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Heterogeneity of three electrophoretically distinct G_o α-subunits in mammalian brain

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So far three splice variants of the α_o-gene coding for two α_o proteins have been identified by molecular cloning, and the corresponding proteins purified. In the present study subtype-specific peptide antibodies revealed the existence of an electrophoretically distinct third form of α_o in mammalian brain membranes which migrates more slowly on SDS-PAGE and shows a more acidic pI value than the other α_o-subunits. Each of the three α_o-subunits is detected as two isoforms when enriched from brain membranes. Rodent α_o-subunits differ from non-rodent species in their electrophoretic mobilities. The results suggest that (i) there may exist a novel α_o-subunit which reacts with an α_{o1}-subunit-specific antibody, (ii) each α_o-subunit may exist in more than one co- or posttranslationally modified isoform in brain membranes, and (iii) differences between α_o-subunits from different species exist which are detectable by gel electrophoretic methods.

G-protein; G_o; Antipeptide antibody; 2-Dimensional-gel electrophoresis

1. INTRODUCTION

Heterotrimeric regulatory GTP-binding proteins (G-proteins) represent an important family of cellular signal transduction components [1]. One member of this family, G_o, is most abundant in mammalian brain tissue but can also be detected in peripheral neuronal, neuroendocrine and endocrine cells. G_o functionally couples various transmembranous receptors (e.g. muscarinic M_2 or M_4 receptors, α_2-adrenoceptors, somatostatin, adenosine A_1, and 5-hydroxytryptamine receptors) to effectors such as calcium channels and possibly potassium channels and phospholipase C [2,3]. So far three splice variants of the α_o-gene coding for two α_o proteins have been identified by molecular cloning [4–7]. Interestingly, purification of the two splice variants revealed the existence of two isoforms of each α_{o1} and α_{o2} [8–11]. Recently, several groups reported on a third form of α_o in brain membranes, using urea-containing SDS-PAGE gels [12–15]. However, there are conflicting reports which have not clarified whether or not this third form is identical with one of the known α_o proteins described so far. Therefore, we investigated the heterogeneity of the α_o family. This study, employing one- and two-dimensional gel electrophoresis and α_o-subunit-specific antibodies, provides evidence for the existence of three gene products of $G_o\alpha$, each of them occurring in two isoforms.

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2. MATERIALS AND METHODS

2.1. *G-Proteins*

Membranes and cholate extracts from brains of different mammalian species (bovine, porcine, murine, rabbit and rat) and of hamster insulinoma HIT-T15, rat insulinoma RINm5F and rat pituitary GH_3 cells were prepared as described [16,17]. G-Proteins from porcine and rat brain membranes were enriched on DEAE-Sepharose [16]. Recombinant α-subunits of G-proteins were prepared as reported [17].

2.2. *Antibodies*

Peptide antibodies AS 8 (anti-α_{common}), AS 6 (anti-$\alpha_{o\ common}$) and AS 201 (anti-α_{o2}) were described previously [15,17]. For generation of anti-α_{o1} antibodies a synthetic peptide specific for α_{o1} (SKNRSPNKEIYCHM) was used to immunize New Zealand white rabbits. The peptide was coupled to keyhole limpet hemocyanine (Calbiochem, Bad Soden, Germany) using sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate (Pierce, Oud Beijerland, The Netherlands) as coupling agent. Antisera were subjected to affinity-chromatography on Affi-Gel 10 (BioRad, München, Germany) to which the respective peptide was coupled.

2.3. *Immunoblot analysis*

Separating gels of one-dimensional SDS gel electrophoresis (SDS-PAGE) contained 6 M urea and 9% (w/v) acrylamide or as indicated [17]. Two-dimensional SDS gel electrophoresis (2D-SDS-PAGE), which separates proteins by isoelectric focussing followed by SDS-PAGE on gels containing 10% acrylamide was performed as detailed previously [16]. Immunoblotting was performed as described, and filter-bound antibodies were visualized by a color reaction catalyzed by alkaline phosphatase [17] or goat anti-rabbit IgG coupled to peroxidase (dilution 1:1000; Sigma, Deisenhofen, Germany) and the chemiluminescence (ECL) Western blotting detection system (Amersham, Braunschweig, Germany). ECL-stained blots were exposed to X-ray films for 1–20 min.

