The tryptic and chymotryptic fragments of the β-subunit of guanine nucleotide binding proteins in brain are identical to those of retinal transducin

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Received 30 January 1985

The 35-kDa β-subunit of transducin purified from rod outer segment membranes is cleaved into 2 major fragments by trypsin, and 7 major fragments by chymotrypsin. Identical fragments are visualized by immunoblotting with transducin-β specific antisera after proteolysis of rod outer segment membranes, purified brain guanine nucleotide binding proteins, and brain membranes. The results indicate that the β-subunits of transducin and of brain guanine nucleotide binding proteins are not only similar structurally, but are also similarly oriented in membranes with respect to accessibility to proteolytic enzymes.

Transmembrane signalling *Adenylate cyclase* *Photoreceptor* *Guanine nucleotide binding protein*

1. INTRODUCTION

A family of G proteins is involved in signal transduction across cell membranes [1,2]. Members of this family include TD, the ROS G protein, G_s and G_i, and a G protein of unknown function, G_o, recently discovered in brain [3–5]. All are heterotrimers [3–6]. The peptide maps of the purified 35-kDa subunits of TD, G_s, and G_i were found to be virtually identical [7]. No information exists, however, on the orientation of the β subunits of TD and other G proteins within ROS and plasma membranes, respectively. We showed previously, that antisera against TD-β are fully cross-reactive with G_s and G_i-β [8]. We now show that anti-TD-β sera cross-react with the β-subunit of purified brain G proteins (comprised predominantly of G_i and G_o [3–5]). Using these antisera, we show, moreover, that the β-subunits of TD in ROS membranes and of G proteins in brain membranes show similar susceptibility to tryptic and chymotryptic cleavage.

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Abbreviations: ROS, rod outer segment; G proteins, guanine nucleotide binding proteins; G_s and G_i, stimulatory and inhibitory G proteins, respectively, associated with adenylate cyclase; G_o, a G protein of unknown function found in brain; TD, transducin, the G protein of retinal ROS; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TPCK, L-1-tosylamide-2- phenylethylchloromethyl ketone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] - 1 - propanesulfonate; GTP-γS, guanosine 5′,3-*O*-triphosphate

2. MATERIALS AND METHODS

2.1. *TPCK-trypsin*

TPCK-trypsin was obtained from Sigma, and α-chymotrypsin from Worthington. GTP-γS was from Boehringer, CHAPS from Pierce, and Blue Sepharose from Pharmacia. The sources of other reagents were described in [8,9].

2.2. *Membrane and protein purifications*

ROS membranes were prepared as in [9] and TD purified as in [8,9] except that GTP-γS was used

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00145793/85/$3.30

for elution. TD-β-γ was purified from the holoprotein by Blue Sepharose chromatography [10], and the β-subunit separated from γ by preparative gel electrophoresis after reduction with mercaptoethanol and denaturing with SDS. The β-subunit purified in this way was dialyzed against Tris-buffered saline to remove SDS before digestion. Brain membranes were prepared from bovine cerebral cortex as in [8,9]. Membranes (10 mg/ml) were extracted with 1% CHAPS (in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl buffer) for 2 h at 4°C. The suspension was spun at 100000 × g for 1 h to remove particulate material. Brain G proteins were purified as in [3].

2.3. *Immunoblots*

SDS-PAGE and immunoblotting were performed as in [8,9]. Gels were 15% acrylamide unless otherwise noted. Antiserum AS/1 [8,9] was used at a dilution of 1/100 for 24 h at room temperature as first antibody immunoblots. Affinity-purified antibodies against TD-β were prepared by adsorbing anti-TD sera onto nitrocellulose strips containing purified TD-β, rinsing the strips, and eluting the bound antibodies with 6 M urea. The eluted antibodies were then dialyzed against Tris-buffered saline to remove urea, and used as first antibody for immunoblots.

