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Fractionation of the β Subunit Common to Guanine Nucleotide-Binding Regulatory Proteins with the Cytoskeleton

KENNETH E. CARLSON, MARILYN J. WOOLKALIS, MARISA G. NEWHOUSE, and DAVID R. MANNING

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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SUMMARY

G_s and G_i are guanine nucleotide-binding proteins that mediate the stimulation and inhibition, respectively, of adenylate cyclase. The extent to which the β subunit common to these proteins may be associated with the cytoskeleton of S49 mouse lymphoma cells was assessed by procedures of differential detergent extraction and immunotransfer blotting. Treatment of cells with 1% Triton X-100 results in nearly quantitative extraction of cellular protein, phospholipid, tubulin, and the catalytic component of adenylate cyclase. Approximately 65% of the β subunit is refractory to extraction. This population of the β subunit, along with a population of actin presumed to originate from actin filaments, is subsequently solubilized with hypotonic medium containing 0.5% sodium deoxycholate and 1% Tween-40. The pattern of distribution of the β subunit among sequential detergent extracts is corroborated by that of generated immunoreactive tryptic fragments. These results are consistent with the interaction of guanine nucleotide-binding proteins with the cytoskeleton.

G_s and G_i are guanine nucleotide-binding proteins that mediate activation and inhibition, respectively, of adenylate cyclase by agonists and other agents (1). Both proteins are associated with the plasma membrane and are heterotrimers consisting of α (45 or 52 kDa for G_s; 41 kDa for G_i), β (35 or 36 kDa), and γ (5–10 kDa) subunits (2–4). Activation of adenylate cyclase by many agents proceeds through the dissociation of G_s into monomeric α- and heterodimeric βγ subunits (5, 6); inhibition of this enzyme probably proceeds through an analogous form of dissociation of G_i (7, 8). Whereas the α subunits of G_s and G_i differ structurally, the β and γ subunits of these two proteins are identical (9, 10). The β subunit, moreover, exhibits a high degree of homology with the β subunits of two other guanine nucleotide-binding proteins, G_o and transducin (9–11).

A possible determinant in the responsiveness of adenylate cyclase to agonists is the interaction of G_s, G_i, or the catalytic component of this enzyme with the cytoskeleton. The occurrence of such interactions has been suggested by the ability of chemical disruptants of the cytoskeleton to enhance agonist- and cholera toxin-effected increases in intracellular levels of cAMP (12–15). An interaction involving G_s is also implied by the resistance of this protein, identified as a substrate for ADP-ribosylation by cholera toxin, to extraction from rat and pigeon erythrocyte ghosts with TX (16, 17). The precise extent to which components of adenylate cyclase interact with the cytoskeleton, however, remains unclear.

Antisera of specificity for the β subunit common to guanine nucleotide-binding regulatory proteins have been recently developed, and these permit a more direct assessment of the extent to which regulatory proteins may interact with the cytoskeleton. In the present study, S49 mouse lymphoma cells were subjected to conventional procedures of differential detergent extraction to achieve isolation of the cytoskeleton and subsequent solubilization of actin filaments. The distribution of the β subunit among the obtained subcellular fractions was determined by procedures of immunotransfer blotting. This distribution, in turn, is compared to those determined for actin, tubulin, and the catalytic component of adenylate cyclase.

Materials and Methods

Protein preparations. Purification of βγ heterodimers from bovine brain and rabbit liver was achieved by chromatography of G_o- and G_i-containing AcA-34 fractions on heptylamine-Sepharose during the course of purification of the latter proteins (3, 11). Transducin was purified from illuminated bovine rod outer segment disks by elution with hypotonic buffer containing GTP as previously described (18). G_s was purified from rabbit liver by procedures of Sternweis *et al.* (2).

