides were from United States Biochemical (Cleveland, OH, USA). The primers for the HLADQb-PCR were a kind gift of Dr. M. Tilanus, Dept. of Pathology, University of Utrecht, the Netherlands. All other chemicals were of analytical grade.

Isolation of platelet total RNA.
Platelet concentrates isolated from blood of 6 donors (Red Cross Bloodbank, Utrecht, the Netherlands) were made leucocyte free by filtration through a PALL50 leucocyte removal filter (PALL Biomedical Ltd., Portsmouth, England). This treatment reduced the leucocyte:platelet ratio from 1:3000 to below $1:10^6$. The platelets were washed twice with phosphate buffered saline supplemented with 1/10 vol ACD (2.5 g tri-sodium citrate, 1.5 g citric acid, 2.0 g D-glucose in 100 ml distilled water) (pH 6.5) by centrifugation/resuspension (20 min, 700*g, 20°C). The pellet was lysed in Solution D (4M Guanidine isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarkosyl, 0.1M β-mercaptoethanol), layered on 5.7 M CsCl + 25 mM Na-acetate and centrifuged for 20 hr at 32.000 rpm in an SW 41 rotor in a L80 Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). The pellet was dissolved in 0.3M Na-acetate. The total platelet RNA was precipitated with 2.5 vol of ice-cold 96% ethanol and stored at -70°C. Before the start of the RT-PCR, the RNA was pelleted (20 min, 14.000*g, 4°C) and dissolved in aqua-dest. The same procedure was used for isolation of total leucocyte-RNA. A possible contamination of the platelet RNA with leucocyte RNA was tested using an RT-PCR with primers specific for a common leucocyte marker, HLADQb. Leucocyte RNA was first treated with DNase to exclude possible contamination with chromosomal DNA. First strand cDNA was synthesized from total platelet- and leucocyte RNA with oligo-$(dT)_{12-18}$ primers using Superscript II reverse transcriptase in a total volume of 40 μl. An aliquot (5 μl) of the first strand cDNA-synthesis reaction mixture was used for amplification of HLADQb (5' primer: 5'-GTC TCA ATT ATG TCT TGG AA-3; 3' primer: 5'-GCC ACT CAG CAT CTT GCT-3'). In the procedure a control RT-PCR was performed using the GeneAMP RNA PCR kit with the pAW 109 RNA template, which contains the IL-1α sequence, and the primers DM151 and DM152 flanking part of the IL-1α sequence. The mixture was heated to 95°C for 2 min and Ampli*Taq* was added. RT-PCR was performed in 35 cycles using a Perkin-Elmer DNA thermal cycler model 480 (Perkin Elmer, Norwalk, CT, USA). During each cycle, samples were denatured at 95°C for 30 s, annealed at 50°C for 1 min and extended at 60°C for 1 min. The PCR-product was analysed on a 1% agarose gel.

RT-PCR of α-subunits of GTP binding proteins.
An aliquot (5 μL) of the first strand cDNA-synthesis reaction mixture was used for the amplification of genes coding for α-subunits. The oligonucleotides used for PCR amplification were designed on the basis of two homologous areas in DNA-sequences of known α-subunits known to code for the guanine nucleotide binding and GTP hydrolysis domains (figure 1):

Primer 1: 5'- GGN AAR AGC ACC ATY GTS AAR CAG ATG - 3'

Primer 2: 5'- DCG YTC DKM BYK CTG RCC HCC BAC RTC -3'

Between these primers a heterogeneous area is located of 490 base pairs, whereas a 540 base pair sequence can be expected if G$_i\alpha$-L is present (1).
This PCR was performed in 30 cycles using *Taq*-polymerase. During each cycle, samples were denatured at 95°C for 1 min, annealed at 55°C for 1 min and extended at 72°C for 2 min.

Cloning and identification of the PCR product.
The PCR-product was purified from a 1% agarose gel using the Prep-A-Gene kit

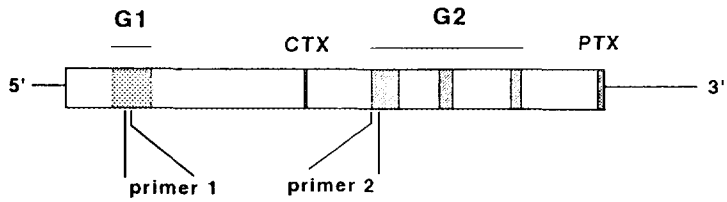

Figure 1.
Location of the degenerate primers on the mRNA of α-subunits of trimeric G-proteins.
Shown is the location of the degenerate primers used for the RT-PCR for amplification of G-protein α-subunits. The black areas represent the homologous areas in the α-subunits thought to function in the guanine nucleotide binding and GTP hydrolysis. CTX: ADP-ribosylation site by cholera toxin; PTX: ADP-ribosylation site by pertussis toxin.