2.4. *Proteolysis*

Stock solutions of proteolytic enzymes were made up in 0.2 N morpholine buffer (pH 8.1). Conditions for proteolysis are described in the figure legends. Proteolysis was stopped by adding SDS-containing sample buffer for PAGE and boiling.

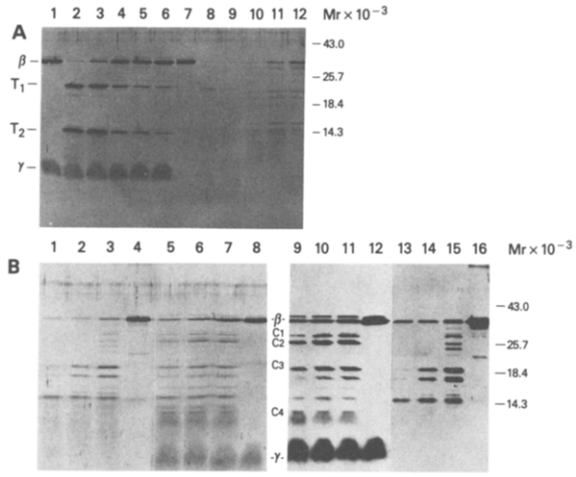

Fig.1. Analysis of the tryptic (A) and chymotryptic (B) fragments of TD-β and of TD-β-γ. In (A), either 8.5 μg purified TD-β-γ (lanes 1–6) or 8.5 μg TD-β (lanes 7–12) were subjected to tryptic proteolysis for 2 min at 37°C (lanes 1 and 7, no trypsin; 2 and 8, 1 μg; 3 and 9, 0.25 μg; 4 and 10, 0.06 μg; 5 and 11, 0.03 μg; and 6 and 12, 0.015 μg trypsin). Peptides were then separated by SDS-PAGE, and the gel stained with Coomassie blue. T1 and T2 indicate the 24- and 14-kDa fragments, respectively, of tryptic cleavage of TD-β. In (B), either 10 μg purified TD-β (lanes 1–4 and 13–16) or 10 μg TD-β-γ (lanes 5–12) were subjected to proteolysis with chymotrypsin (1 μg) for 1 (lanes 3,7,11,15), 3 (lanes 2,6,10,14) or 5 (lanes 1,5,9,13) min at 37°C. No chymotrypsin was added to samples in lanes 4,8,12 and 16. The peptides were then separated by SDS-PAGE and either stained with Coomassie blue (lanes 1–8) or blotted onto nitrocellulose paper and immunostained with a 1/100 dilution of antiserum AS/1 (lanes 9–16), as described in section 2. C1–C4 indicate 4 major chymotryptic fragments of TD-β of approx. molecular mass: 27,25,18 and 12 kDa, respectively.

3. RESULTS

Under nondenaturing conditions, the β-subunits of TD and other G proteins are associated with low molecular mass γ-subunits [6]. We subjected purified TD-β-γ and TD-β to tryptic and chymotryptic digestion and compared the resultant proteolytic fragments (fig.1). Several points are worth noting. The γ-subunit is apparently cleaved neither by trypsin nor by chymotrypsin. Recent work on the sequence of the γ-subunit [15] indicates several chymotryptic fragments with more extensive proteolysis. Tryptic cleavage of TD-β-γ yields 2 major fragments of β of approx. 24 and 14 kDa, comparable to those shown by Fung and Nash [11]. In contrast, the isolated β-subunit shows greater susceptibility to tryptic proteolysis (cf. lanes 9 and 3) and yields many more fragments. Coomassie blue staining demonstrates at least 7 major chymotryptic fragments of TD-β. Virtually all of these are also visualized on immunoblot, but 4 of approx. 27, 25, 18 and 12 kDa are labeled because of their relation to bands seen in subsequent experiments (see fig.2). Again, the isolated β-subunit shows greater susceptibility to