Preparation of antisera. Rabbit antisera of specificity for the β

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ABBREVIATIONS: G_s and G_i, guanine nucleotide-binding proteins that mediate stimulation and inhibition, respectively, of adenylate cyclase; G_o, a guanine nucleotide-binding protein isolated from bovine brain; TX, Triton X-100; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaDodSO$_4$-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); DOC, sodium deoxycholate; Tw, Tween-40.

subunit were prepared by multiple intradermal injections of a preparation of $\beta\gamma$ heterodimer obtained from bovine brain (19). The preparation consisted of heterodimer (80%), α subunit of G_o (10%), and minor contaminants. Reported data were obtained with an antiserum containing particularly high titers of antibodies directed toward the β subunit and cross-reacting minimally with other cellular proteins (antiserum 5357). As shown in Fig. 1, the immunoreactivities of β subunits isolated either as $\beta\gamma$ heterodimers from bovine brain and rabbit liver or as transducin from bovine retina are nearly equivalent; although both the 35-and 36-kDa forms of this subunit (2, 11) are discerned, the antiserum exhibits a 3-fold selectivity for the latter form. Results pertaining to the subcellular distribution of the β subunit were corroborated with a previously described antiserum (antiserum U49; Ref. 20) generously provided by Dr. Susanne M. Mumby, University of Texas Health Science Center at Dallas. A rabbit antiserum of specificity for the α subunits of G_s, G_i, G_o, and trasnsducin (antiserum 6458) was generated by multiple intradermal injections of the conjugated, synthetic peptide designated $G_{\alpha,common}$ in Ref. 20. Monoclonal antibodies to chicken gizzard actin were the generous gift of Dr. James L. Lessard, University of Cincinnati College of Medicine, and monoclonal antibodies to tubulin were obtained from Amersham Corp.

Differential detergent extraction. Procedures of differential detergent extraction were patterned after those developed by Lenk *et al.* (21) and were conducted at 0° with buffers containing freshly added phenylmethylsulfonyl fluoride (2 mM). Approximately 2×10^8 S49 mouse lymphoma cells (2×10^6 cells/ml) were collected by centrifugation, washed, and resuspended in 2.5 ml of 300 mM sucrose, 10 mM HEPES (pH 6.8), 100 or 300 mM KCl, 2.5 mM $MgCl_2$, and 10 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TX was added to a concentration of 1%. After a 5–20-min period of incubation with occasional swirling, the suspension was subjected to centrifugation at $500 \times g$ for 3 min. The supernatant fraction (TX-extract) was collected, and the pellet was resuspended by homogenization in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5% DOC, and 1% Tw either immediately or after two washes. After 30 min, the suspension was subjected to centrifugation. The supernatant fraction (DOC/Tw-extract) was removed, and the final pellet was homogenized in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM $CaCl_2$, then incubated at 37° for 20 min with 50 μg/ml each of DNase I, RNase A, and micrococcal nuclease. Where cited in the text, cells were incubated for 1 hr at 37° in serum-free medium containing either cytochalasin D (20 μg/ml), colchicine (50 μM), taxol (5 μM), or vehicles (dimethyl sulfoxide or H_2O) prior to extraction.

Electrophoresis and immunotransfer blotting. Samples were subjected to discontinuous $NaDodSO_4$-PAGE as described by Laemmli (22). Gels were either stained with Coomassie brilliant blue or used in procedures of immunotransfer blotting employing either rabbit antisera or monoclonal antibodies and horseradish peroxidase-conjugated secondary antibody as previously described (19).

Assay for adenylate cyclase. The activity of the catalytic component of adenylate cyclase was assessed with purified, hepatic G_s activated with GTPγS as previously described (19). Assays contained 10–50 μg of extract protein and 0.08–0.8 μg of activated G_s in a volume of 100 μl. Calculated subcellular distributions of activity were identical for all concentrations of G_s utilized, and activities expressed in the absence of G_s were less than 10% of those expressed in its presence. Production of cAMP was linear with respect to the duration of the assay (i.e., < 40 min) and the amount of included extract.

