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Structural Characteristics of the 35- and 36-kDa Forms of the β Subunit Common to GTP-Binding Regulatory Proteins

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SUMMARY

The β subunit common to GTP-binding regulatory proteins can be resolved into immunologically distinct proteins typically referred to as 35- and 36-kDa forms of the subunit. The extent to which these two forms are structurally related was examined by isoelectric focusing, nonequilibrium pH gradient electrophoresis (NEPHGE), and two-dimensional peptide mapping. Each of the two forms of the β subunit isolated from rabbit liver was found to comprise species of protein having isoelectric points of 5.7, 5.8, and 5.95; those having an isoelectric point of 5.8 were the most prominent. The β subunits isolated from bovine brain and rod outer segments exhibited species having identical isoelectric points. Similar patterns of species were also noted for forms of the β subunit detected immunologically within diverse types of cells. Analysis of the 35- and 36-kDa forms of the β subunit by NEPHGE confirmed the absence, with only minor exceptions, of other constituent proteins. Subjection of chymotryptic peptides to high voltage electrophoresis and chromatography on cellulose revealed similarities, as well as differences, between the two forms of the β subunit. These data provide evidence that the 35- and 36-kDa forms of the β subunit are indeed structurally related and are conserved among diverse types of cells.

G_s, G_i, G_o, and transducin constitute a family of GTP-binding proteins that mediate the actions within membranes of agonist- and light-activated receptors (1). Each of these proteins has been identified upon purification as a heterotrimer consisting of α (39–52 kDa), β (≃35 kDa), and γ (≃10 kDa) subunits. While the α subunits have been demonstrated to have distinct primary structures and activities (2–7), the β subunits are overtly similar (2, 8). The γ subunits of G_s, G_i, and G_o appear to be homologous but differ from the γ subunit of transducin (8). Differences in the γ subunits among tissues have also been suggested (9).

The β subunit common to GTP-binding regulatory proteins may be resolved electrophoretically into one or both of two closely migrating proteins typically referred to as 35- and 36-kDa forms of the subunit (10, 11). When isolated from rabbit liver, the β subunit comprises approximately equal amounts of the two forms; that obtained from bovine brain or ROS, however, consists predominantly or entirely of the 36-kDa form. The extent to which the two forms of the subunit differ in primary structure is unclear. Antibodies generated against the β subunit isolated from bovine brain or ROS typically recognize the 36-kDa form preferentially, if not exclusively (12). Yet, those generated ostensibly against the 35-kDa form purified from human placenta may recognize both forms (9). Proteolysis of the two forms within polyacrylamide gels reveals both similarities and differences. Thus, the two forms of the β subunit may constitute either two distinct proteins or a single protein subject to limited post-translational modification.

In the present study, procedures of IEF, NEPHGE, and two-dimensional peptide mapping are used to further characterize the primary structures of each of the two purported forms of the β subunit. These procedures provide information pertaining to the homogeneity of the forms as typically isolated and the extent to which they are structurally related. It is demonstrated that the 35- and 36-kDa forms of the β subunit have identical isoelectric properties and proportions of constituent species; nevertheless, some differences in primary structure are evident. It is also confirmed that only marginal differences exist among β subunits either purified from or detected within diverse types of tissue and cultured cells.

Materials and Methods

Purification of protein. The β subunit was isolated from rabbit liver by three different means: 1) as a component of G_s purified to homogeneity by the methods of Sternweis *et al.* (10), 2) as a nearly

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ABBREVIATIONS: G_s and G_i, stimulatory and inhibitory GTP-binding proteins of adenylyl cyclase that contain 45- or 52-kDa (G_s) and 41-kDa (G_i) α subunits; G_o, a GTP-binding protein isolated from bovine brain containing a 39-kDa α subunit; ROS, rod outer segments; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient electrophoresis; CEF, chicken embryo fibroblasts; NRK, normal rat kidney; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, tosyl lysyl chloromethyl ketone.

(>90%) homogeneous βγ heterodimer eluting prior to G_s during purification of the latter on hydroxyapatite as described by Bokoch *et al.* (13), and 3) as one of several proteins (purity 60%) eluting after G_s during the course of purification of G_s and G_i by chromatography on heptylamine-Sepharose in the absence of fluoride. The β subunit was isolated from bovine brain as a component of G_o and G_i or as a nearly (80%) homogeneous βγ heterodimer eluting after G_s upon chromatography on heptylamine-Sepharose by the methods of Sternweis and Robishaw (11). Transducin was purified to homogeneity from illuminated bovine ROS by elution with a hypotonic solution containing GTP as previously described (14).

