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CHARACTERIZATION OF G25K, A GTP-BINDING PROTEIN CONTAINING A NOVEL PUTATIVE NUCLEOTIDE BINDING DOMAIN

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Amino acid sequences were obtained for four peptides (p1, -2, -3 and 4) generated by chemical or proteolytic cleavage of a 25 kDa GTP-binding protein purified from human placental and platelet membranes. The peptides shared sequence similarities with those contained in several of the ras-related GTP-binding proteins. Peptide p2, a 12-mer, was homologous with a region of the GTP-binding proteins that contains a structural motif proposed to contribute to the nucleotide binding site. However, whereas nearly all GTP-binding proteins exhibit the residues NKXD as this motif, p2 contains TQID. Antisera (Ap1 and Ap3) raised against synthetic peptides corresponding to p1 and p3 specifically reacted on Western blots with the 25 kDa GTP-binding protein purified from human placenta, human platelet and bovine brain as well as with a 25 kDa polypeptide in various cell lines. These results demonstrate the widespread existence of an abundant 25 kDa GTP-binding protein which contains a putative nucleotide binding domain that is chemically distinct from that described for all GTP-binding proteins of known primary structure.

It is becoming increasingly apparent that there exists a large and diverse family of ras-related GTP binding proteins with molecular masses of ~20-30 kDa. Several cDNAs encoding for proteins homologous to the N- (1), K- (2), and H-ras (3) gene products have been isolated from a variety of cell types (4-10). The amino acid sequences deduced from these cDNAs all contain consensus elements proposed to form the structural domains involved in the binding and hydrolysis of GTP (11-14). The purification of low molecular mass GTP-binding proteins initially led to the isolation of a 25,000 dalton protein from human placental membranes (15). This protein, originally designated Gp but subsequently renamed G25K, was also purified from bovine brain (16). In addition, ARF, a 21 kDa GTP-binding protein which serves as a cofactor for cholera toxin-catalyzed ADP-ribosylation, has been purified (17) and the cDNA encoding this protein has

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Abbreviations

G25K, a 25 kDa GTP-binding protein originally purified from human placenta and designated Gp; G protein, a family of heterotrimeric GTP-binding regulatory proteins; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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been isolated (18). More recently, at least six GTP-binding proteins of low molecular mass were identified in detergent extracts of bovine brain membranes (19). Three of these proteins, with molecular masses of 20, 21, and 24 kDa, have been identified as the gene products of rho (20), c-Ki-ras (21), and rab 3 (22), respectively, while a 22 kDa protein is encoded by a novel gene with 55% homology to c-Ha-ras (23,24).

Given the recent plethora of cDNAs reported to encode for low molecular mass GTP-binding proteins and the purification of several proteins that remain to be definitively associated with such cDNAs we sought to clarify the identity of G25K. To this end approximately 30% of the primary structure of placental G25K was obtained. Here we report on the relationship of G25K to the ras-related gene products. We also describe antisera raised against specific peptide sequences derived from this protein.

METHODS

Immunological Procedures. Peptides were synthesized with a cysteine residue at the amino terminus and conjugated to soy trypsin inhibitor using maleimidobenzoyl-N-hydroxysuccinimide (25). Rabbits received intradermal injections of 0.5 mg of conjugated peptide in complete Freund's adjuvant and were boosted at two week intervals thereafter for eight weeks. Antibody affinity supports were prepared by coupling the peptides to cyanogen bromide-activated Sepharose beads as described by the manufacturer (Pharmacia, Inc.). Antibody affinity purification and Western blotting procedures were carried out as described by Mumby *et al.* (26). In all cases, antibodies Ap1 and Ap3 were used at dilutions of 1:1000 and 1:500, respectively. The secondary antibody was 2.5 x 10^5 cpm/ml ^{125}I-labeled donkey-anti rabbit IgG (Amersham).