Miscellaneous procedures. S49 mouse lymphoma wild-type cells (subclone 24.3.2; Cell Culture Facility, University of California, San Francisco) were maintained in suspension culture as described by Ross *et al.* (23). Cellular membranes (i.e., the $43,000 \times g$ pellet) and plasma membranes (i.e., combined 20/30 and 30/40% sucrose interfaces) were obtained by procedures of nitrogen cavitation and centrifugation (23). Limited tryptic digestion was performed according to the method of Fung and Nash (24). Phospholipid was measured for twice-washed cell suspensions and TX-resistant subcellular fractions by the methods of Hess and Derr (25); both fractions were detergent-free. Protein was quantitated by staining with Amido black (26). Optical intensities of adsorbed chromogen (4-chloro-1-naphthol) following immunotransfer blotting were determined by densitometric analysis (19). All values are expressed as means ± standard error.

Results

Differential detergent extraction. TX has been demonstrated to effect rapid solubilization of plasma and intracellular membranes and thereby enable isolation of detergent-resistant arrays of actin and intermediate filaments (21, 27–29). Typically, such arrays are referred to as the cytoskeleton. In the present study, S49 mouse lymphoma cells were extracted with 1% TX at a detergent:phospholipid ratio of 25:1 (w/w). KCl was included in the extraction medium at a concentration of either 100 or 300 mM: the lower concentration is similar to that occurring within the cell, whereas the higher concentration prevents disruption of the cytoskeleton upon repeated washing of collected TX-insoluble material. Data obtained at the two concentrations of KCl are equivalent (see below). The initial extraction with TX resulted in the release of approximately 80% of total protein into a low-speed supernatant fraction (Table 1); 79% of phospholipid and greater than 90% of tubulin (assessed by immunotransfer blotting) were also released (data not shown). Subsequent extraction of sedimentable material with 0.5% DOC and 1% Tw in hypotonic medium yielded 2–5% of cellular protein. The remaining insoluble material (pellet) was recovered by resuspension and incubation with nucleases.

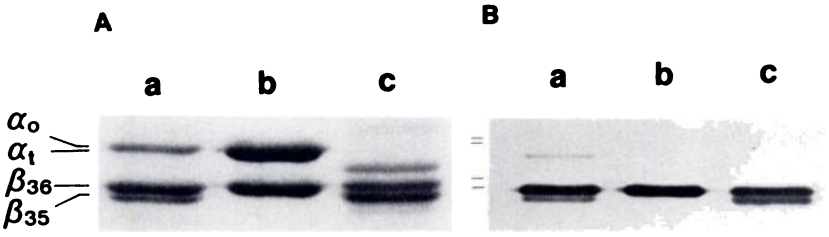

Fig. 1. Immunoreactivities of β subunits isolated from brain, retina, and liver. Preparations of $\beta\gamma$ heterodimer from bovine brain (*a*), transducin from bovine retina (*b*), and $\beta\gamma$ heterodimer from rabbit liver (*c*) were alkylated (11) and subjected to $NaDodSO_4$-PAGE (11% acrylamide) followed by: A, Coomassie blue-staining, or B, immunotransfer blotting utilizing antiserum 5357. Volumes of samples containing proteins to be visualized with Coomassie blue were adjusted to provide approximately 3 μg of β subunit. Samples for immunoblotting were prepared in an identical manner but were diluted 1:15. The α subunits of G_o and transducin are denoted α_o and α_t, respectively, and two forms of the β subunit are denoted β_{35} and β_{36}. The optical intensities of Coomassie blue and adsorbed chromogen associated with the β subunits are linear with respect to the amount of protein subjected to analysis. No immunoreactive protein is detected in regions of the nitrocellulose membrane not shown.

TABLE 1

Distribution of protein upon differential detergent extraction of S49 mouse lymphoma cells

S49 mouse lymphoma cells were solubilized with 1% TX at either 100 or 300 mM KCl as described in Materials and Methods. The 500 × *g* supernatant fraction (designated TX-extract) was collected, and the pellet was extracted in hypotonic medium containing 0.5% DOC and 1% Tw either immediately or after washes in buffer containing 0.1% TX. The subsequent 500 × *g* supernatant fraction (DOC/Tw-extract) was collected, and the insoluble material (Pellet) was resuspended and incubated with nucleases. The amounts of total protein, β subunit, and activity of the catalytic component of adenylate cyclase in each fraction are expressed as percentages ± standard error of totals for all fractions. Values in parentheses refer to the number of determinations.

