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Distribution of polypeptides binding guanosine 5′-[γ-[35S]thio]triphosphate and anti-(ras protein) antibodies in liver subcellular fractions

Evidence for endosome-specific components

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The subcellular distribution in rat liver of polypeptides binding guanosine 5′-[γ-[35S]thio]triphosphate ([35S]GTP[S]) and seven antibodies against ras oncoproteins was evaluated. Multiple low-M_r (21000–28000) GTP-binding proteins were detected, but their relative distribution among the membrane fractions varied. A more specific compartmentation of polypeptides which bind antibodies generated against ras proteins was evident, with an M_r-28000 polypeptide and a probable M_r-56000 dimer, identified by six of the antibodies tested, being confined mainly to endosomes. An M_r-23000 polypeptide was detected by some of the antibodies in all of the membrane fractions, but especially in the plasma membranes.

INTRODUCTION

A family of heterotrimeric GTP-binding proteins (G-proteins) regulate signal transmission at the plasma membrane (Gilman, 1987). A further class of low-M_r G-proteins (20000–30000) that bind and hydrolyse GTP have been identified. These may correspond to the products expressed by the *ras* proto-oncogenes (Barbacid, 1987; Santos & Nebreda, 1989) as well as an expanding list of other gene products (Burgoyne, 1989; Kawata *et al.*, 1989; Mizoguchi *et al.*, 1989). Low-M_r components that may correspond to ras-related G-proteins have been identified by their property of specifically binding GTP after separation in SDS/polyacrylamide gels followed by transfer to nitrocellulose sheets in, for example, platelets and rat tissue homogenates (Bhullar & Haslam, 1987), and in various mammalian cell lines (Comerford & Dawson, 1989). These low-M_r G-proteins are thought to be implicated in secretion (Bourne, 1988), and studies with transport-deficient yeast mutants add credence to these views (Goud *et al.*, 1988; Segev *et al.*, 1988; Beckers & Balch, 1989). More recently, it has become evident that low-M_r G-proteins are also involved in endocytosis (Mayorga *et al.*, 1989*a*,*b*) and may be more widely implicated in the regulation of membrane trafficking.

We have already shown that there is a widespread distribution of the α and β subunits of G-proteins in rat liver plasma membranes, endosomes and Golgi membranes (Ali *et al.*, 1989). In the present work, we identify seven GTP-binding polypeptides (M_r 21000–28000) in various rat liver subcellular fractions. Furthermore, using seven antibodies against ras oncoproteins, we have examined the distribution of these proteins in the subcellular fractions and show that an M_r-28000 polypeptide is compartmentalized mainly to endosomes, where it is positioned to feature in the regulation of membrane trafficking.

EXPERIMENTAL

Materials

Guanosine 5′-[γ-[35S]thio]triphosphate ([35S]GTP[S]) (1320 Ci/mmol) was from New England Nuclear–Du Pont. The Y-13-259 monoclonal antibody was donated by Dr. A. I. Magee of this Institute. Sheep polyclonal anti-peptide antibody to an *N*-terminal amino acid sequence common to the aligned sequences of c-Ki-*ras*-1, -2A and -2b and conjugated to keyhole-limpet haemocyanin was from Cambridge Research Biochemicals. E_{546} and E_{557} mouse monoclonal anti-p21^{ras} antibodies were given by Dr. R. J. A. Grand, CRC Laboratories, University of Birmingham, Birmingham, U.K. RAS-10, RAS-11 and 6B7 anti-p21^{ras} monoclonal antibodies were obtained from Dr. C. J. Marshall, Institute of Cancer Research, Chester Beatty Laboratories, London, U.K. ^{125}I-labelled Protein A was from Amersham International. Other chemicals were from Sigma or BDH. Recombinant p21$^{N\text{-}ras}$ expressed by *Escherichia coli* was provided by Dr. M. Webb of this Institute.

