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Water-soluble GTP-binding protein from rat olfactory epithelium

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Abstract Rat olfactory epithelium and ciliar cytosol of olfactory cells contained the water-soluble 45 kDa protein which was revealed by antibodies against a peptide fragment of the α-subunits common to the G-proteins. No analogous proteins were found in other rat tissues. According to the photo-affinity labeling, the 45 kDa protein possessed a high affinity to GTP; it also exhibited a low GTP hydrolytic activity.

Key words: GTP-binding protein; α-Subunit of G-protein; Olfactory epithelium; Cilia; Rat

1. Introduction

It is generally accepted that guanine nucleotide binding proteins (G-protein) are involved in the olfactory transduction [1]. With different approaches, several categories of G-proteins have been identified in the olfactory epithelia of vertebrates and insects [2–5]. This set includes the stimulatory G-protein (G_s), the inhibitory G-protein (G_i), the olfactory specific G-protein (G_{olf}). Recently, using antibodies raised against peptide fragment common to the α-subunits of the G-proteins, we have found a water-soluble GTP-binding protein which has properties different from those of the listed G-proteins. In the present paper we describe some properties of this GTP-binding protein.

2. Materials and methods

2.1. Materials

[α-^{32}P]GTP, [γ-^{32}P]GTP (> 2000 Ci/mmol) was from Physics and Energy Inst., Obninsk, Russia; peroxidase-conjugated goat anti-rabbit Ig from Amersham, UK; GTP, Triton X-100, 3',3-diaminobenzidine, chemicals for electrophoresis from Sigma (St. Louis, USA); and Immobilon PVDF from Millipore, Bedford, USA.

2.2. Preparation of anti-peptide antisera

The sequence of a synthetic peptide for the generation of antisera against α_{common} (CGAGESGKSTIVKQMK) was deduced from published sequences of cDNA encoding the α-subunits of G-proteins [6]. Peptide synthesis and coupling to keyhole limpet hemocyanin were performed as described before [7]. Female New Zealand white rabbits were used for the generation of the antisera.

2.3. Preparation of tissue extracts

Male Wistar rats were killed by decapitation. Nasal turbinates were dissected, pooled and washed in Dulbecco's modified Eagle's Medium (DME), pH 8.0, at 4°C. The tissue was centrifuged at 1,000 × *g* for 5 min, the supernatant was collected ('washing extract'), and the pellet was resuspended in DME. The olfactory cilia were detached by a calcium shock procedure raising the Ca^{2+} concentration in DME media to 10 mM [8]. The deciliated epithelia were removed by centrifugation for 5 min at 1,000 × *g*. The supernatant containing the detached cilia was centrifuged for 15 min at 12,000 × *g*. The supernatant was collected ('Ca^{2+}-extract'), and the pellet containing the isolated cilia was washed twice in DME medium. To obtain cilia cytosol and ciliar membrane, the pellet was resuspended in 10 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 8.0 and centrifuged for 30 min at 20,000 × *g*. The supernatant ('ciliar cytosol') and the pellet ('ciliar membrane') were collected.

To obtain the detergent extracts, the nasal turbinates were carefully washed with DME media and incubated in 0.3% Triton X-100 in DME medium for 1 or 5 min (1 ml detergent solution per mucosa) at 4°C, and medium was collected and centrifuged at 20,000 × *g* for 30 min. The same procedures were performed with hamster and guinea pig olfactory epithelia.

Protein concentrations were measured according to the method of Bredford [9] using bovine serum albumin as a standard.

2.4. Gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis was performed after treatment of the samples with 2-mercaptoethanol on 10% slab gels in a discontinuous buffer system according to Laemmli [10].

For immunoblotting the electrophoretic transfer onto a Immobilon PVDF membrane was performed in a 2051 Midget Multiblot Transphor Unit (LKB, Sweden) in 100 mM Tris, 100 mM boric acid, pH 8.2–8.3, for 1 h at 10°C. The incubation with primary antibody at appropriate dilution was in 10 mM sodium phosphate buffer, 0.9% NaCl and 0.05% Tween 20 for 3 h at 4°C. The bound antibody was visualized by the formation of a complex with rabbit Ig horseradish peroxidase-linked antibody. The complexes were visualized in 10 mM sodium phosphate buffer and 0.9% NaCl, pH 7.5, containing 0.5 mg/ml 3',3-diaminobenzidine tetrachloride and 0.015% hydrogen peroxide.

2.5. Photo-affinity labeling

Direct photo-affinity labeling of protein with [α-^{32}P]GTP was carried out according to Antonoff et al. [11]. Protein samples were irradiated with the source having a peak emission at 253.7 nm. Affinity-labeled proteins were analysed by SDS-gel electrophoresis.