Protein	Subcellular fraction			
	TX-extract	Wash	DOC/Tw-extract	Pellet
	% total			
100 mM KCl				
Total (11)	75 ± 2	–[a]	5 ± 1	20 ± 2
β-Subunit (9)[b]	35 ± 6	–[a]	63 ± 6	2 ± 1
Catalyst (5)[c]	94 ± 2	–[a]	6 ± 1	
300 mM KCl				
Total (8)	86 ± 2	2 ± 1	2 ± 1	10 ± 2
β Subunit (8)[b]	28 ± 4	1 ± 1	69 ± 4	2 ± 1
Catalyst (6)[c]	89 ± 1	5 ± 1	6 ± 1	

[a] Washes were not performed for material refractory to extraction with TX at 100 mM KCl.

[b] Typically, the amounts of protein subjected to immunotransfer blotting followed by densitometric analysis were 100, 50, 25, and 25 μg for TX-extract, wash, DOC/Tw-extract, and pellet, respectively. Percentage of total is calculated according to the equation $[(A/mg)_i \times (\%\ \text{total protein})_i / \sum (OD/mg)_i \times (\%\ \text{total protein})_i)] \times 100$, where *i* represents individual fractions.

[c] Values represent percentages of enzymic activity associated with the TX-extract and the resultant pellet was dispersed by homogenization in extraction medium containing TX. Activity present within a suspension of TX-solubilized cells is completely recovered in these two fractions.

Analysis of the obtained fractions by $NaDodSO_4$-PAGE followed by Coomassie blue staining (Fig. 2A) or immunotransfer blotting (data not shown) demonstrates a substantial enrichment of the DOC/Tw-extract in actin. This population of actin represents approximately 30% of that initially present within the cell.

Subcellular distribution of the β subunit. The distribution of the β subunit among the obtained subcellular fractions was ascertained by immunotransfer blotting utilizing antiserum 5357. The predominant immunoreactive protein is present in the DOC/Tw-extract and has an electrophoretic mobility equivalent to that of the β subunit (i.e., 35/36 kDa; Fig. 2B); often this protein can be resolved into two species (Fig. 2C). The 35/36-kDa immunoreactive protein was not detected with preimmune serum or antisera directed toward the α subunits of known guanine nucleotide-binding regulatory proteins. All other observed immunoreactive proteins were detected with preimmune serum or occurred in a variable fashion. Approximately 65% of the 35/36-kDa immunoreactive protein, presumed to be the β subunit (see below), was present in the DOC/Tw-extract (Table 1). Attempts to alter the distribution of this protein, as well as that of actin, with cytochalasin D have not been successful; colchicine and taxol similarly have no effect, with the exception that taxol causes an appreciable redistribution of tubulin into the DOC/Tw-extract.

The efficiency of transfer of the presumptive β subunit is equivalent for all subcellular fractions: the optical intensity of adsorbed chromogen is a linear function of the amount of protein analyzed and proves to be additive upon mixing of fractions prior to analysis. The absence of relevant proteolytic activity is demonstrated by the stability of purified βγ heterodimer included in TX-extracts for up to 2 hr. Moreover, the distribution of the β subunit is identical regardless of the time of incubation of cells in TX prior to extraction with DOC/Tw.