and cloned in the pGEM-T vector making use of the overhanging 3' A introduced by *Taq*-polymerase. A total of 180 clones were isolated and a first screening was performed by T-track analysis using the di-deoxychain termination method according to Sanger (17) using T7-DNA polymerase. The result revealed 72 different T-tracks. All clones were bi-directionally sequenced with the di-deoxychain termination method. Clones were identified by comparing the sequences with known sequences of α-subunits of GTP-binding proteins deposited in the EMBL-databank using the GCG sequence analysis software package (18). Sequences were considered identical with a homology >99%.

Results and Discussion

Fig.2A illustrates the result of the RT-PCR amplification. Compared with the markers (lane A), lane B shows the expected band of 495 bp and an extra band of 540 illustrating the presence of $G_z\alpha$-L. Amplification of part of the leucocyte-specific HLADQb RNA from total platelet RNA gave no product (Fig.2B, lane B). In contrast, amplification of part of the IL-1α sequence from the control RNA (lane C) and HLADQb amplified from total leucocyte RNA resulted in the expected bands of 308 and 702 bp, respectively. Together these findings indicate that the fragments amplified from total platelet RNA were of platelet origin.
After cloning and sequencing the PCR-product, seven α-subunits could be identified on the basis of their base pair composition. We identified the α-subunits $G_i\alpha$-2 and $G_s\alpha$-S as well as $G_z\alpha$. These findings confirm earlier identification by Western-blotting of platelet proteins (9,10,16). $G_i\alpha$-2 is the predominant species of the G_i-family in platelets (9,19) and mediates the coupling between the seven transmembrane thrombin receptor (19) and the α_{2A}-adrenergic receptor with adenyl cyclase (20,21). $G_s\alpha$-S couples the prostacyclin receptor to adenyl cyclase

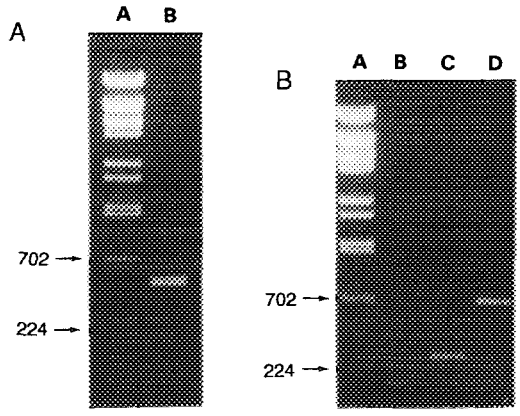

Figure 2A.
RT-PCR of α-subunits of GTP-binding proteins.
Identification of RT-PCR on total platelet RNA. Lane A shows the marker (λBstEII; marker size indicated on the left). Lane B shows RT-PCR product with two bands with a length of 495 and 540 base pairs.

Figure 2B.
Purity control of total platelet RNA.
RT-PCR was performed on total platelet RNA (lane A), control RT-PCR on Paw 109 (lane B) and total RNA from leucocytes isolated from peripheral blood (lane C) using primers specific for a leucocyte specific marker HLA-DQb. Indicated on the left are the marker-bands of λBstEII.

(22). $G_z\alpha$ has been detected in platelets and megakaryoblastic cell lines Dami and Hel by Northern- and Western blot analysis and in megakaryocytes using in situ hybridization (15,16). Despite a high homology with α-subunits of the G_i-family, the protein lacks sensitivity for pertussis toxin due to a Cys to Ile replacement (16,23,24). The most prominant feature of $G_z\alpha$ is that it becomes phosphorylated by protein kinase C after platelet activation with α-thrombin or phorbol ester (15,16). However both the function of G_z and the importance of the phosphorylation remains uncertain. Upon transfection in Swiss 3T3 cells (25) or human embryonic kidney cells, G_z mediates the inhibition of adenyl cyclase by the κ-opoid-, α_2-adrenergic-, dopamine-, adenosine A_1-, and the complement C5a receptor (25-27). G_z possibly also functions in the inhibition of adenyl cyclase by the α_2-adrenergic- and adenosine A_1 receptor in platelets.

In addition to the short type of $G_z\alpha$ ($G_z\alpha$-S), we found $G_z\alpha$-L containing 45 extra base pairs located on exon 3 (28,29). Nanoff et al (30) used recombinant forms of both G_z subtypes and found a 3-10 fold higher activation of adenyl cyclase by $G_z\alpha$-L in isolated platelet membranes which were depleted of platelet G-proteins,

confirming previous results using a reconstitution system (31). This suggests that the $G_s\alpha$-L type is the primary determinant of cyclic AMP formation in platelets, assuming an almost equal abundance of $G_s\alpha$-S and $G_s\alpha$-L.