3. RESULTS

The deduced amino acid sequences of the splice variants of α_o, α_{o1} and α_{o2}, differ only in their C-terminal thirds (i.e. exons 7 and 8), the assumed receptor recognition site (Table I) [4–7]. The α_{o1}-peptide used for generation of the antibody AS 248 differs in 5–6 residues from the corresponding region of α_{o2} [7]. To determine the specificity of peptide antibodies, immunoblots with recombinant α-subunits of G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_{o2}, G_s and G_z were performed (Fig. 1). AS 248 exclusively reacted with recombinant α_{o1} whereas AS 201 detected only recombinant α_{o2}. Thus both antibodies are subtype-specific. In contrast, AS 6 specifically recognized both forms of recombinant α_o [15].

We assessed the α_o-composition of various mammalian brain membranes, using urea-containing SDS-PAGE. As shown in Fig. 2A, all species tested exhibited 3 differently migrating anti-α_o-immunoreactive bands, which were stained by AS 8 and AS 6. The fastest migrating protein sensitive to the AS 6 antibody was also recognized by the α_{o2}-specific antibody AS 201, which was detectable when cholate extracts from rat and mouse brain membranes were tested. On the other hand, the broad middle and upper bands stained with AS 6 were also immunoreactive to AS 248, which is specific for α_{o1}. The antibody AS 248 detected protein bands in all species tested. In contrast, AS 201 only detected bands in rodent species. In cholate extracts of HIT T15 (Fig. 2B), GH_3 and RINm5F (not shown) cell membranes, only the anti-α_{o2}-immunoreactive and broad anti-α_{o1}-sensitive protein bands could be detected. Thus, at least these three cell lines appear to be devoid of the upper anti-α_{o1}-immunoreactive protein band.

Interestingly, the extraction procedure employed for porcine brain G-proteins led to the appearance of additional anti-α_o-sensitive proteins. In membrane preparations the broad middle band was obviously composed of two bands of which the faster migrating one was faint, whereas the additional lower and upper bands were single bands (Fig. 2B). In cholate extracts the upper and lower bands appeared as doublets. Further-

Table I

Differences in the deduced amino acid sequences of α_{o1} and α_{o2} and sequences of synthetic peptides employed for production of antibodies

(a)	
$\alpha_{o\text{-common}}$-peptide	(C) **NLKEDGISAAKDVK**
α_{o1}-(a,b,c)	*IEK***NLKEDGISAAKDVK***LLL*
α_{o2}-(a,b,c)	*IEK***NLKEDGISAAKDVK***LLL*
(b)	
α_{o1}-peptide	**SKNRSPNKEIYCHM**
α_{o1}-(a,b,c)	*QFES***KNRSPNKEIYCHM***TCA*
α_{o2}-(a)	*QYES***KNKSAHKEIYTHP***TCA*
α_{o2}-(b)	*QYES***KNKSAHKEVYSHV***TCA*
α_{o2}-(c)	*QYES***KNKSAHKEIYSHV***TCA*
(c)	
α_{o1}(a,b)	*EYTGSNTYEDAAAYIQAQF**ESK***
α_{o2}(a,b)	*EYTGPSAFTEAVAHIQGQY**ESK***
α_{o2}(c)	*EYTGPSAFTEAVAYIQAQY**ESK***
α_{o2}-peptid e	(C) **GPSAFTEAVAHIQGQY**

$\alpha_{o\text{-common}}$ peptide=residues 22–35 of α_{o1} and α_{o2}; α_{o1} peptide=residues 310–323 of α_{o1}; α_{o2} peptide=residues 293–308 of α_{o2}. Amino acid sequences are derived from (a) HIT, (b) mouse and rat (c) human cDNA [4–7]