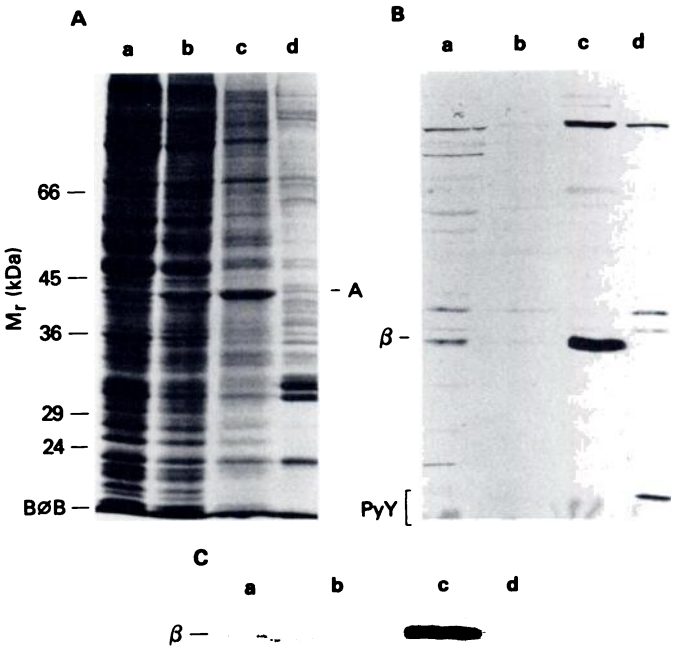

Fig. 2. Distribution of β subunit among subcellular fractions obtained by differential detergent extraction. S49 mouse lymphoma cells were extracted sequentially with TX (300 mM KCl) and DOC/Tw as described in Materials and Methods. Individual fractions were subjected to $NaDodSO_4$-PAGE (11% acrylamide) followed by Coomassie blue-staining (A) or immunotransfer blotting utilizing antiserum 5357 (B and C). Fractions depicted in A and B are identical; C represents a portion of an immunoblot for another preparation of fractions that is enlarged to illustrate the doublet of immunoreactive proteins that have mobilities equivalent to that of the β subunit. Samples are: *a*, 100 μg of TX extract; *b*, 50 μg of wash; *c*, 25 μg of DOC/Tw-extract; and *d*, 25 μg of final pellet. Actin is denoted by *A*. The mobility of the β subunit was determined with the subunit isolated as a heterodimer from bovine brain. Molecular weight standards are bovine serum albumin (66 kDa), ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), and trypsinogen (24 kDa). *BØB* (bromphenol blue) and *PyY* (Pyronin Y) denote dye fronts; the mobility of Pyronin Y is slightly greater than that of bromphenol blue in gels containing 11% acrylamide.

The subcellular distribution of the β subunit is corroborated by the distribution of immunoreactive 14- and 26-kDa tryptic fragments generated upon incubation of fractions with trypsin under nondenaturing conditions (Fig. 3). These fragments have been previously described as originating from the β subunit (24). Although the recovery of immunoreactive material following proteolysis was approximately one-half that anticipated from studies with purified subunit (data not shown), the distribution of the fragments was comparable to that observed for the presumptive β subunit: approximately 60% of each of the generated fragments was present within the DOC/Tw-extract. The relative optical intensities of the adsorbed chromogen for the 14- and 26-kDa fragments are similar to those for fragments of purified β subunit. The intensities for the individual fragments within TX- and DOC/Tw-extracts proved to be additive upon combination of these two extracts prior to digestion and analysis.

The presumptive β subunit was detected within preparations of total cellular and plasma membranes (Fig. 4); this protein is