Purification of membranes. Crude (i.e., 100,000 × *g* pellet) and purified plasma membranes were prepared from S49 mouse lymphoma wildtype cells (subclone 24.3.2; Cell Culture Facility, University of California, San Francisco) by differential and sucrose-density centrifugation following nitrogen-cavitation (15). Membranes were obtained from CEF, NRK cells, and human placental cytotrophoblasts by hypotonic lysis and differential centrifugation as previously described for CEF (16). Membranes were obtained from mouse sperm as described by Kopf *et al.* (17). Cytotrophoblasts and membranes of sperm were the generous gifts of Drs. Jerome F. Strauss III, and George L. Gerton, respectively, University of Pennsylvania School of Medicine.

SDS-PAGE, IEF, and NEPHGE. Samples were subjected to discontinuous SDS-PAGE (11% acrylamide) as described by Laemmli (18). IEF in one dimension and SDS-PAGE in a second dimension were performed as previously described (16), with the following exceptions: 1) samples were treated with 4% Lubrol PX, 0.2% sodium deoxycholate, 8 mM K_2CO_3, 70 mM dithiothreitol, and 3.3% Bio-Lytes 5/7 (Bio-Rad Laboratories); samples were then clarified by centrifugation, and solid urea was added to a final concentration of 9.5 M; and 2) focusing gels contained 2% Lubrol PX (rather than 2% NP-40) and 4% Bio-Lytes 5/7. Established gradients in pH typically extended from 4.5 to 7.0 and were linear from 5.3 to 6.9. NEPHGE followed by SDS-PAGE was performed according to the method of O'Farrell *et al.* (19). NEPHGE was conducted for 1600 V-hr using 4% 3/10 Bio-Lytes; gradients in pH were linear from 4.0 to 9.0. Solubilization and equilibration buffers for IEF and NEPHGE contained 40 mM dithiothreitol and were freshly prepared; reduction and alkylation of protein were not routinely performed. Recovery of protein following focusing or NEPHGE was monitored by inclusion of an identical amount of sample in a well adjacent to the first-dimension tube gel for electrophoresis in the second dimension.

Immunotransfer blotting. Immunotransfer blotting entailed electrophoretic transfer of proteins separated by IEF/SDS-PAGE or NEPHGE/SDS-PAGE and consecutive incubations of blotted nitrocellulose membranes with rabbit antisera and horseradish peroxidase-conjugated goat anti-rabbit IgG as described (16). Rabbit antisera were generated using either the βγ heterodimer isolated from bovine brain (i.e., antisera 5356 and 5357) or a conjugated, synthetic peptide corresponding to the amino terminal sequence of a 27-kDa tryptic peptide of the β subunit [i.e., antiserum U49 (12)]. Procedures of immunization are described in Refs. 12 and 16. Antiserum U49 was kindly supplied by Dr. Susanne M. Mumby, University of Texas Health Science Center at Dallas. The specificities of antisera 5357 and U49 for the 35- and 36-kDa forms of the β subunit have been previously documented (12, 20); antiserum 5356, like U49 but differing from 5357, does not enable detection of the 35-kDa form.

Two-dimensional peptide mapping. Procedures of radioiodination, proteolysis, and peptide mapping were performed according to the method of Elder *et al.* (21), as modified by Glenney and Glenney (22). The 35- and 36-kDa forms of the β subunit (ca. 5 μg) purified from rabbit liver were resolved by SDS-PAGE and directly visualized with Coomassie blue. Portions of the polyacrylamide gel containing individual forms were carefully excised, dried, and incubated for 1 hr at room

