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Amino acid sequence determination of the novel forms of $G_o\alpha$ purified from bovine brain membranes

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Previously we have reported that there are at least four different forms of $G_o\alpha$ in bovine brain membranes which can be distinguished by their elution profiles from Mono Q column and their immunological reactivities. The four α-subunits are referred to as α_o1, α_o2, α_o3 and α_o4 in their elution orders from the column. Partial amino acid sequences of the purified α_o1 and α_o2 were determined and compared with the predicted sequences of two classes of $G_o\alpha$ cDNAs, termed $G_o\alpha$-1 and $G_o\alpha$-2. There were at least two unique fragments corresponding with the predicted amino acid sequence of the $G_o\alpha$-2 cDNA but different from that of the $G_o\alpha$-1 cDNA upon tryptic digestion of α_o1- or α_o2-subunit. The α_o3- and α_o4-subunits, but not α_o1-and α_o2-subunits, were recognized by an antibody raised against a unique amino acid sequence predicted from $G_o\alpha$-1 cDNA. These results suggest that $\alpha_o1,2$ subunits and $\alpha_o3,4$ subunits are encoded by $G_o\alpha$-2 cDNA and $G_o\alpha$-1 cDNA, respectively.

GTP-binding protein; Pertussis toxin (IAP); Partial amino acid sequence

1. INTRODUCTION

Heterotrimeric GTP-binding proteins (G proteins) function as transducers carrying signals from activated receptors to effectors, such as enzymes or ion channels [1]. G proteins composed of α-, β- and γ-subunits have been purified from plasma membranes of various tissues. Among the G protein family, G_o-type protein was present in a relatively large quantity in bovine brain membranes. Although the precise function of G_o is still unclear, it may be a stimulator of phospholipase C [2], an inhibitor of neuronal Ca^{2+} channels [3] and a stimulator of neuronal K^+ channels [4].

The two classes of $G_o\alpha$ cDNAs have been cloned, $G_o\alpha$-1 (referred also as α_{o1} or $G_oA\alpha$) and $G_o\alpha$-2 (α_{o2} or $G_oB\alpha$) [5–8]. The $G_o\alpha$-1 cDNA codes for a major α-subunit in mammalian brains which was identical to that cloned previously from rat and bovine tissues. On the other hand, $G_o\alpha$-2 cDNA has recently been cloned from libraries of hamster insulin-secreting tumor (HIT) cells [5], mouse brain [6] and rat [8]. The new $G_o\alpha$-2 cDNA codes for an α-subunit that is identical to $G_o\alpha$-1 product in its first two-thirds but differed in the remaining carboxyl-terminal one-third of the molecule. Thus, the two cDNAs appeared to be derived from alternatively spliced mRNAs. In the previous studies [9–11], we have reported that there are four types of G_o (or G_o-like) α-subunits cross-reacting with a polyclonal anti-α_o antibody in bovine brain membranes. However, it was not clear whether those α-subunits differed in their primary sequences or in the post-translational modifications. In this paper, the partial amino acid sequences of the purified α_o-subunits are determined and compared with the sequences deduced from the two $G_o\alpha$ cDNAs.

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2. MATERIALS AND METHODS

2.1. *Materials*

Antisera, ONI, IMI, OCI and RV3, of which antigens were GCTLSAEERAALERSK, NLKEDGISAAK, ANNLRGCGLY and purified bovine brain G_o, respectively, were generous gifts from Dr. G. Milligan, University of Glasgow. AP3 and NP3 were affinity-purified rabbit polyclonal IgG raised against synthetic peptides of GAGESGKSTIVKQMK and GSNTYEDAAA, respectively. Horseradish peroxidase-conjugated anti-rabbit IgG was purchased from Amersham. POD Immunostain-kit was purchased from Funakoshi Co.

2.2. *Purification of G_o-type proteins*

Four G_o-type proteins were purified from bovine brain membranes as described in [9,10]. The one, α_o1, was purified without βγ-subunits, and other (G_o2, G_o3 and G_o4) were as heterotrimers. The α-subunits of the purified heterotrimers were obtained as described in [10]. The purified proteins were quantitated by staining with amido black with bovine serum albumin as a standard protein [12].

2.3. *Analysis of partial amino acid sequences of the purified α-subunits*

The purified α_o1- and α_o2-subunits (approximately 40 μg) were

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boiled for 5 min and then digested with tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin at a protein/protease ratio of 10 at 37°C for 1 h. The digested peptides were applied to a column of PepRPC HR5/5 (Pharmacia-LKB) that had been equilibrated with 0.1% (v/v) trifluoroacetic acid and then eluted with a 30-ml linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min using the Pharmacia-LKB FPLC system. Peptides eluted from the column were detected by measuring absorbance at 215 nm and sequenced by automated Edman degradation using a gas-phase sequencer (Model 477A Applied Biosystems) with an on-line phenylthiohydantoin-amino acid analyzer (Model 120A Applied Biosystems).