Electrophoretic Procedures. Two dimensional PAGE was by the method of O'Farrell (27) using pH 3.5 - 10 ampholytes (Pharmacia LKB Biotechnology, Inc). Isoelectric focusing was performed in tube gels (190 cm x 1.5 mm) for 20 h at 700 volts. Isoelectric points of proteins visualized in a Coomassie Blue stained tube gel were obtained by interpolation using a calibration curve constructed by measuring the pH gradient of a separate gel focused in the same experiment. SDS-PAGE was performed in 12% polyacrylamide gels according to Laemmli (28).

Proteins and Peptides. Human placental G25K protein was purified as we have described previously (15). The fraction containing G25K from bovine brain membranes (Fig. 1) represents the descending portion of the GTPγs binding peak from the AcA34 chromatography that we and others have previously alluded to (15). The activity was further chromatographed through hydroxylapatite. The human platelet G25K protein (Fig. 1) was purified using methods essentially as described (15). The preparation shown resulted from chromatography of the human platelet cholate extract through DEAE-Sephacel, Ultrogel AcA34, Heptylamine-Sepharose, Hydroxylapatite, DEAE-Fractogel, and finally Hydroxylapatite.

G25K purified from placental membranes was resolved from the G protein β-subunits by SDS-PAGE. The 25 kDa protein band was visualized staining briefly in Coomassie Blue, excised from the gel and then electroeluted into Tris/glycine (20 mM/192 mM) buffer containing 0.1% (w/w) sodium dodecyl sulfate. The protein was precipitated with acetone, redissolved in 0.1 M NH_4HCO_3, pH 8.5, containing 0.1% (w/v) sodium dodecyl sulfate and then digested overnight with 1% (w/w) lysine C protease or 2% (w/w) trypsin. For cyanogen bromide digestion the precipitated protein was dissolved in 70% formic acid containing 60 mg/ml cyanogen bromide and incubated overnight at room temperature in the dark. The purified platelet G25K, 184 μg/ml in 50 mM Hepes, pH 7.2, 1 mM dithiothreitol, 100 mM NaCl and 0.1% (w/v) lubrol, was diluted with one volume of 1% (w/v) NH_4HCO_3 pH 8.5, and digested overnight with TPCK-treated trypsin (5% w/w) at 37°C. Trypsin (5% w/w) was again added and the sample incubated for 6 hours at 37°C. The peptide fragments were resolved by reversed phase high performance liquid chromatography using a C-4 microbore column (Synchropak RP4-4000, Synchrom, Inc.) developed with a linear gradient (1%/min) of acetonitrile in 0.1% TFA from 0-60%. Peptide

sequencing was carried out using an Applied Biosystems Model 470A Sequencer with on-line detection of phenylthiohydantoin-derivatives. For homology searches a list was compiled containing recently published amino acid sequences of low molecular mass GTP-binding proteins: human H-ras (3); human and Aplysia rho (9); bovine smg-25A,B and C (21); rat rab 1-4 (10); Simian ral (8); yeast RAS 1 and 2 (29,30); yeast SEC4 (6); yeast YPT (4); yeast RHO1 and 2 (5); bovine and yeast ARF (18). The sequences of the G25K fragments were compared to these protein sequences using a computer and by inspection.

RESULTS AND DISCUSSION

Partial Amino Acid Sequence of G25K. The G25K protein purified from human placental membranes was digested with either trypsin, lysine C, or cyanogen bromide and the amino acid sequences of four isolated peptide fragments (p1, -2, -3 and -4) were determined (Table 1). The sequences of two of these peptides, p1 and p2, were identical to the sequences determined for two peptides derived from the platelet G25K protein suggesting that the placental and platelet proteins are identical. We compared the amino acid sequences of the 4 peptide fragments with the amino acid sequences deduced from cloned cDNAs reported to encode for low molecular mass GTP-binding proteins. Most striking is the homology observed with the hexameric sequence DTAGQE contained in peptide p4 (residues 11-16). This sequence is absolutely conserved in all of the ras-related GTP-binding proteins (10) and constitutes one of the four homology boxes proposed to contribute to the formation of the GTP-binding site (11-14). Although p4 contains this strictly conserved sequence, the amino terminal portion of the peptide (residues 1-10) is quite dissimilar to the corresponding sequences contained in other low molecular mass GTP-binding proteins.