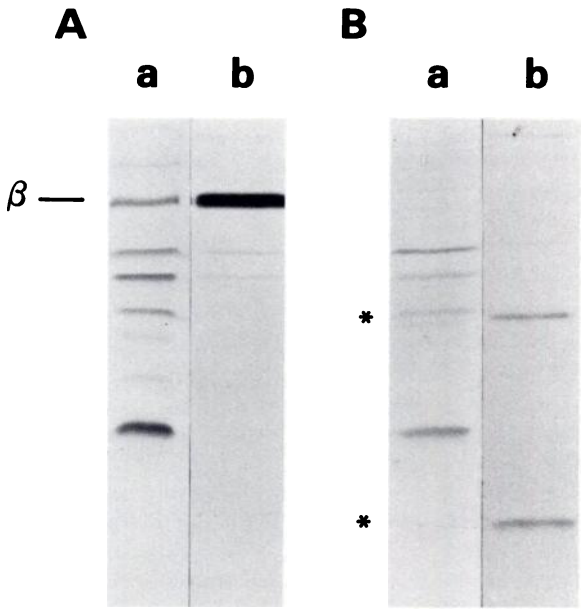

Fig. 3. Immunoreactive tryptic peptides generated in TX- and DOC/Tw-extracts. S49 mouse lymphoma cells were subjected to differential detergent extraction, and TX- and DOC/Tw-extracts were incubated for 10 min at 23° under nondenaturing conditions either without (A) or with (B) trypsin at a 1:60 (w/w) ratio of trypsin:protein. Extracts were then subjected to $NaDodSO_4$ -PAGE (15% acrylamide) followed by immunotransfer blotting utilizing antiserum 5357. Samples are: *a*, 150 μg of TX-extract or corresponding tryptic digest, and *b*, 20 μg of DOC/Tw-extract or corresponding tryptic digest. *, peptides co-migrating with the 14- and 26-kDa tryptic peptides of purified β subunit. The DOC/Tw-extract depicted in this figure represents 6% of cellular protein; results have been replicated with four other preparations of extracts.

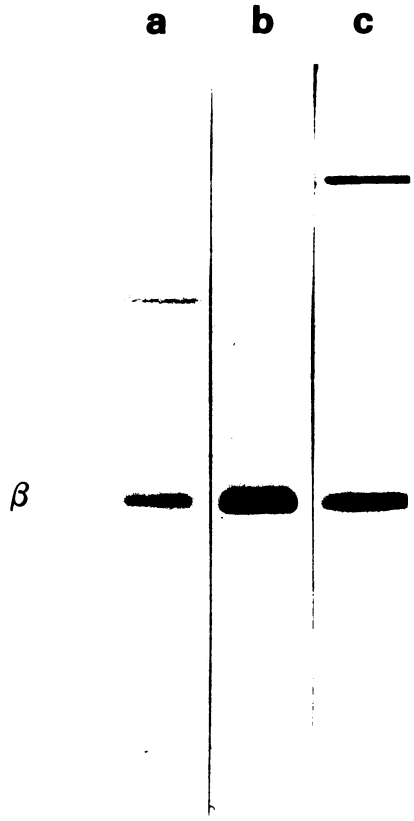

Fig. 4. Relative amounts of β subunit within membranes and DOC/Tw-extract. Preparations of cellular and plasma membranes were obtained from S49 mouse lymphoma cells by differential and sucrose-density centrifugation following nitrogen cavitation. Fifteen μg of total cellular membrane (a), plasma membrane (b), and a DOC/Tw-extract (c) were subjected to $NaDodSO_4$-PAGE (11% acrylamide) followed by immunotransfer blotting utilizing antiserum 5357. The DOC/Tw-extract depicted in this figure represents 2.1% of cellular protein.

not detected within the cytoplasm. Enrichment of the plasma membrane in the immunoreactive protein was approximately 10-fold greater than that of total cellular membrane. Using purified β subunit to calibrate the optical intensity of adsorbed chromogen with protein mass, the immunoreactive protein was found to constitute approximately 0.4% of the protein within plasma membrane.

Distribution of other components of adenylate cyclase. The distribution of the catalytic component of adenylate cyclase was assessed with purified, GTPγS-activated G_s as an activator (Table 1). Activation of the catalyst in a TX-solubilized suspension of S49 cells was half-maximal at 2 μg/ml of activated G_s, and the maximum specific activity exhibited by the suspension was 150 pmol of cAMP/min/mg of protein. Approximately 90% of catalytic activity was recovered in the TX-extract; the remaining 5–10% was found within material refractory to extraction with TX.

Attempts have also been made to assess the subcellular distribution of the α subunits of G_i and G_s by immunotransfer blotting utilizing antiserum 6458. Although the data are consistent with the existence of at least 60% of the α subunit of G_i in the DOC/Tw-extract (not shown), mixing of extracts prior to analysis indicates that the transfer efficiency for this subunit in TX-extracts is poor. The α subunit of G_s is apparently not present in amounts sufficient for detection.