Isolation of plasma and intracellular membrane fractions

Membrane fractions originating from the three plasma membrane domains of hepatocytes (sinusoidal, lateral and bile canalicular) and endosomes were isolated from the livers of Sprague–Dawley rats (150–200 g) as described previously (Ali *et al.*, 1989). 'Early' and 'late' endosomes, separated by isopycnic centrifugation in Nycodenz gradients, were defined kinetically according to the location of ligands endocytosed 2–3 min or 10 min after liver perfusion and were of density 1.115 and 1.09 g/cm^3 respectively. 'Heavy' endosomes, also resolved in these gradients, were ligand-free membranes of density 1.18 g/cm^3 (Evans & Flint, 1985). Residual microsomes were obtained during the isolation of sinusoidal plasma membranes as described by Wisher & Evans (1975). Three Golgi fractions designated 'light', 'intermediate' and 'heavy' were prepared as described by Bergeron (1979), as modified by Evans (1985) to deplete these fractions of endosomal vesicles. Subcellular fractions were routinely prepared in the presence of proteolytic inhibitors, e.g. pepstatin, chymostatin, antipain and leupeptin (5 μg/ml).

Electrophoresis, electroblotting and Western blotting

Membrane fractions (50 μg of protein) were resolved in 12.5% or 9–18% gradient polyacrylamide gels in the presence of SDS (Laemmli, 1970) and electrophoretically transferred to

Abbreviations used: G-protein, GTP-binding protein; GTP[S], guanosine 5′-[γ-thio]triphosphate.

nitrocellulose paper (0.1μm; Schleicher and Schüll) using a Bio-Rad Transblot (Burnette, 1981). Western blot analysis was performed as described previously (Ali *et al.*, 1989). With monoclonal antibodies, blots were incubated with a second antibody [anti-(rat IgG) or anti-(mouse IgG)] before incubation with ^{125}I-Protein A.

Identification of G-proteins by [^{35}S]GTP[S] binding

Low-M_r G-proteins, transferred to nitrocellulose paper, were identified as described (Bhullar & Haslam, 1987; Comerford *et al.*, 1989). Nitrocellulose sheets were equilibrated in buffer A (50 mM-Tris/HCl, pH 7.5, 0.3% Tween-20, 12 μM-$MgCl_2$ and 1 mM-dithiothreitol) for 10 min at 20 °C and then for 30 min in buffer A containing 10 μM-ATP and 1 nM-[^{35}S]GTP[S] (1–2μCi/ml of buffer). Specificity of GTP binding was monitored by incubating the sheets in 10 μM unlabelled GTP[S] or 30μmM-GDP. Nitrocellulose sheets were washed three times for 10 min in buffer A, air-dried and autoradiographed using Kodak X-AR films.

RESULTS

Subcellular fractions derived mainly from the three plasma membrane domains of the hepatocyte, the endocytic compartment and the Golgi apparatus were examined by SDS/PAGE followed by Western blotting on to nitrocellulose sheets. Seven GTP-binding polypeptides (M_r 21000–28000) were resolved by the [^{35}S]GTP[S] overlay procedure (Fig. 1). The binding of [^{35}S]GTP[S] was specific, for it was abolished in the presence of 10 μM-GTP[S] or 30 μM-GDP (results not shown). It was evident that each of the liver subcellular fractions examined by this method contained different proportions of the various GTP-binding polypeptides. The most prominent GTP-binding polypeptides were those of M_r 25000–28000, with an M_r-28000 polypeptide being especially evident in endosomes and with an M_r-26000 polypeptide in Golgi and sinusoidal plasma membranes. A minor M_r-23000 GTP-binding polypeptide was observed in plasma membranes and endosomes.

In view of the multiplicity of the GTP-binding polypeptides in the membranes originating from the various membrane compartments in liver, the analysis was extended by probing each of the fractions using a range of antibodies generated to ras oncoproteins. A biochemical property common to all p21$^{N\text{-}ras}$ proteins is the ability to bind and hydrolyse GTP, a feature considered to be essential for their roles in signal transduction at the inner surface of the plasma membrane (Santos & Nebreda, 1989). The antibodies used in this study and their epitope specificity, where known, are listed in Table 1.