Comparison of peptide p2 with the low molecular mass GTP-binding proteins (Table 2) reveals homologies with a region known to contain the GTP-binding consensus element NKXD (where X is any amino acid). This consensus element is thought to be critical in defining the guanine specificity of the GTP-binding site (11,13). With the exception of yeast RHO1 and RHO2, this sequence is found in all of the low molecular mass GTP-binding proteins as well as in the elongation factors and in the α-subunits of the trimeric G proteins; G_o, G_i, G_s and transducin.

Table 1. Sequences of peptides derived from the degradation of placental and platelet G25K

p1	NVFDEAILAALEPPEPK[a]
p2	PFLLVGTQIDLR[a]
p3	GIQTPETAEK[b]
p4	GGEPYTLGLFDTAGQEDYDR[c]

[a]Derived from tryptic cleavage of placental and platelet G25K, and lysine C cleavage of placental G25K.

[b]Derived from tryptic cleavage of placental G25K.

[c]Derived from cyanogen bromide cleavage of placental G25K.

Table 2. Sequence homology of G25K peptide p2 to the low molecular mass GTP-binding proteins

G25K-p2	P	F	L	L	V	G	T	Q	I	D	L	R	(1-12)
Human H-ras	-	M	V	-	-	-	N	K	C	-	-	A	(109-121)
Human Rho	-	I	I	-	-	A	N	K	K	-	-	-	(110-122)
Bovine SMG-A	Q	V	-	-	-	-	N	K	C	-	M	E	(128-141)
Bovine SMG-B	Q	V	I	-	-	-	N	K	C	-	M	E	(128-141)
Bovine SMG-C	Q	V	I	-	-	-	N	K	C	-	M	E	(128-141)
RAB-1	N	K	-	-	-	-	N	K	C	-	-	T	(116-129)
RAB-2	V	I	M	-	I	-	N	K	S	-	-	E	(112-125)
RAB-3	Q	V	-	-	-	-	N	K	C	-	M	E	(128-141)
RAB-4	V	I	I	-	C	-	N	K	K	-	-	D	(115-128)
Simian Ral	-	-	-	-	-	-	N	K	S	-	-	E	(109-122)
Yeast Ras1	-	V	V	V	-	-	N	K	L	-	-	E	(116-129)
Yeast Ras2	-	I	V	V	-	-	N	K	S	-	-	E	(116-129)
Yeast Sec4	Q	L	-	-	-	-	N	K	S	-	M	E	(126-139)
Yeast Ypt1	L	K	-	-	-	-	N	K	C	-	-	K	(114-127)
Yeast Rho1	-	I	I	-	-	-	C	K	V	-	-	-	(115-128)
Yeast Rho2	-	I	V	-	-	-	L	K	K	-	-	-	(112-125)
Bovine Arf	V	L	-	V	F	A	N	K	Q	-	-	P	(119-132)
Yeast Arf	A	W	-	V	F	A	N	K	Q	-	-	P	(119-132)
Aplysia Rho	-	I	I	-	-	-	N	K	K	-	-	-	(110-123)

Hyphens indicate residues identical to those found in the corresponding position of p2. Values in parenthesis refer to the position of the amino acids as they appear in the complete protein sequence with the exception of p2.

In contrast, p2 contains the sequence TQID in the position corresponding to the NKXD consensus element. This sequence was determined in four separate analyses; twice each for p2 derived from placental and platelet G25K. The amino acid sequences of peptides p1 and p3 were also compared to low molecular mass GTP-binding proteins. Weak homologies (29-35%) between p1 and the yeast, Aplysia, and human rho gene products were detected while peptide p3 was similar (50%) only to the carboxy-terminal region of the yeast RAS-1. Thus, G25K shares the characteristics of the ras-related family of GTP-binding proteins but is clearly distinct, particularly in a region known to be critical for GTP binding. Single amino acid substitutions in the NKXD consensus sequence of p21 ras results in a dramatic loss of GTP binding (reviewed in ref. 29). This is consistent with the three dimensional structures of EF-Tu (11) and p21 ras (13,14) which predict that the NKXD sequence constitutes one of two noncontiguous sequences which forms the binding pocket for the guanine base. Therefore, the presence of residues T and Q, found at the positions of N and K, respectively, in this consensus sequence may impart unique guanine nucleotide binding properties to the G25K protein. Indeed, recent evidence[2] suggests that G25K, purified from human placental membranes, will not exchange bound GDP for GTP in the presence of Mg^{2+}. Similar properties have also been noted for a 24 kDa GTP-binding protein purified from porcine brain (32).