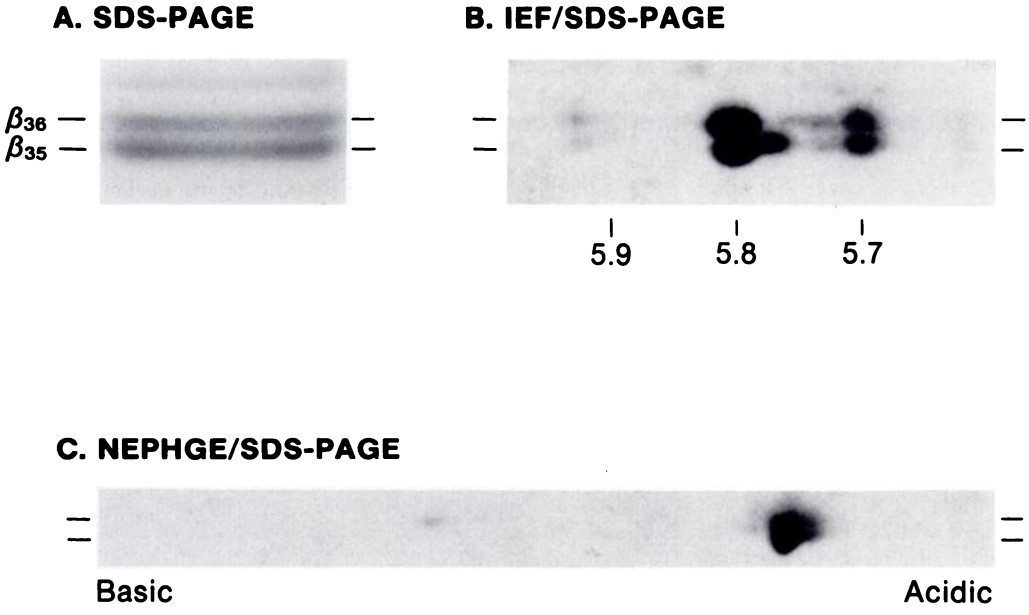

Fig. 1. Electrophoretic analysis of β subunit from rabbit liver. The βγ heterodimer isolated from rabbit liver (3–10 μg) was subjected to SDS-PAGE alone (A), IEF, then SDS-PAGE (B), or NEPHGE, then SDS-PAGE (C). Protein was visualized with Coomassie Blue R-250, and those proteins corresponding to the two forms of β subunit are denoted β_{35} and β_{36}. For IEF, only the relevant portion of the gel is shown; pH values noted for the horizontal dimension were determined for IEF gels run in parallel without protein.

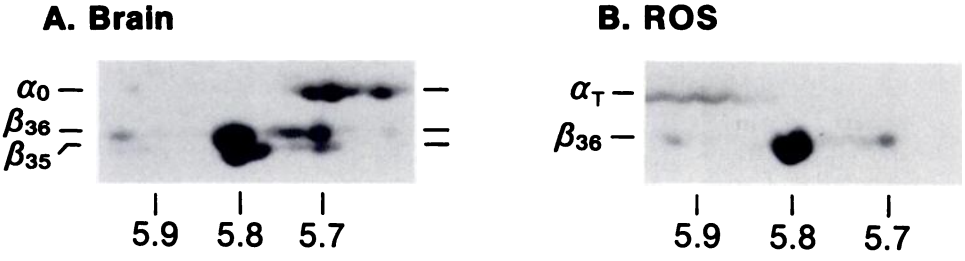

Fig. 2. IEF of β subunits from bovine brain and ROS. Five μg of βγ heterodimer isolated from bovine brain (A) and transducin isolated from bovine ROS (B) were subjected to IEF and SDS-PAGE. Protein was visualized with Coomassie Blue R-250. The two forms of the β subunit, as well as the α subunit of G_o (α_o) and transducin (α_T), are indicated.