2.4. *Immunoblot analysis*

The purified proteins were separated by SDS-PAGE (12% gel) and transferred to PVDF membrane at a constant voltage of 50 V for 1 h. The membranes, after being blocked with 2% of BSA for 1 h, were incubated with various polyclonal antibodies at 30°C for overnight and then treated with horseradish peroxidase-conjugated anti-rabbit IgG. The detection was carried out with POD immunostain-kit.

3. RESULTS AND DISCUSSION

In the previous papers [9–11], we have reported that there were at least four forms of G_o in bovine brain membranes. The four G_o-type proteins, termed α_o1, G_o2, G_o3 and G_o4, were purified as one α-monomeric form and three $\alpha\beta\gamma$-trimeric forms. For simplicity, their α-subunits are henceforth referred to as α_o1, α_o2, α_o3 and α_o4, respectively. Analysis of their peptide mappings revealed that they were classified into two groups such as α_o1, α_o2 and α_o3, α_o4 [11]. In the present study, we first determined the partial amino acid sequences of the one group, α_o1 and α_o2, though it remained to be determined the significant difference between each of the two groups.

After complete digestion of the two α-subunits with trypsin, the fragments were separated by a reverse-phase FPLC system. Nine tryptic fragments obtained from α_o1 and seven ones from α_o2 were sequenced by a gas-phase sequencer. The partial amino acid sequences, which covered 103 and 68 amino acid residues of α_o1 and α_o2, respectively, were compared with the sequences deduced from two $G_o\alpha$ cDNAs, $G_o\alpha$-1 and $G_o\alpha$-2 [8]. Predicted amino acid sequences from the two cDNAs were identical in their first two-thirds but different from each other in the remaining carboxyl-terminal one-third. As shown in Fig. 1, eight independent fragments (*a–h*) from α_o1 or α_o2 exactly fitted with the first two-thirds common sequences of the two cDNAs. However, the other two fragments, *i* and *j*, corresponded fairly well with the remaining carboxyl-terminal region in $G_o\alpha$-2 cDNA. Lys in fragment *i* was the 249th amino acid residue of $G_o\alpha$-2, though there was Met in the same position of $G_o\alpha$-1. Ile and Glu in fragment *j* were also the 274th and 276th residues of $G_o\alpha$-2, respectively, though there were Leu^{274} and Gly^{276} in $G_o\alpha$-1. Thus, it is very likely that both α_o1 and α_o2 are encoded by $G_o\alpha$-2 cDNA.

We next investigated the immunoreactivities of α_o-subunits of the purified four proteins by means of

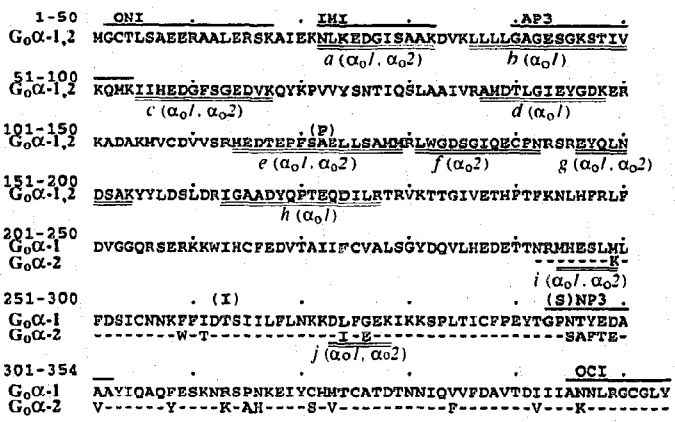

Fig. 1. Comparison of the partial amino acid sequences of α_o1 and α_o2 with predicted sequences deduced from two $G_o\alpha$ cDNAs, $G_o\alpha$-1 and $G_o\alpha$-2. The decuced amino acid sequence of human $G_o\alpha$-1 [8] is shown by the standard one-letter abbreviation code. Hyphens (-) represent an amino acid residue of the $G_o\alpha$-2 [8] that is identical to the residue shown in the $G_o\alpha$-1. One-letter codes in parentheses represent an amino acid residue of bovine α_o cDNA [14] that is different from the residue shown in the $G_o\alpha$-1. Partial amino acid sequences obtained from the tryptic fragments (*a–j*) of α_o1 or α_o2 are indicated by double underlinings with the originated subunits. Synthetic peptides used for antigens are shown by flat lines with the abbreviations of antibodies.