We also demonstrated for the first time the presence of $G_{16}\alpha$ in human platelets. This heterotrimeric G-protein is exclusively found in hematopoietic cells, such as T-cells (32) and progenitors of B-cells (33). G_{16} is a member of the pertussis toxin insensitive G_q-family and therefore a candidate for toxin insensitive phospholipase C activation observed after stimulation of the thromboxane receptor (34,35). Coprecipitation from platelet lysates revealed complexes between the thromboxane receptor and members of the G_q family (36). In reconstitution experiments $G_{16}\alpha$ activated phospholipase C-β1, -β2 and -β3 (37). In addition, G_{16} might function in regulating cell growth. Swiss 3T3 cells transfected with a mutational inactive $G_{16}\alpha$ grew slower than their normal counterparts (38). In a parallel study we found that $G_{16}\alpha$ was absent in the immature megakaryoblastic cell line MEG-01 and came to expression in the more mature Dami and CHRF 288-11 cell lines, suggesting that it may serve in megakaryocyte maturation (van der Vuurst et al, unpublished results). We identified variants of both $G_s\alpha$-S and $G_s\alpha$-L that contained the CAG triplet (designated $G_s\alpha^*$, figure 3). For $G_s\alpha$-S this sequence results from an insertion between nucleotides A^{+224} and T^{+225}, whereas for $G_s\alpha$-L an insertion between G^{+257} and T^{+258} is the basis for the extra codon. Besides the insertion of the Ser-residue the amino acid sequence remains undisturbed. The relative abundance of the different $G_s\alpha$ subtypes in platelets is 23,33,33 and 11 % for $G_s\alpha$-S, $G_s\alpha$-S*, $G_s\alpha$-L and $G_s\alpha$-L*, respectively, based on the abundance of the different G_s-variants in the clones that were sequenced. A similar heterogeneity in $G_s\alpha$ subtypes has previously been demonstrated in human brain (29). The structure of the human $G_s\alpha$ gene suggests that the four variants may be generated from a single $G_s\alpha$ gene by alternate use of exon 3

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          N   G                          D     E   K
Gsα-S:    AAT GGA ---------- GA----T GAG AAG
          N   G                  D   S   E   K
Gsα-S*:   AAT GGA ---------- GAC AGT GAG AAG

          N   G              D     G     E   K
Gsα-L:    AAT GGA - 45 bp - GAT GG----T GAG AAG
          N   G              D   G   S   E   K
Gsα-L*:   AAT GGA - 45 bp - GAT GGC AGT GAG AAG
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Figure 3
DNA sequence and amino acid sequence alignment of the G_s-variants.
The extra bases CAG and the resulting Ser residue are in bold .

and/or of two 3' splice sites of intron 3 where an unusual splice junction sequence (TG) instead of the consensus (AG) is used (28,29).

The presence of CAG in $G_s\alpha$-S* and $G_s\alpha$-L* results in the introduction of a Ser-residue leading to a consensus sequence for protein kinase C phosphorylation (Ser-X-Lys, figure 3). This implies that about half of the $G_s\alpha$ molecules are potential substrates for protein kinase C in agreement with the findings by Pyne et al (39), who demonstrated that this potential phosphorylation site is indeed recognized by protein kinase C. The phosphorylation site lies in the N-terminal region of the area that is believed to interact with adenyl cyclase and is important for the activation of the enzyme (40,41). An inhibitory effect of protein kinase C on G_s stimulated adenyl cyclase was shown by Murphy et al (42). Recently we showed that complete suppression of PGI_2-induced cyclic AMP formation in platelets requires maximal activation of both G_i and protein kinase C, whereas the action of each protein alone is not sufficient (43). The present data are in line with this observation and suggest that phosphorylation of $G_s\alpha$-S* and $G_s\alpha$-L* makes these GTP-binding proteins inactive whereas $G_s\alpha$-S/$G_s\alpha$-L mediated adenyl cyclase activation remains intact.

Notably absent in our RT-PCR preparations were two members of the G_i family, $G_i\alpha$-1 and $G_i\alpha$-3 and the recently described $G_{12}\alpha$ and $G_{13}\alpha$ which were detected using Western-blots (7,9,11,13,14,44). The use of platelet-rich plasma and cross reactivity of antibodies might be causes for these discrepancies. The presence of $G_{11}\alpha$ in platelets has been described (7,11) and denied (45) and coding sequences for this α-subunit were absent in our RT-PCR analysis.

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