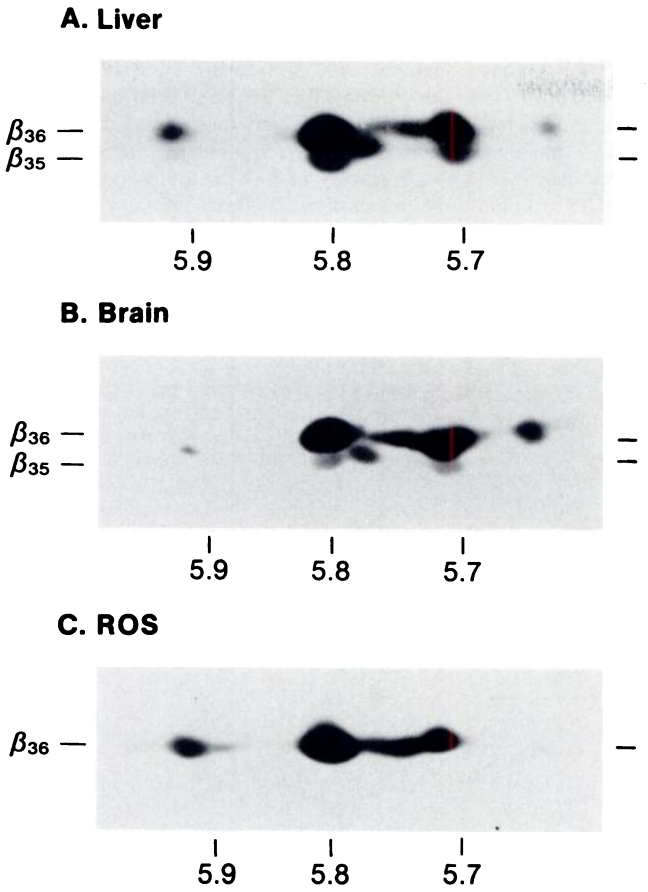

Fig. 3. Immunotransfer blot following IEF of β subunit. Approximately 500 ng of the βγ heterodimers isolated from rabbit liver (A) and bovine brain (B) and of transducin (C) were subjected to IEF and SDS-PAGE, then to procedures of immunotransfer blotting utilizing antiserum 5357. Only the relevant portions of the nitrocellulose membranes are shown.

temperature in 25 μl of 0.5 M sodium phosphate, pH 7.5, 0.2 mg/ml chloramine T, and 300 μCi $Na^{125}I$. Following extensive washing with 10% methanol and drying, gel slices were hydrated and incubated for 20 hr at 37° with 50 μg of TLCK-treated α-chymotrypsin (Sigma Chemical Co.) in 0.5 ml of 50 mM NH_4HCO_3. Soluble material was dried using a Speed-Vac concentrator (Savant), dissolved in H_2O, and dried again. This material was redissolved in 20 μl of H_2O/acetic acid/formic acid (80:15:5). Three μl of the redissolved material were spotted on cellulose-coated thin layer chromatography plates, and electrophoresis (1 kV) was performed at 10° in H_2O/acetic acid/formic acid (80:15:5) until basic fuchsin had migrated 10 cm. Plates were dried overnight, and peptides were subjected to ascending chromatography in a second dimension in butanol/pyridine/acetic acid/H_2O (97:75:15:40). Plates were dried and exposed to Kodak XAR film at room temperature for 2–8 hr. Incubation of peptide digests with an additional 50 μg of chymotrypsin for a further 20 hr had no effect on subsequently generated peptide maps. No peptides were discerned if chymotrypsin was omitted from the digestion protocol, nor were they observed if slices of gel adjacent to (but not containing) the β subunit were submitted to procedures of radioiodination and proteolysis.

Results

Analysis of β subunits visualized by protein staining. As previously reported (10, 11), the β subunit isolated from rabbit liver can be resolved upon SDS-PAGE into two forms having apparent molecular weights of 35,000 and 36,000 (Fig. 1A). Each of these forms consists of several differently charged species of protein: upon IEF, those having isoelectric points of approximately 5.7, 5.8, and 5.95 were discerned (Fig. 1B).[1] With the exception of a relatively minor protein having an isoelectric point of approximately 5.77 ($M_r \simeq 35.2$), the patterns of species for the two forms of the β subunit are identical. The absence of species other than those detected by IEF was corroborated by NEPHGE (Fig. 1C). The yield of Coomassie blue-staining protein following either IEF or NEPHGE was 50–90%, and the ratio of the 35- and 36-kDa forms of subunit was maintained relative to that observed upon SDS-PAGE alone. Results were identical for three different preparations of β subunit from rabbit liver.

The β subunits isolated from bovine brain and ROS displayed patterns of constituent species similar to that observed for the β subunit of rabbit liver (Fig. 2). Common isoelectric points were confirmed by combination of β subunits from liver, brain, and ROS prior to analysis. The 35- and 36-kDa forms of the β subunit isolated from brain comprised species having identical isoelectric points, with the exception previously noted for the β subunit of rabbit liver. As reported elsewhere (4), the β subunit isolated from ROS does not exhibit the 35-kDa form. No other species were observed for the two subunits upon NEPHGE.

Analysis of β subunits visualized by immunotransfer blotting. Species of the 35- and 36-kDa forms of the β subunits were also detected by procedures of immunotransfer blotting utilizing rabbit antiserum 5357 (Fig. 3). This antiserum enabled recognition of both forms of the β subunit, although the 36-kDa form was detected preferentially (20). No appreciable differences in immunoreactivities of the differently charged species of each form were noted. Species of the 35-kDa form of the β subunit were not detected with either antiserum 5356 or U49.