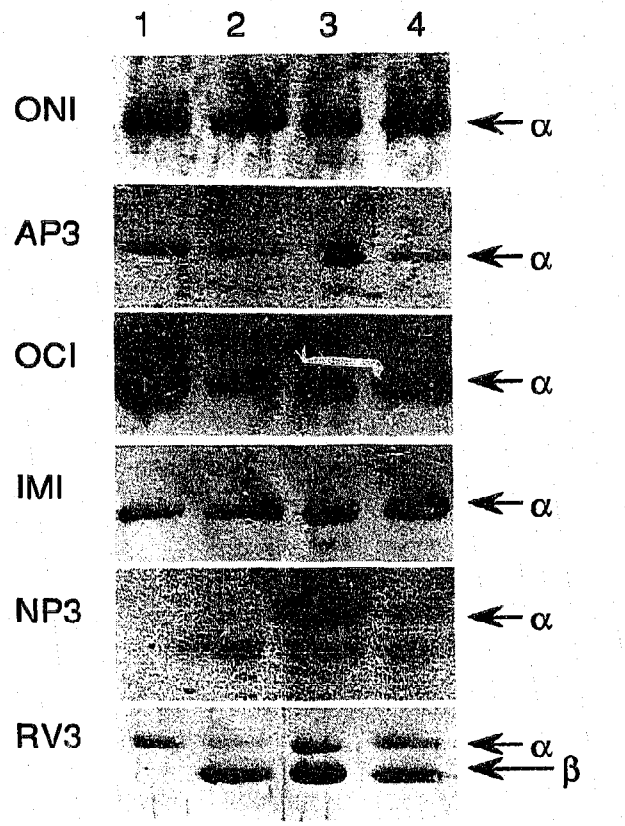

Fig. 2. Immunoblot analysis of the four α_o-subunits. The four G_o-type proteins, approximately 175 ng of α_o1 (lane 1) and 350 ng of G_o2 (lane 2), G_o3 (lane 3) and G_o4 (lane 4), were separated by SDS-PAGE (12% gel) and subjected to immunoblot analysis as described in section 2.4.

Western blot analysis (Fig. 2). ONI, IMI and AP3 antibodies, whose antigens were synthetic peptides corresponding with the common amino-terminal region of the two $G_o\alpha$ cDNAs (see Fig. 1), reacted with all of the four α-subunits. These subunits were also recognized by OCI and RV3 antibodies raised against the carboxyl terminus of the $G_o\alpha$-1 cDNA-predicted sequence (Ala^{345}-Tyr^{354}) and purified bovine brain G_o. However, NP3 antibody, of which antigenic peptide was designed to fit with a specific sequence (Gly^{293}-Ala^{302}) in $G_o\alpha$-1 cDNA, selectively reacted with α_o3 or α_o4; α_o1 or α_o2 was not recognized by the $G_o\alpha$-1-specific antibody. The antibody OCI raised against the synthetic peptide predicted from $G_o\alpha$-1 cDNA reacted with α_o3 and α_o4 as well as α_o1 and α_o2. This may be due to the difference of only one amino acid residue between $G_o\alpha$-1 and $G_o\alpha$-2 in the region of the synthetic peptide for OCI antibody. These results suggest that $\alpha_o1,2$ subunits and $\alpha_o3,4$ subunits are encoded by $G_o\alpha$-2 cDNA and $G_o\alpha$-1 cDNA, respectively.

The difference between the two forms of $G_o\alpha$ cDNA occurs in the carboxyl-terminal one-third of the molecule. This appears to be the region identified by Masters et al. [13] as being critical for specificity of interaction with both receptors and effectors. In the previous paper [10], we have reported that one of the multiple forms of G_o α-subunit, α_o2, could inhibit G_s-stimulated adenylate cyclase of S49 cyc^- membranes, though its potency was lower than that observed with G_i α-subunits. It may be interesting to point out here that α_o1 and α_o2 presumably encoded by $G_o\alpha$-2 cDNA have an amino acid sequence homologous to the α-subunits of G_i in the carboxyl-terminal one-third of the molecule [8].

In the present study, we did not address a significant difference between α_o1 and α_o2 or α_o3 and α_o4. However, the two α-subunits of the first (α_o1 and α_o2) or the second (α_o3 and α_o4) group which were indistinguishable from each other at least in the present amino acid sequence analysis could be classified into further two distinct entities with respect to their properties of GTPase activity [11]. Demonstration of the functional interaction of the heterogenous α-subunits with specific receptors and/or effectors, if present, is a crucial next step in confirmation of their physiological differences in signal transductions.

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