A polyclonal antibody raised to an *N*-terminal amino acid sequence of p21$^{N\text{-}ras}$ bound mainly to an M_r-28000 polypeptide in the three endosome fractions, especially heavy endosomes,

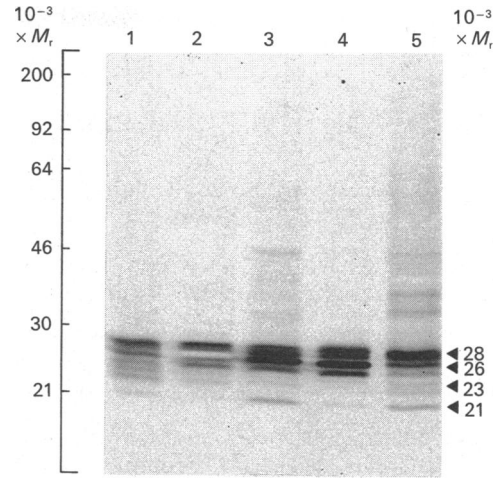

Fig. 1. Distribution of GTP-binding polypeptides in various rat liver subcellular fractions

Membrane fractions (50 μg of protein) were resolved in 9–18% polyacrylamide gels in the presence of SDS, electrophoretically transferred to nitrocellulose sheets and incubated with [^{35}S]GTP[S] as described in the Materials and methods section. Lanes: 1, canalicular plasma membranes; 2, lateral plasma membranes; 3, sinusoidal plasma membranes; 4 Golgi; 5, late endosomes.

Table 1. Antibodies used to identify ras-related polypeptides in the liver subcellular fractions

n.d. = not determined; – indicates little or no reaction.

		Major polypeptide(s) recognized ($10^{-3} \times M_r$)				
				Plasma membranes		
Antibody	Epitope specificity	Endosomes	Golgi	Sinusoidal	Lateral	Canalicular
Anti-peptide polyclonal	p21$^{N\text{-}ras}$ *N*-terminus	28/44	–	–	–	
Y-13-259 rat monoclonal	Conserved region; amino acids 63–73	26/52, 28/56	26/28/52	26/52	26/52	26/52
E_{546} mouse monoclonal	Unknown; recognizes p21$^{N\text{-}ras}$	28	–	–	–	–
E_{557} mouse monoclonal	Unknown; does not recognize p21$^{N\text{-}ras}$	28	–	–	–	–
RAS-10 mouse monoclonal	Unknown; recognizes all ras-proteins of M_r 21000–23000	23/28	23	23	n.d.	23
RAS-11 mouse monoclonal	Unknown; recognizes all ras-proteins of M_r 21000–23000	23/28	23	23	n.d.	23
6B7 mouse monoclonal	Effector domain of ras; amino acids 29–44	23/56	23	n.d.	23	23

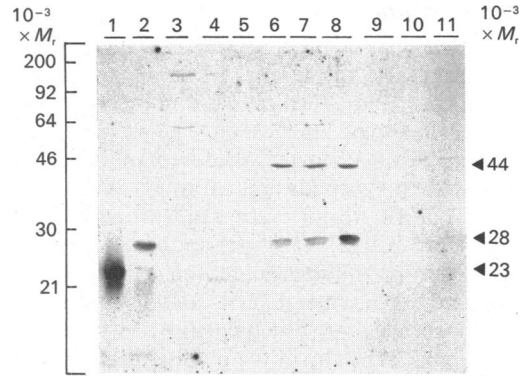

Fig. 2. Western blots showing distribution of ras-related polypeptides in N-ras recombinant protein expressed in *E. coli*, T-15 cell membranes and various rat liver subcellular fractions

Membrane fractions (50 μg of protein) were resolved in SDS/12.5%-polyacrylamide gels, electrophoretically transferred to nitrocellulose sheets and stained with polyclonal anti-p21$^{N\text{-}ras}$ antibodies. Lanes: 1, N-ras recombinant protein; 2, T-15 membranes; 3, 4 and 5, canalicular, lateral and sinusoidal plasma membranes respectively; 6, 7 and 8, late, early and heavy endosomes respectively; 9, 10 and 11, light, intermediate and heavy Golgi respectively.

with little or no binding to the plasma membrane and Golgi fractions examined concurrently (Fig. 2). This antibody bound to a recombinant ras protein (M_r 23000), and also to an M_r-28000 polypeptide in T-15 cell membranes used as a control (Gutierrez *et al.*, 1989). The antibody also identified an M_r-44000 polypeptide in the three endosome fractions. This polypeptide is unlikely to be a dimer of the M_r-28000 polypeptide and may correspond to M_r-46000 polypeptides shown to be ADP-ribosylated in liver membranes (Toki *et al.*, 1989).