GTPase activity was determined according to [12].

3. Results and discussion

Fig. 1a shows the immunoblotting of SDS gel electrophoresis patterns of extracts obtained at different steps of cilia isolation. Ca^{2+}-extracts contained the water-soluble protein of 45 kDa which was revealed by the antibodies against α_{common}. This protein was also found in the ciliar cytosol which was obtained after hypotonic shock of isolated cilia. In the membrane of purified cilia, the antibodies against α_{common} revealed only one main band (about 40 kDa) which is likely to represent the well-known G-proteins of olfactory epithelium [1–3]. So, the presence of the water-soluble 45 kDa protein in Ca^{2+}-extracts seems to be caused by a partial destruction of cilia during the Ca^{2+} treatment of mucosa which results in the shedding from the cilia of some cytosol protein. The same cause obviously explains the identification of the 45 kDa protein in the washing solution.

The 45 kDa protein can be extracted from the rat olfactory epithelium by a short detergent treatment of olfactory mucosa: one minute treatment with 0.3% Triton X-100 mainly extracted

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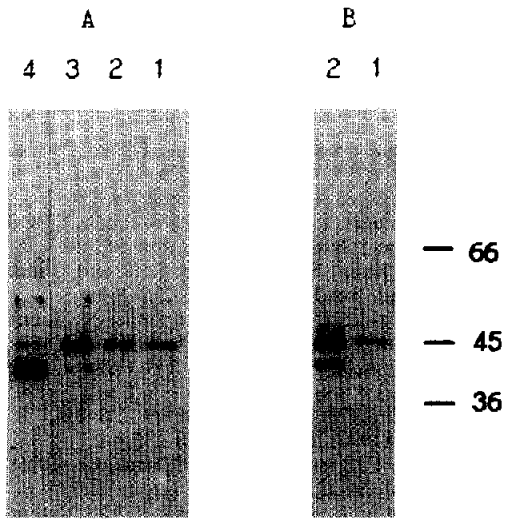

Fig. 1. Immunoblots of various extracts from rat olfactory epithelium with rabbit antisera against α_{common} peptide. (A) Extracts obtained at different steps of cilia isolation: 1, mucosa washing with DME medium; 2, 'Ca^{2+}-extract' of mucosa; 3, cytosol of isolated cilia; 4, membrane of isolated cilia. (B) Triton X-100 extracts of rat olfactory epithelium: 1, one-minute treatment of mucosa, only 45 kDa protein was revealed; 2, five min treatment of mucosa, two proteins were revealed – 45 kDa and 40 kDa.

45 kDa protein (Fig. 1b). Repeated detergent treatment (for 5 min) also extracted the 40 kDa membrane protein. It is likely that the 5-min Triton X-100 treatment of the mucosa solubilized ciliar membrane, while the one-minute detergent treatment only partially destroyed the ciliar membrane. On the other hand, the 45 kDa protein might have a weak binding to the ciliar membrane and therefore might be easily extracted by a short treatment with detergents as in the case of the specific olfactory protein, Olfactomedin [13]. Therefore, it is not to be

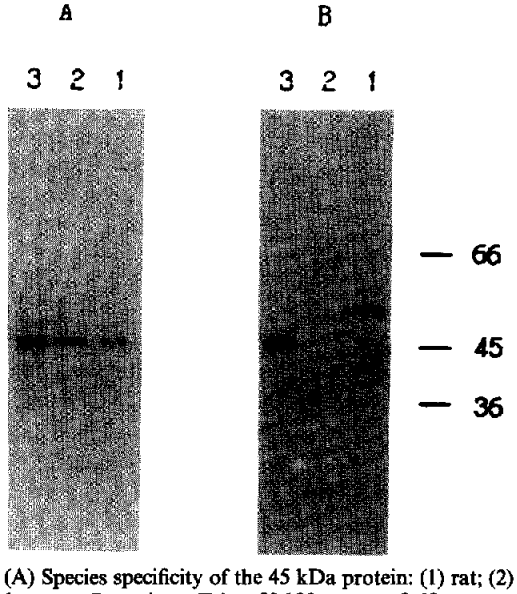

Fig. 2. (A) Species specificity of the 45 kDa protein: (1) rat; (2) guinea pig; (3) hamster. One minute Triton X-100 extract of olfactory epithelia. (B) Tissue specificity of 45 kDa protein: (1) 'Ca^{2+}-extract' of rat olfactory epithelium, (2) liver cytosol, (3) brain cytosol. Visualisation by rabbit antisera against α_{common} peptide.