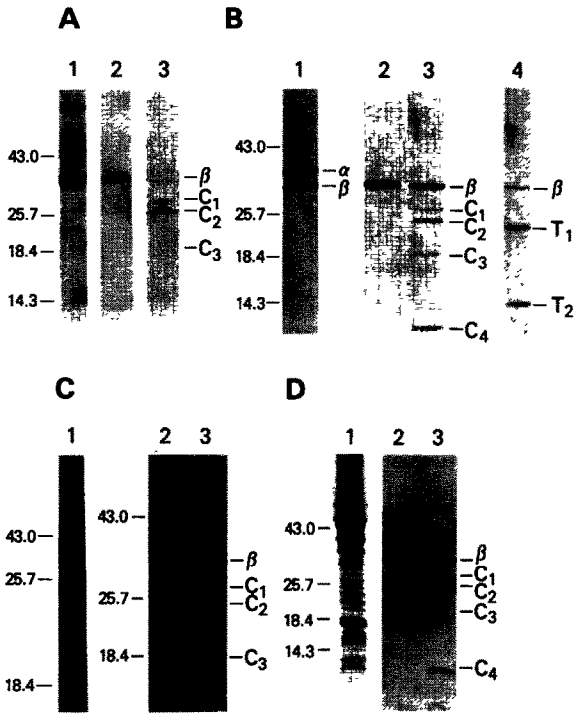

Fig.2. Analysis of the chymotryptic fragments of (A) TD-β in rod outer segment membranes; (B) the β-subunit of purified brain G proteins; (C) the β-subunit of G proteins in a CHAPS extract of bovine cerebral cortical membranes; (D) the β-subunit of G proteins in bovine cerebral cortical membranes. In (A), 200 μg ROS membrane protein/lane were separated by SDS-PAGE and either stained with Coomassie blue (lane 1), or blotted onto nitrocellulose and immunostained with affinity purified antibodies against TD-β (lanes 2 and 3), as described in section 2. Lanes 1 and 2 were undigested and lane 3 was digested with 5 μg chymotrypsin for 1 min at 37°C. In (B) 2.5 μg purified brain G proteins/lane were separated by SDS-PAGE and either stained with Coomassie blue (lane 1) or immunostained with a 1/100 dilution of AS/1 (lanes 2–4). Lanes 1 and 2 were undigested, lane 3 was digested with 2.5 μg chymotrypsin for 20 min at 37°C, and lane 4 was digested with 2 μg trypsin for 5 min at 37°C. In (C), 135 μg CHAPS extract of brain membranes/lane were separated by SDS-PAGE and either stained with Coomassie blue (lane 1), or blotted to nitrocellulose and immunostained with a 1/100 dilution of AS/1 (lanes 2 and 3). Lanes 1 and 2 were undigested, and lane 3 was digested with 1 μg chymotrypsin for 5 min at 37°C. In (D) 165 μg brain membrane protein/lane were separated by SDS-PAGE (on a 12.5% gel) and either stained with Coomassie blue (lane 1), or blotted onto nitrocellulose and stained with a 1/100 dilution of AS/1 (lanes 2 and 3). Lanes 1 and 2 were undigested, and lane 3 was digested with 5 μg chymotrypsin for 1 min at 37°C. C1–C4 and T1,T2 indicate the major chymotryptic and tryptic fragments, respectively, of the β-subunit (see fig.1). Molecular mass markers (in kDa) are indicated to the left of each gel lane.

chymotryptic cleavage than does TD-β-γ, and yields a somewhat different set of fragments (cf. fig.1B, lanes 11 and 15).

We next analyzed the products of tryptic and chymotryptic cleavage of TD-β in ROS membranes. This was done by immunoblotting digested ROS membranes using affinity-purified antibodies against TD-β (see section 2). We found that TD-β in ROS membranes was readily cleaved by chymotrypsin and trypsin, and that the products were identical (figs 2A and 4) to those seen with purified TD-β-γ. The inability to detect additional chymotryptic fragments (fig.2A, lane 3) is presumably a function of the sensitivity of the affinity-purified antibodies used for immunoblotting. The results with trypsin and ROS membranes agree with the findings of Halliday et al. [12].