A number of monoclonal antibodies have been generated against ras proteins (Table 1). One such antibody (Y-13-259), to a conserved sequence, bound to an M_r-26000 polypeptide and to a probable dimeric form (M_r-52000) in all of the membrane fractions examined (Fig. 3) as well as to an M_r-23000 ras protein expressed in *E. Coli* ras (results not shown). A minor band (M_r 28000) was also observed in the fractions, and this was most

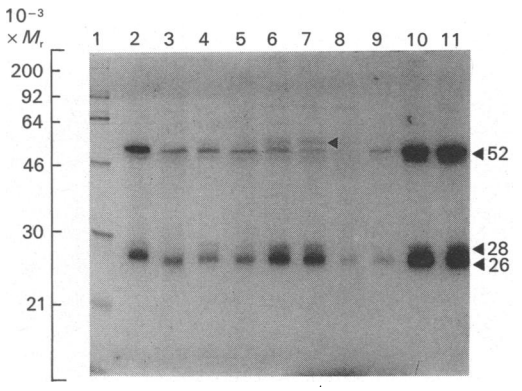

Fig. 3. Western blots showing distribution of ras-related polypeptides stained by monoclonal Y-13-259 antibodies in various rat liver subcellular fractions

The arrowhead points in lane 7 to an M_r-56000 polypeptide in endosomal fractions. For further details, see the legend to Fig. 2. Lanes: 1, M_r markers; 2, 3 and 4, canalicular, lateral and sinusoidal plasma membranes respectively; 5, residual microsomes; 6, 7 and 8, late, early and heavy endosomes respectively; 9, 10 and 11, light, intermediate and heavy Golgi respectively.

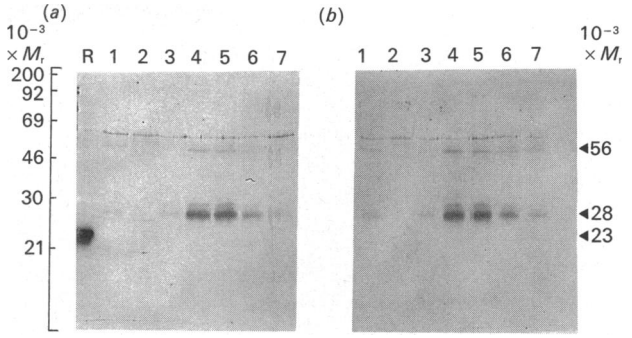

Fig. 4. Western blots showing distribution of ras-related polypeptides in recombinant protein expressed in *E. Coli* and various rat liver subcellular fractions using monoclonal antibodies E_{546} and E_{557}

Membrane fractions (50 μg of protein) were stained with anti-ras monoclonal antibodies E_{546} (*a*) and E_{557} (*b*). For further details, see the legend to Fig. 2. Lanes: R, recombinant ras; 1, 2 and 3, canalicular, lateral and sinusoidal plasma membranes respectively; 4, 5 and 6, late, early and heavy endosomes respectively; 7, Golgi.

evident in the endosomal fraction in the probable dimeric form (M_r 56000) identified by this antibody. Two further monoclonal antibodies (E_{546} and E_{557}) also identified primarily an M_r-28000 polypeptide in endosomes, with little or no labelling of polypeptides in the other subcellular fractions examined (Fig. 4); presumed dimers (M_r 56000) were also detected in the endosome fractions. In contrast with antibody E_{546}, antibody E_{557} did not bind to the recombinant ras protein of M_r 23000. Monoclonal antibodies RAS-10 and RAS-11 identified in the fractions an M_r-23000 protein, coincident with the recombinant ras product (Fig. 5). However, these antibodies also identified an M_r-28000 polypeptide in late endosomes. A further antibody (mAB 6B7) identified an M_r-23000 polypeptide in all of the membrane fractions examined as well as the recombinant ras polypeptide; this antibody, which bound relatively weakly to the membrane polypeptides compared with the others examined in this work, did however identify an M_r-56000 polypeptide in endosome fractions that may correspond to the dimer of the M_r-28000 polypeptide identified by the other monoclonal antibodies. This presumed dimer was not apparent in two plasma membrane