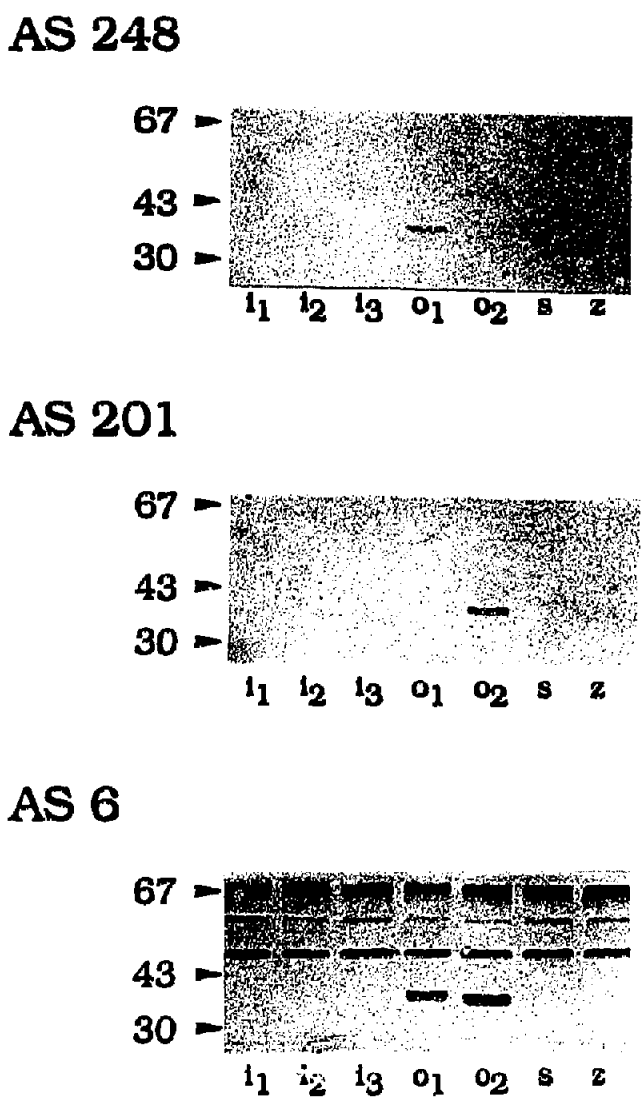

Fig. 1. Subtype specificity of α_o-antibodies. Comparable amounts of recombinant α-subunits were applied as total cell lysates of *E. coli* to SDS-gels (8% acrylamide), separated in the presence of 4.3 M urea and blotted onto nitrocellulose filters. Filters were incubated with AS 248 (α_{o1}-peptide antibody, diluted 1:25), AS 201 (α_{o2}-peptide antibody, diluted 1:25) and AS 6 ($\alpha_{o\text{-common}}$-peptide antibody, diluted 1:50). Molecular masses (kDa) of marker proteins are indicated on the left. Alkaline phosphatase was used for visualization of filter-bound antibodies. Proteins applied: $\alpha_{i1}=i_1$; $\alpha_{i2}=i_2$; $\alpha_{i3}=i_3$; $\alpha_{o1}=o_1$; $\alpha_{o2}=o_2$; $\alpha_s=s$; $\alpha_z=z$). The proteins of >43 kDa immunostained with AS 6 are not related to G-proteins since they were present in all lysates irrespective of the transfected α-subunit.