Discussion

Procedures of differential detergent extraction are widely employed in the isolation of components of the cytoskeleton and associated proteins (30–34). The efficacy of these procedures appears to extend to S49 mouse lymphoma cells: treatment of cells with TX results in nearly quantitative extraction of cellular protein, phospholipid, tubulin, and the catalytic component of adenylate cyclase; approximately 30% of actin is refractory to extraction with TX but is solubilized in hypotonic medium containing sodium deoxycholate and Tween-40. The TX-soluble and TX-resistant forms of actin are presumed to represent globular and filamentous populations, respectively. The maximum amount of protein co-fractionating with filamentous actin is 5% of that contained within the cell (Table 1). This amount is probably an overestimate of that specifically associated with the cytoskeleton, however, since some of the protein present within the DOC/Tw-extract may be attributable to carry-over.

The protein, or doublet of proteins, most readily detected among subcellular fractions with antiserum 5357 is identified as the β subunit: this protein has an electrophoretic mobility identical to that of purified β subunit, exists within membranes obtained by conventional procedures of cell lysis and differential centrifugation, and is not recognized with a preimmune serum. Moreover, the amount of immunoreactive protein within plasma membranes (ca. 0.4%) is similar to that calculated for the β subunit by assays of deactivation of G_s (ca. 0.2%; Ref. 35). The basis for the frequent appearance of this protein as a

doublet is not clear, but probably involves the existence of isozymic or otherwise modified forms of the subunit (2, 11).

Approximately 65% of the total population of the β subunit is associated in a stable manner with the cytoskeleton. Specifically, this population of subunit co-fractionates with actin that is presumed to originate from actin filaments. The extent of this association is corroborated by the distribution of generated immunoreactive tryptic peptides. The subcellular distribution of the β subunit is also consistent with that previously inferred for the α subunit of G_s in erythrocytes (16, 17). Measurements of the latter subunit, however, are compromised by uncertainties pertaining to the stoichiometries of ADP-ribosylation and the effects *per se* of this modification on interactions with the cytoskeleton. It is important to point out that the β subunit observed in this study may not originate solely from G_s and G_i, but from other types of GTP-binding regulatory proteins yet to be characterized. Heterogeneity of such proteins may account for the relative amounts of β subunit found within TX- and DOC/Tw-extracts.

The basis for co-fractionation of the β subunit with actin remains to be determined. It is possible that the β subunit is associated either directly with actin filaments or indirectly with these filaments via actin-associated proteins. Indirect associations could include those mediated by α or γ subunits. It is also conceivable that the β subunit exists in polymerized or otherwise aggregated forms intrinsically resistant to extraction with TX but not DOC/Tw. Small domains of the plasma membrane resistant to extraction with nonionic detergents have been intimated to exist (34, 36), and localization of the β subunit to these regions constitutes a third possibility for co-fractionation; the catalytic component of adenylate cyclase, however, is readily extracted with TX and would therefore not be constrained to such regions. Associations of the β subunit with the plasma membrane and cytoskeleton need not be mutually exclusive. Typical means of preparing plasma membranes (e.g., those employing homogenization or pressure-cavitation) would be expected to disrupt certain elements of the cytoskeleton; portions of these and other elements may remain attached to the plasma membrane during purification. Actin, for instance, is a major protein in highly purified preparations of plasma membrane (e.g., Ref. 34).

Regardless of the basis of co-fractionation of the β subunit with the cytoskeleton, the implied physical restraint of this and associated subunits is likely to be a determinant in the responsiveness of adenylate cyclase to stimulatory and inhibitory agonists. Restraint of guanine nucleotide-binding regulatory proteins may also have a bearing on other agonist-effected processes, such as the influx of Mg^{2+} and the metabolism of phosphatidylinositol (37, 38). Finally, interaction of guanine nucleotide-binding regulatory proteins with the cytoskeleton may enable participation of these proteins in receptor-mediated endocytosis, down-regulation of receptors, and spatial orientation of second-messenger systems.

Acknowledgments

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Send reprint requests to: Dr. David R. Manning, Department of Pharmacology, University of Pennsylvania School of Medicine, 36th and Hamilton Walk, Philadelphia, PA 19104-6084.