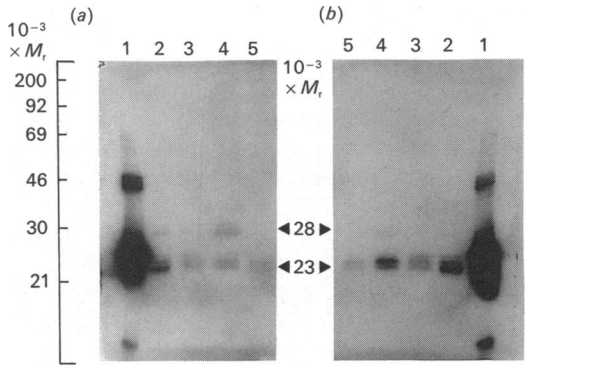

Fig. 5. Western blots showing distribution of ras-related polypeptides in recombinant protein expressed in *E. Coli* and various rat liver subcellular fractions using monoclonal antibodies E_{546} and E_{557}

Membrane fractions (50 μg of protein) were stained with anti-ras monoclonal antibodies RAS-10 (*a*) or RAS-11 (*b*). For further details, see the legend to Fig. 2. Lanes: 1, recombinant ras; 2 and 3, canalicular and sinusoidal plasma membranes respectively; 4, late endosomes; 5, Golgi.

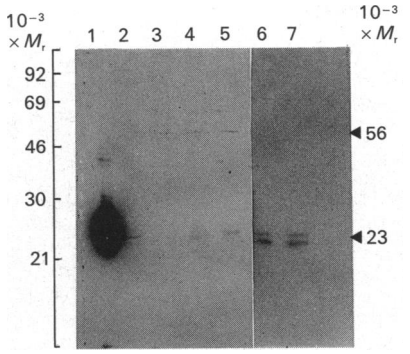

Fig. 6. Western blot showing distribution of ras-related polypeptides in recombinant protein and rat liver subcellular fractions using antibody 6B7

Membrane fractions (50μg of protein) were stained with anti-ras monoclonal antibody 6B7. For further details, see the legend to Fig. 2. Lanes: 1, recombinant ras; 2, Golgi; 3, 4 and 5, heavy, early and late endosomes respectively; 6 and 7, canalicular and lateral plasma membranes respectively.

fractions, even after extended autoradiographic exposure (Fig. 6).

DISCUSSION

The present work shows that several low-M_r G-proteins were identified by specific binding of [^{35}S]GTP[S] after their separation by SDS/PAGE and transfer to nitrocellulose sheets. Seven closely migrating GTP-binding polypeptides (M_r 21000–28000) were resolved by this procedure in the three plasma membrane, Golgi and endosome fractions isolated from liver homogenates. These fractions have been characterized in detail previously (Shears *et al.*, 1988; Ali *et al.*, 1989). Many reports have shown, mainly using [^{32}P]GTP, the complexity of low-M_r G-proteins in erythrocyte membranes (M_r 22000–27000) (Damonte *et al.*, 1990), liver microsomes (M_r 20000–25000 (Lanoix *et al.*, 1989; Nigam, 1990), Golgi (M_r 20000–25000) (Toki *et al.*, 1989), secretory granules (M_r 18000–24000) (Burgoyne & Morgan, 1989) and brain membranes (M_r 24000) (Kikuchi *et al.*, 1989). The present work, in agreement with that of Comerford *et al.* (1989), shows that good resolution of these closely migrating GTP-binding polypeptides was achieved by using the [^{35}S]GTP[S] analogue. The results also point to the conclusion that an M_r-28000 GTP-binding polypeptide was enriched in endosomes and an M_r-26000 protein was enriched in Golgi membranes. The three plasma membranes fractions examined contained different proportions of GTP-binding polypeptides, with an M_r-26000 polypeptide being enriched in sinuosidal domain membranes, probably reflecting the cross-contamination of this fraction with Golgi components (Wisher & Evans, 1975). The overall distribution in liver of the low M_r G-proteins appears to be different to that determined for the heterotrimeric G-proteins, especially the α and β subunits that were shown, by antibody binding and toxin labelling, to be most highly enriched in canalicular plasma membranes (Ali *et al.*, 1989). These polypeptides are generally not identified by the use of labelled GTP after gel electrophoresis. A similar subcellular distribution of the enzymes which hydrolyse inositol trisphosphate and tetrakisphosphate has also been noted (Shears *et al.*, 1988).