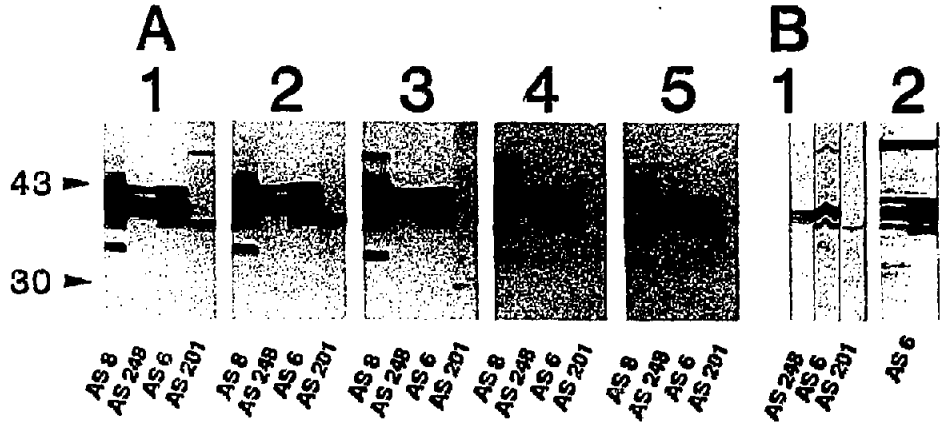

Fig. 2. (A) Comparison of α_o-subunits in cholate extracts of murine, rat, rabbit, bovine and porcine brain. Cholate extracts (750 μg) of murine (1), rat (2), rabbit (3), porcine (4) and bovine brain (5) were precipitated with acetone and loaded on gels (6 M urea, 9% acrylamide, 13 cm length, 3.5 cm width of slots). After blotting, nitrocellulose filters were cut into strips (6 mm) and incubated with the indicated antibodies. Dilutions of antibodies were: AS 8, 1:25, AS 248, 1:7.5; AS 6, 1:10; AS 201, 1:7.5. Molecular masses (kDa) of marker proteins are indicated on the left. The ECL system was used for detection of filter-bound antibodies. (B) Identification of α_o-subunits in membranes of HIT T15 cells and porcine brain. (1) Acetone-precipitated HIT T15 membrane proteins (2 mg, 35 mm slots) or (2) porcine brain membranes (80 μg, 4 mm slots) or cholate extracts from porcine brain membranes (50 μg, 4 mm slots) were loaded on gels and processed as described (see A) except that alkaline phosphatase-coupled antibodies were used and AS 6 (serum diluted 1:200) was applied to visualize protein bands.

more, the lower component of the middle anti-α_o-immunoreactive protein doublet was enhanced. The fastest migrating protein doublet was not detected by α_{o1}-specific antibodies (not shown). For more detailed studies, we employed 2D-SDS-PAGE. All species tested (i.e. mouse, rat and pig) showed similar patterns after staining with AS 6 (Fig. 3B and data not shown). Three major spots were detected with pI values of about 6.05, 5.6 and 5.45. The most basic protein was subsequently identified as α_{o2} which corresponded to the lower band in urea-containing SDS-PAGE whereas the acidic anti-α_{o1} immunoreactive spots were equivalent with the middle and upper bands of the urea-containing SDS-PAGE (see Figs. 2B and 3). Furthermore, all three major spots showed small satelite spots. These doublets, composed of a major and a minor spot, presumably correspond to the doublets on urea-containing SDS-PAGE. Evaluating the mobilities and pI values of the α_o proteins from various species, we detected a decreased mobility of α_o-subtypes in rodents in comparison to non-rodent species (Fig. 2A); additionally, the pI values of all three rat α_o-proteins were apparently more acidic than porcine α_o proteins.