Procedures of immunotransfer blotting following IEF were used to characterize β subunits existing within membranes isolated from CEF, NRK cells, S49 mouse lymphoma cells, human placental cytotrophoblasts, and mouse sperm (Fig. 4). Depending upon the cell, the β subunit appeared as either the 36-kDa form alone or the 35- and 36-kDa forms together. Species having an isoelectric point of 5.8 were always detected, and those having isoelectric points of approximately 5.7 and 5.95 were observed for CEF, NRK, and S49 membranes. Common isoelectric points for these major immunoreactive species were confirmed by combination of various membranes with each other or with purified β subunits prior to analysis. The protein previously noted to have an isoelectric point of 5.77 ($M_r \simeq 35.2$) was absent in illustrated membranes but has been observed in those of human platelets and neutrophils (data not shown). CEF membranes contained an additional immunoreactive protein having an isoelectric point of 6.0, and NRK membranes consistently exhibited very small amounts of 36- and 38-kDa proteins having more basic net charges (e.g., Fig. 4B). Yields of immunoreactive protein upon IEF or NEPHGE were quantitative.

Two-dimensional peptide mapping. Results obtained from IEF demonstrate that the 35- and 36-kDa forms of the β subunit are identical in net electrical charge. The implied structural similarity was further investigated by two-dimen-

[1] The word "form" is used to denote one or the other of the 35- and 36-kDa proteins, whereas "species" refers to proteins resolved by IEF or NEPHGE.