Brain has been found to contain more G protein in general [4], and more β-subunit in particular [8,9] than other tissues including liver, kidney and heart. The majority of brain G protein is accounted for by G_i and G_o which copurify from cholate extracts of bovine brain [3–5]. We subjected a purified brain G protein preparation, containing G_i and G_o ([3], kindly supplied by G. Milligan and W. Klee) to tryptic and chymotryptic proteolysis, and detected the β-subunit and its cleavage products by immunoblotting with TD-β antiserum. The antiserum used readily detects the β-subunit (fig.2B, lane 2) but does not cross-react with either G_i or G_o-α ([8], unpublished). The immunoblots clearly show that the tryptic and chymotryptic fragments of purified brain G-β are identical to those of TD-β (fig.2B, lanes 3 and 4). Essentially the same results were obtained with immunoblots of trypsin (fig.3) and chymotrypsin (fig.2C,D) digested detergent extracts of brain membranes, or of unextracted brain membranes.

We next compared the susceptibility of solubilized vs membrane-bound brain G proteins to tryptic cleavage. The immunoblot in fig.3 clearly shows that solubilized G-β is more sensitive to trypsin. Finally, we compared the susceptibility of

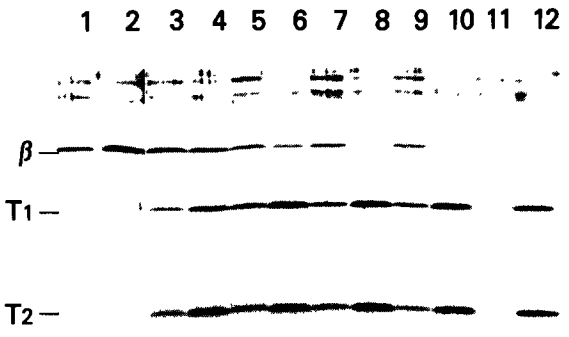

Fig.3. Comparison of the susceptibility of the β-subunit of brain G proteins to tryptic proteolysis in either membranes or a detergent (CHAPS) solubilized extract; 87 μg/lane of either brain membrane protein (lanes 1,3,5,7,9,11) or CHAPS extract of brain membranes (lanes 2,4,6,8,10,12) were separated by SDS-PAGE and blotted onto nitrocellulose and then immunostained with a 1/100 dilution of AS/1. Lanes 1 and 2 were undigested, 3 and 4 and 5 and 6 digested with 0.5 μg trypsin for 1 and 5 min, respectively, 7 and 8 and 9 and 10 with 1 μg trypsin for 5 and 10 min, respectively, and 11 and 12 with 5 μg trypsin for 1 min, all at 37°C. T1 and T2 indicate the tryptic fragments of the β-subunit.

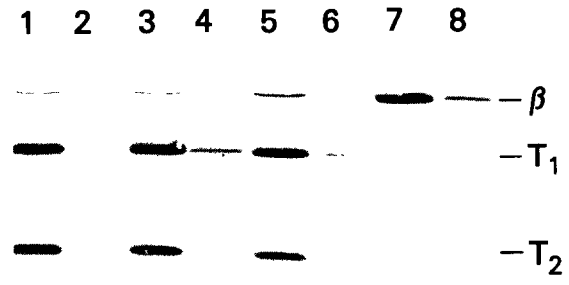

Fig.4. Comparison of the susceptibility of TD-β in ROS membranes and of the β-subunit of G proteins in brain membranes to tryptic proteolysis; 87 μg/lane of either ROS membrane protein (lanes 1,3,5,7) or brain membrane protein (lanes 2,4,6,8) were separated by SDS-PAGE and blotted onto nitrocellulose and then immunostained with affinity-purified antibodies against TD-β. Lanes 7 and 8 were undigested, 5 and 6 digested with 0.5 μg trypsin for 2 min, 3 and 4 digested with 0.5 μg trypsin for 5 min, and 1 and 2 digested with 1 μg trypsin for 5 min, all at 37°C. T1 and T2 indicate the tryptic fragments of the β-subunit.