4. DISCUSSION

Recently, three splice variants of $G_o\alpha$ have been cloned, coding for two distinct α_o-subunits [4–7]. Purification of the corresponding proteins revealed the existence of two isoforms of each α_{o1} and α_{o2} [8–11]. Additionally, a third α_o form was detected in brain membranes, using specific antibodies [12–15]. However, it is not clear whether this third form is identical with one of the known α_o proteins [14,18]. Therefore, we investigated the heterogeneity of the α_o-subunits. Using specific antibodies, we showed that all mammalian brain membranes tested express both splice variants of α_o. AS 248 (an anti-α_{o1}-specific antibody) identified the middle band in urea-containing SDS-PAGE as the most abundant α_o protein in mammalian brain, having a pI of 5.6. AS 201 appears to distinguish between α_{o2} protein bands of rodent and non-rodent species, recognizing only the former (Fig. 2). This phenomenon can be due to species differences in the chosen peptide sequence (see Table 1). Nevertheless, taking together the results obtained with α_o-specific antibodies, e.g. AS 6 and AS 248, and with urea-containing SDS-PAGE and 2D-SDS-PAGE, it is obvious that adult bovine, porcine and rabbit membranes do contain significant amounts of an α_{o2}-like form (see Fig. 3A and B). Moreover, using these gel systems in combination with immunoblot analysis we showed that there are differences in the electrophoretic mobility of rodent and non-rodent α_o-isoforms. These differences may be the result of small changes in the amino acid composition and/or different co- or posttranslational modifications [7,19]. All membranes obtained from whole brain tissue but not membranes from distinct cell lines tested show a third $G_o\alpha$-protein with a pI value of about 5.45 which is detected by α_{o1}- and $\alpha_{o\text{-common}}$-specific antibodies but not by α_{o2}-specific antibodies. The most acidic α_o-form on 2D-SDS-PAGE and the slowest migrating α_o protein on urea-containing SDS-PAGE is identical with neither the two α_{o2} nor two α_{o1} isoforms reported previously [10,11]. Therefore, it is not G_{39}* described by Goldsmith et al. [8], which actually is G_{o2} [20], with a pI of about 6.05. All three α_o proteins appear as 2 isoforms in two different gel systems. Since myristoylation slightly

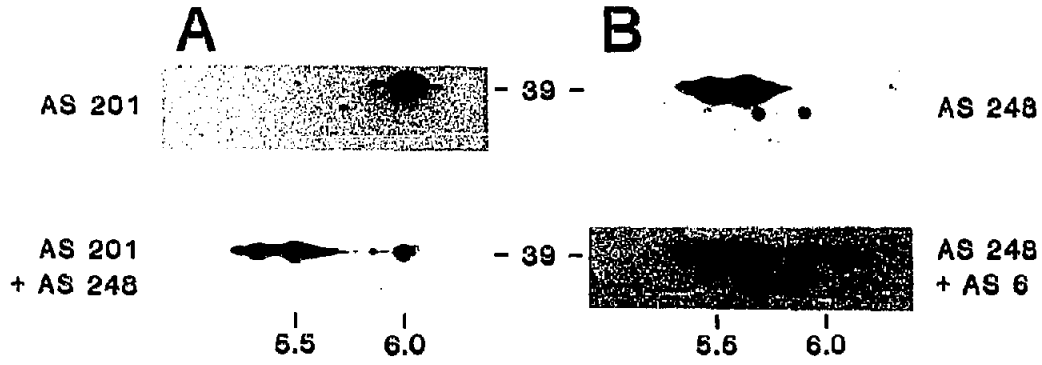

Fig. 3. 2D-SDS-PAGE of rat and porcine α_o-proteins. Rat (A) and porcine (B) acetone-precipitated brain membrane proteins (350 μg and 250 μg, respectively) enriched on DEAE-Sepharose were loaded on 2D-SDS-PAGE. After blotting of proteins, nitrocellulose filters were first incubated with AS 201 (A, dilution 1:7.5) or AS 248 (B, dilution 1:10). Detection of filter-bound antibodies visualizing α_{o2}-subunits (A) and α_{o1}-subunits (B) was performed with the ECL system (upper panel). Subsequently, the identical nitrocellulose filters were incubated with AS 248 (A, dilution 1:10) or AS 6 (B, serum diluted 1:200) in order to identify α_{o1}-subunits (A) and α_o-subunits (B) not detected with AS 248. After incubation with two different subunit-specific antibodies both species show similar G_o α-subunit-pattern (lower panel). pI values are shown under the figure, and molecular masses between panels A and B.

changes the mobility of α_o proteins in urea-containing SDS-PAGE [19], it is feasible that the two isoforms of the proteins found in this study, by Katada's group [10,11], and Goldsmith et al. [8] differ in their co- or posttranslational modifications. Experiments are underway to evaluate this question and to prove whether the third α_o-form found is a novel gene product or result of a different co- or posttranslational modification.

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