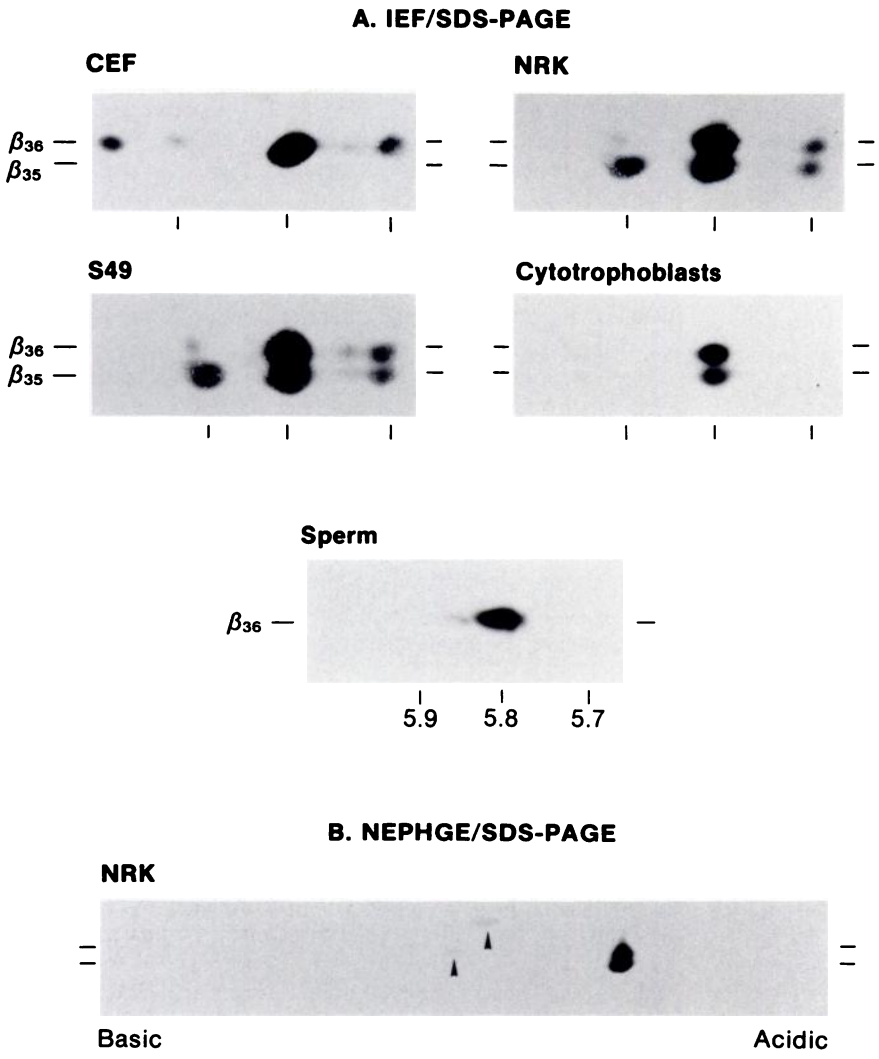

Fig. 4. Isoelectric properties of the β subunit within various cells. A. One hundred μg of membrane obtained from CEF, NRK cells, S49 mouse lymphoma cells, human placental cytotrophoblasts, and mouse sperm were subjected to IEF and SDS-PAGE, then to procedures of immunotransfer blotting utilizing antiserum 5357. Only the relevant portions of the nitrocellulose membranes are shown. B. One hundred μg of membrane obtained from NRK cells were similarly analyzed, but resolution was effected by NEPHGE/SDS-PAGE. *Arrowheads* denote minor immunoreactive proteins discussed in the text.

sional mapping of radioiodinated chymotryptic peptides. The 35- and 36-kDa forms of the β subunit isolated from rabbit liver provided peptide maps exhibiting both similarities and differences (Fig. 5): at least 10 peptides derived from the two forms were found to have identical mobilities, while at least 5 were unique to one or the other form. Peptides generated from the predominant species of the 35-kDa form (pI = 5.8) and from the β subunit isolated from ROS exhibited patterns virtually identical to those of the illustrated 35- and 36-kDa forms, respectively.

Discussion

Procedures of IEF, NEPHGE, and peptide mapping employed in this study provide basic information pertaining to the structure and homogeneity of the 35- and 36-kDa forms of the β subunit. These procedures are considerably more rigorous than those of amino acid determination and proteolysis within polyacrylamide gels. IEF and NEPHGE, for example, are capable of resolving proteins differing by less than one unit in net electrical charge (23). Moreover, IEF and NEPHGE permit detection of conceivably diverse, yet minor, populations of protein. The possibility that only selected species of the β subunit were detected by IEF and NEPHGE was precluded by the ranges in pH examined and the quantitative yields of Coomassie blue-staining protein. Finally, two-dimensional mapping of peptides generated with chymotrypsin is often sensitive to subtle changes in primary structure (24).

The 35- and 36-kDa forms of the β subunit appear by IEF and NEPHGE to be both similar in structure and conserved among cells. First, the two forms of the subunit are virtually indistinguishable by net electrical charge; moreover, the relative proportions of constituent species for these two forms are identical. Second, the β subunits isolated from rabbit liver, bovine brain, and bovine ROS behave identically upon analysis, differences in the quantities of 35- and 36-kDa forms notwithstanding. Third, similarities in electrophoretic behavior are found to extend to β subunits identified within various types of cells by immunological means. The nature of approximately 0.1 pH-unit differences among species of each of the two forms of the β subunit is unknown, but such differences are consistent with the loss or gain of a charged amino acid or a modification conferring a single charge at a pH slightly less than neutrality. The origin of the relatively minor protein having an isoelectric point of 5.77 ($M_r \simeq 35.2$) and noted particularly in purified preparations of β subunits is also unclear. The existence of even limited heterogeneity within the two forms of the β subunit, however, warrants consideration in the characterization of antibodies and other probes of specificity for this subunit.