TD-β in ROS membranes vs G-β in brain membranes to trypsin. Equivalent amounts of membrane protein were digested. The difference in amount of β-subunit in the two samples (lane 7 vs 8) is not due to differences in antibody reactivity with TD- vs G-β [8], but reflects instead, the higher concentration of TD in ROS membranes compared with G proteins in brain membranes. Irrespective of the initial amount of β-subunit, more than 90% of β in each set of membranes is cleaved within 5 min of digestion with 0.5 μg trypsin.

4. DISCUSSION

TD, G_s, and G_i are members of a family of G proteins involved in signal transduction across cell membranes. A new G protein of unknown function, G_o, has recently been discovered in brain, where together with G_i, it accounts for almost all of the G proteins [3–5]. G proteins are heterotrimers [3–6]; studies with TD antisera have shown that TD-α and TD-γ differ from other G-αs and G-γs, but that the β-subunits are immunochemically related [8]. Peptide maps of the *Staphylococcus aureus* V8 protease and elastase digests of purified TD, G_s, and G_i β-subunits were virtually identical [7]. Although these studies indicate that G protein β-subunits are very closely related, if not identical, there is very little data available comparing G protein β-subunits in their native state within membranes. Since G protein β-subunits in the native state interact with different α- and γ-subunits, the latter could result in differences in β-subunit exposure within membranes.

To probe the accessibility of β-subunits within the membrane, we subjected ROS and brain membranes to tryptic and chymotryptic digestion and detected β and its proteolytic fragments by immunoblotting with TD-β specific antisera. The tryptic and chymotryptic fragments of TD-β in ROS membranes are the same as those obtained from digestion of purified TD-β-γ. In agreement with prior studies [11,12], this indicates that the β-subunit in ROS membranes is exposed to proteolytic enzymes and that interaction with the α-subunit does not alter its susceptibility to proteolysis.

Digestion of purified brain G proteins (G_i + G_o) with trypsin and chymotrypsin yields fragments of the β subunits identical to those of TD-β. This pro-

vides further evidence for the similarity of G protein β-subunits, and indicates that interaction with different α- and γ-subunits does not alter the susceptibility of the β-subunit to tryptic and chymotryptic digestion. We cannot exclude the possibility, however, that digestion of brain G protein β yields additional fragments unrelated to those of TD-β and not detected with our antisera.

The β-subunit of G_s and G_i was found to be almost totally resistant to trypsin and chymotrypsin in previous studies employing conditions similar to ours [14]. Activity (inhibition of G_s activation), rather than structure, was assessed in those studies. Since it is clear from our results that G-β is readily cleaved by both enzymes, it seems likely that proteolytically cleaved β-subunit retains activity. A similar conclusion was reached for TD-β cleaved by trypsin, with respect to ability of β to interact with rhodopsin [11].

Digestion of detergent extracts of brain membranes and of unextracted membranes with trypsin and chymotrypsin resulted in the same fragments of the β-subunit obtained from digests of the purified proteins. The β-subunit in detergent extracts of membranes was more susceptible to proteolysis than when membrane-bound, but the difference was relative rather than absolute. Within the limits of the immunoblotting method, moreover, there was no major difference in susceptibility to proteolysis of TD-β in ROS membranes vs G-β in brain membranes. In conclusion, G protein β-subunits are not only closely related structurally, they are similar in orientation within membranes, at least with respect to accessibility to tryptic and chymotryptic cleavage.

ACKNOWLEDGEMENTS

We are grateful to C. Woodard and R. Vinitzky for expert technical assistance, and to G. Milligan and W. Klee for providing purified brain G proteins. M.P. is on sabbatical leave from the Volcani Agricultural Research Center, Rehovoth, Israel. P.G. was supported in part by a grant (Gi 138/1-1) from the Deutsche Forschungsgemeinschaft.

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