[2] A. Tamir, A. Fawzi, H. Tamir, T. Evans, and J. Northup, manuscript in preparation.

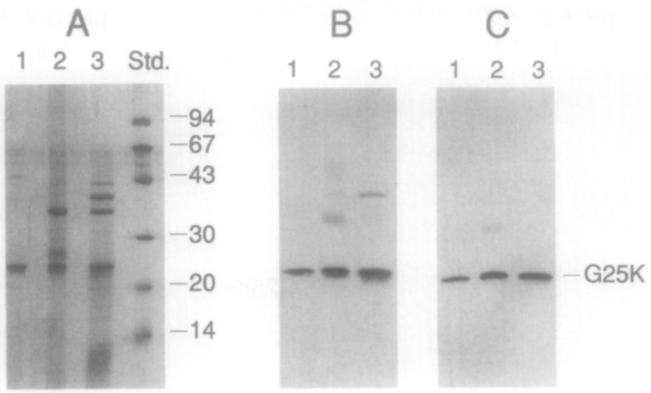

Figure 1. Immunological cross-reactivity of antibodies Ap1 and Ap3 with G25K purified from platelet, placenta, and brain. **A**, SDS-PAGE of purified preparations of G25K. Proteins were visualized with Coomassie Blue stain. **B** and **C**, autoradiograms of Western blot transfers immunoblotted with antibodies Ap1 or Ap3, respectively. Lane 1, platelet G25K, 1 μg total protein; lane 2, placental G25K, 3 μg total protein; lane 3, brain G25K, 5 μg total protein. Standard proteins (std) and their relative molecular weights (x 10^{-3}) are shown at right in **A**. SDS-PAGE and Western blotting are described under METHODS. Autoradiograms were developed following a 1 hour exposure at -70°C in the presence of an enhancing screen.

Specific Antibodies to G25K. Peptides p1, -2, and -3 were coupled to soy trypsin inhibitor and the conjugates were used to immunize rabbits. High titre antibodies, designated Ap1 and Ap3, were raised against p1 and p3, respectively, and then purified using either a p1 or p3-agarose affinity support. Both of the affinity purified antibodies reacted strongly on Western blots with the G25K protein purified from human placenta, platelets, and bovine brain (Fig. 1). We have demonstrated previously that G25K purified from these same three sources was also indistinguishable based on peptide mapping analysis and cross reactivity to polyclonal antisera raised against platelet G25K (16).

Antisera raised against peptide p2 reacted only weakly with the purified proteins (not shown). The reasons for this are not clear, although similar results were reported recently in an immunological study of ARF (17). Antibodies raised against a 16 residue ARF peptide containing the NKXD consensus sequence had only limited reactivity to the ARF holoprotein.

Antibodies Ap1 and Ap3 were also used to probe a purified preparation of G25K from platelets for the presence of subforms or unrelated protein contaminants. Because G25K migrates as a poorly resolved doublet with a molecular mass of 24-25 kDa on SDS-polyacrylamide gels we resolved the protein further using two dimensional gel electrophoresis. Two polypeptides with approximate pI values of 6.2 and 6.7 were resolved by isoelectric focusing but migrated with apparently identical mass on SDS-PAGE (Fig. 2A). Immunoblots of the two dimensional gels revealed that both polypeptides reacted strongly with antibody Ap1 (Fig. 2B) and Ap3 (not shown). These results demonstrate that G25K, as purified from human platelet membranes, exists as two immunologically indistinguishable isoelectric forms.