Since the *ras* oncogene family constitutes a major class of GTP-binding proteins of similar M_r values to those of polypeptides detected by this and similar approaches, a survey of the polypeptides identified in the liver subcellular fractions by a panel of antibodies to ras proteins was undertaken. Ras proteins were initially thought to be associated mainly with the inner surface of the plasma membrane, where they are involved in signal transduction, but a further role in intracellular membrane trafficking is increasingly appreciated. The present work, on the basis of the results obtained with the seven antibodies, indicates that an M_r-28000 polypeptide and/or an M_r-56000 polypeptide (probably a dimeric form) were resident predominantly in endosome membranes. Three of the antibodies recognized this M_r-28000 protein specifically in endosomal fractions. This M_r-28000 polypeptide thus emerges as a candidate for a endosome-specific component. An M_r-23000 polypeptide, detected in all of the fractions, and especially in the plasma membranes by three monoclonal antibodies, probably corresponds to the ras polypeptide functioning at the cell surface. The results obtained with antibody Y-13-259 were more difficult to analyse, for this antibody was raised to a conserved region of the ras protein and is thus likely to identify other members of this family of proteins. Although this antibody recognized an M_r-26000 polypeptide in the fractions, it also recognized an M_r-56000 polypeptide that may correspond to a dimeric form of the M_r-28000 polypeptide in the endosome fractions.

Overall, the results show that an M_r-23000 polypeptide is a ras-related polypeptide present in liver plasma membranes, Golgi and endosomal membranes and is probably involved in inter-compartmental membrane trafficking (Klausner, 1989). Indeed, monoclonal antibodies RAS-10 and RAS-11 indicated that the highest level of an M_r-23000 doublet was present in canalicular plasma membranes, a result that mirrors the distribution of the heterotrimeric G-proteins (Ali *et al.*, 1989). It has been suggested, for example, that small G-proteins are involved in signalling via the phosphatidylinositol cycle in liver plasma membranes (Urumow & Wieland, 1990), although this continues to be a controversial area. The results also indicate that a mainly Golgi location for an M_r-26000 polypeptide and an endosomal location for the M_r-28000 polypeptide identified would allow assignment of these two polypeptides to a role in the endocytic and exocytic arms of intracellular trafficking. This is consonant with the evidence accruing from studies with yeast mutants (Goud *et al.*, 1988; Segev *et al.*, 1988; Beckers & Balch, 1989) for a role of low-M_r G-proteins in trafficking along the secretory pathway and for GTP-binding proteins in the transport of molecules along the endocytic pathway (Mayorga *et al.*, 1989*a*,*b*). A characteristic feature observed with some of the antibody-stained gels was the presence of closely migrating doublets. This is unlikely to indicate proteolytic degradation of these constituents, as inhibitors were included during preparation, and is possibly related to post-translational processing such as phosphorylation (Lapetina *et al.*, 1989) or acylation (Buss *et al.*, 1989; Gutierrez *et al.*, 1989).

The antibodies used in the present work were generated to various parts of p21^{ras} including an *N*-terminal region, a conserved region and the effecter domain (Sigal *et al.*, 1986). Significantly, one of the antibodies that did not recognize recombinant ras protein (E_{557}) identified an M_r-28000 polypeptide in endosomes. This observation is highly suggestive that these polypeptides, which correspond in electrophoretic mobility to those that bind GTP and are shown to be highly enriched in endosomes, are members of the expanding ras family that show various degrees of sequence similarity and are now recognized to be present in a variety of mammalian and other membranes (Haldar *et al.*, 1989; Cross, 1990; Santos & Nebreda, 1990).

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