Two-dimensional mapping of chymotryptic peptides sup-

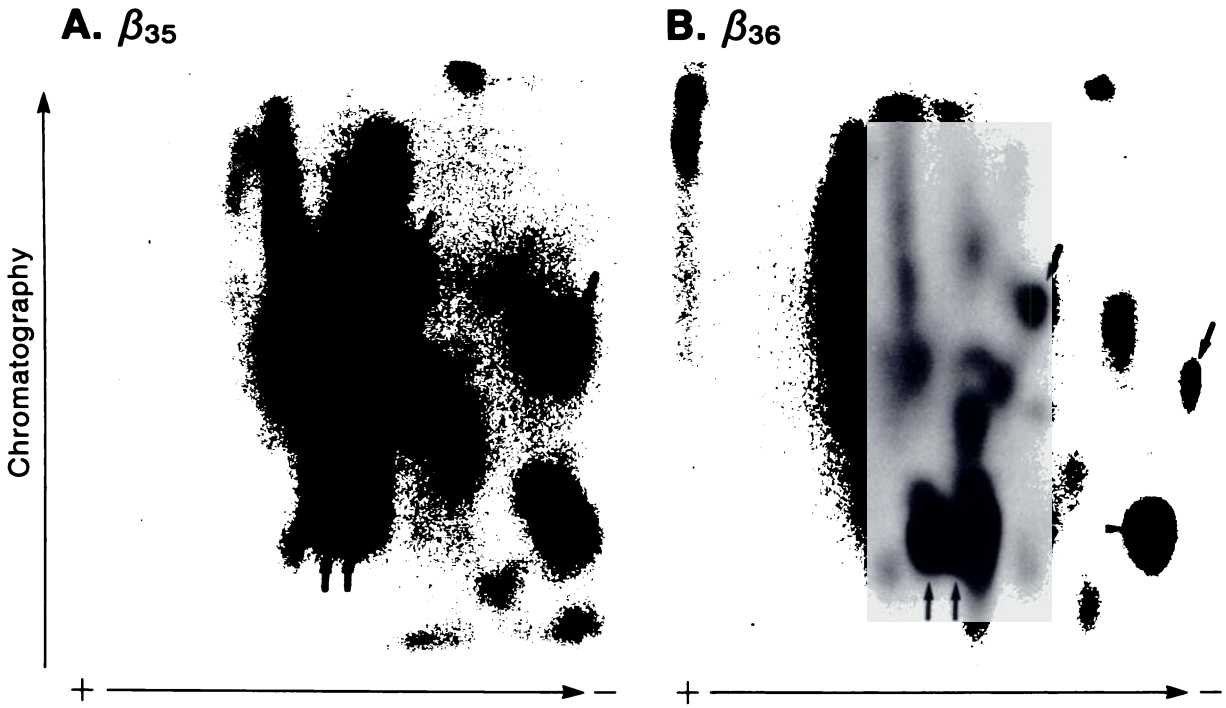

Fig. 5. Two-dimensional peptide map for 35- and 36-kDa forms of β subunit. Radioiodinated 35- and 36-kDa forms of the β subunit purified from rabbit liver were digested with α-chymotrypsin, and the resultant peptides were subjected to high voltage electrophoresis and chromatography on cellulose thin layer plates in horizontal and vertical dimensions, respectively. Shown are autoradiograms for peptides derived from the 35-kDa form (A) and the 36-kDa form (B) of the subunit. For reference, *arrows* depict several prominent peptides having identical mobilities, and *arrowheads* depict prominent peptides virtually unique to one or the other form of the subunit. Results were identical for two different preparations of β subunit isolated from rabbit liver.

ports the existence of homology between the 35- and 36-kDa forms of the β subunit, but clearly demonstrates some differences in primary structure. These results are consistent with those recently reported by Evans *et al.* (9) utilizing procedures of proteolysis described by Lam and Kasper (25). They are also consistent with observed differences in immunoreactivities of the two forms (12, 20). It is important to note that differences in peptide maps obtained following digestion with chymotrypsin may in part reflect charge-neutral, and therefore possibly conservative, substitutions of amino acids (24).

The basis for the previously reported and confirmed differences in apparent molecular weights and immunoreactivities of the 35- and 36-kDa forms of the β subunit is still conjectural. It is conceivable that these differences are due simply to conservative amino acid substitutions that affect binding of SDS and occur within antigenic determinants. Substitutions that alter charge are less likely, but could conceivably occur if balanced exactly by substitutions elsewhere of opposite charge. Post-translational modifications that do not impart greater than approximately 0.2-charge differences (e.g., acylation at cysteine residues) might also explain the differences in the two forms of the β subunit. Regardless of the nature of substitution or modification, however, such alterations must occur minimally at one internal site of the subunit, since an antiserum specific for residues 130–145 of the 36-kDa form (i.e., U49) does not recognize the 35-kDa form (cf. Refs. 12 and 26). Additional sites of subsitution/modification are implied by the number of chymotryptic peptides of the two forms that differ in electrophoretic and chromatographic behavior.

The data strongly indicate that the 35- and 36-kDa forms of the β subunit are indeed related and may both be considered to be relevant to the function of GTP-binding regulatory proteins. Furthermore, the data provide constraints for postulated primary structures. Specifically, structures proposed for the 35- and 36-kDa forms of the β subunit may only exhibit differences that are not manifest as alterations in net electrical charge. Such differences must also affect the dominant epitopes of the 36-kDa form of the subunit. Unique forms of the β subunit (e.g., those discerned within extracts of certain membranes and perhaps that having an isoelectric point of 5.77) may constitute the only exceptions to these constraints.

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