The existence of electrophoretic subforms appears to be a common phenomenon for low molecular mass GTP-binding proteins. In the case of p21 ras, the plasma membrane-bound form of the protein exhibits a faster electrophoretic mobility than the cytosolic form (33,34). ARF migrates as a closely spaced doublet on SDS-polyacrylamide gels (35) and a 22 kDa GTP-binding

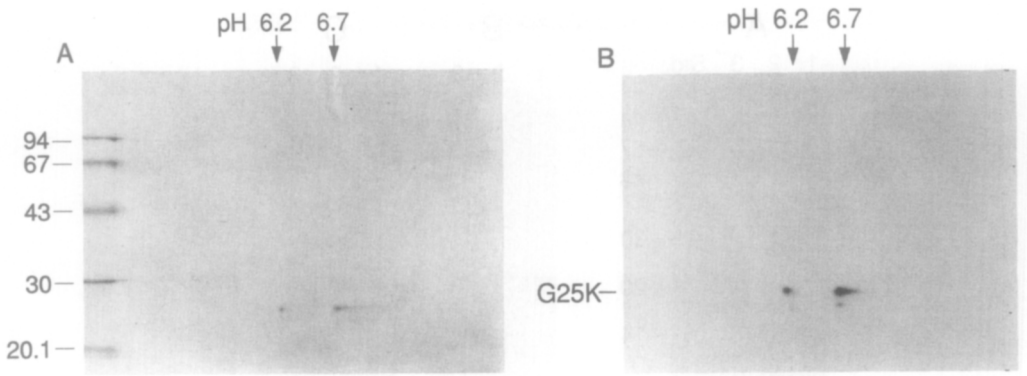

Figure 2. Two dimensional gel electrophoresis and immunoreactivity of G25K purified from human platelets. Duplicate samples of purified G25K (5 μg each) were subjected to 2-D gel electrophoresis as described in METHODS. **A**, 2-D gel stained for protein with Coomassie Blue. Values at left indicate molecular masses of protein standards applied to the SDS-PAGE gel. Isoelectric points estimated for the two G25K forms are shown at the top. A duplicate 2-D gel was subjected to Western blotting and the transfer was incubated with antibody Ap1. **B**, Autoradiogram of the Western blot developed after a 1 hour exposure at -70°C in the presence of an enhancing screen.

protein serving as a substrate for botulinum toxin has been resolved into two isoelectric forms (36). Although ARF and p21 ras are known to be acylated by fatty acids (17,34), there is no direct evidence to indicate this as the basis for the heterogeneity of these proteins. In fact, there is evidence to the contrary for p21 ras (33). The heterogeneity described here for the G25K protein most likely results from a post-translational modification, however, it cannot be ruled out that the two isoelectric forms represent related but distinct gene products.

We have previously isolated a low mass GTP-binding protein from human leukemic (HL60) cells (37) and have found it to be cross reactive with antisera Ap1[3]. We therefore tested the specificity of Ap1 on immunoblots of differentiated HL60 cell membrane proteins resolved by two dimensional polyacrylamide gel electrophoresis. The antisera detected only a single band with relative molecular mass of ca. 25 kDa (Fig. 3). To assess the relative distribution of G25K we used antibodies Ap1 and Ap3 to screen crude extracts from a variety of cell lines. Ap1 and Ap3 reacted with a 25 kDa polypeptide in all of the cell lines with the exception of the primary sertoli cell (Fig. 4). Interestingly, a second polypeptide with a molecular mass of ~26.5 kDa was also recognized by Ap1. This Ap1-reactive band, which is detectable in the rat TR1 endothelial and primary sertoli cell lysates is not reactive to Ap3 (Fig. 4, compare upper and lower panels). It is possible that the 26.5 kDa protein is a precursor form of G25K, however, Ap3 would be expected to react with this precursor as well. The most direct interpretation is that the 26.5 kDa protein is distinct and contains the epitope for Ap1 but not for Ap3. That we have never observed the copurification of a 26.5 kDa polypeptide with G25K also suggests that it is a distinct protein.

These results demonstrate that antibodies Ap1 and Ap3, when used in combination, can specifically identify the G25K protein present in plasma membrane preparations or crude cell

[3] P. Polakis, T. Evans, and R. Snyderman, unpublished observation.

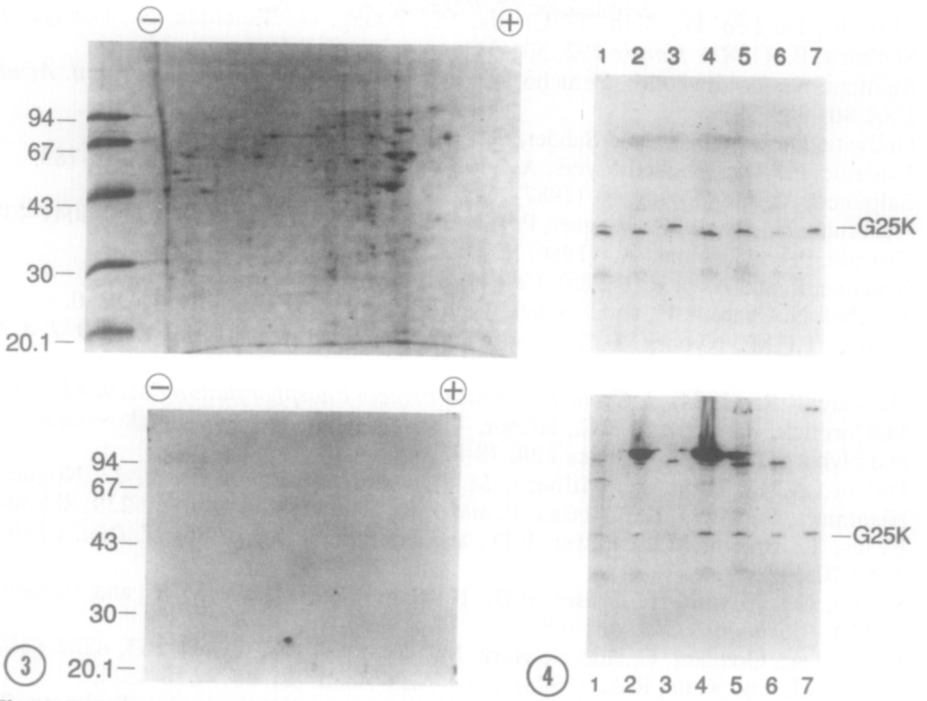

Figure 3. Reactivity of antisera Ap1 with HL60 cell membrane proteins. Duplicate samples of HL60 cell plasma membranes (50 µg, total protein each) were subjected to two dimensional PAGE as described in METHODS. One gel was stained for protein using Coomassie Blue (upper). A second gel was subjected to electroblotting and the nitrocellulose transfer was incubated in antisera Ap1 (1/1000 dilution) followed by ^{125}I-labeled secondary antibody. An autoradiogram (12 hour exposure) is presented (lower). Values at left indicate molecular masses (x10^{-3}) of standard proteins. Negative and positive symbols indicate the positions of the cathodic and anodic ends of the IEF gel.

Figure 4. Reactivity of antibodies Ap1 and Ap3 with proteins present in various cell lines. Approximately 2.5 x 10^5 cells of each of the following cell lines were solubilized in 75 µl of SDS-PAGE sample buffer and following centrifugation (5 min. IEC Centra-M) the supernatants were subjected to SDS-PAGE. Lanes: 1, TR1 rat endothelial (35); 2, rat lung; 3, primary sertoli; 4, fetal hamster lung; 5, TM3 murine leydig (35); 6, TR-M rat myoid (36). Lane 7 is HL60 cell plasma membranes (10 µg protein). SDS-PAGE and immunoblotting with antibodies Ap1 (upper) and Ap3 (lower) were carried out as described under METHODS. The autoradiograms presented were developed following a 24 hour exposures at -70°C in the presence of an enhancing screen.

lysates. Antibodies Ap1 and Ap3 as well as the peptide sequences reported here will be useful in identifying specific cDNAs which encode for the G25K protein.

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