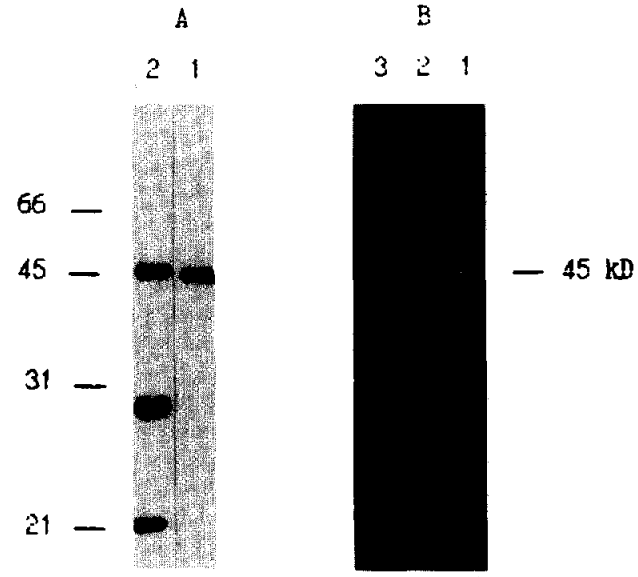

Fig. 3. (A) SDS-electrophoresis patterns of partially purified 45 kDa protein from rat olfactory epithelium. (1) Visualisation by rabbit antisera against common α-subunits of G-proteins; (2) Coomassie R-250 staining. 45 kDa protein was purified by DEAE ion-exchange chromatography on DEAE-Sepharose followed by gel-chromatography on Sephacryl S-200. The 20 kDa protein is likely to represent the odor-binding protein (OBP) ([14]; Fig. 2). (B) Photo-affinity labeling of the 45 kDa protein by [^{32}P]GTP. (1) 7 μCi [^{32}P]GTP and UV; (2) 7 μCi [^{32}P]GTP without UV; (3) 7 μCi [^{32}P]GTP, 1 μM GTP and UV. Samples: 10 μg of 45 kDa protein in 200 μl of 100 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.4; the UV-irradiation was 1 h.

concluded that the 45 kDa protein is primarily present in the rat olfactory mucus.

To determine the tissue and species specificities of the 45 kDa protein, some attempts were made to reveal this protein in other tissues using the same procedure of tissue extract preparation and antibodies against α_{common}. No analogous proteins were found in other rat tissues (Fig. 2a); on the other hand the same protein was detected in olfactory epithelium extracts of guinea pig and hamster (Fig. 2b).

The 45 kDa protein was isolated from Ca^{2+}-extract and purified by ion-exchange chromatography on DEAE-Sepharose, gel-exclusion chromatography and isoelectrofocusing (manuscript in preparation). Fig. 3a shows SDS-patterns of partially purified 45 kDa protein from the rat olfactory epithelium. It was isolated as a single polypeptide with a molecular mass of about 45 kDa and an isoelectric point of about 5.35.

In the following experiments we tried to elucidate some properties of the 45 kDa protein. We used the antibodies generated in rabbits by immunization with a peptide fragment common to the α-subunits of most G-proteins (P region of the G-protein sequence). This region, with the consensus sequence motif, is common not only in GTP-binding proteins, but also in many nucleotide triphosphate-utilizing enzymes [15]. Since our protein was revealed by antibodies against α_{common} it might be expected that it could also bind to GTP. Fig. 3b shows the data on photo-affinity labeling of the 45 kDa protein by [^{32}P]GTP. As shown in the figure, the 45 kDa protein possessed a high affinity to GTP, but not to ATP. The purified 45 kDa protein also exhibited a low GTP hydrolytic activity (less than

10 nmol/mg/min). Thus, considering these properties of the isolated 45 kDa protein, we assigned it as 'water-soluble GTP-binding 45 kDa protein'.

The yield of the 45 kDa protein isolated from one rat mucosa by Ca^{2+} shock was about 1–2 μg. On the other hand, immunohistochemical staining of olfactory mucosa using the antibodies against α_{common} showed that the 45 kDa protein is mostly located in mucus layer (data not shown). So, the local concentration of the 45 kDa protein is rather high.

It is tempting to suppose that the water-soluble GTP-binding 45 kDa protein from the rat olfactory mucosa is a member of G-protein family (GTP-binding, low GTP-ase activity, interaction with antibodies generated against α_{common} of G-protein). However, some of its properties prove to disagree with this hypothesis. Firstly, this protein is not a membrane protein and could easily be isolated without detergents. (For known G-protein, there is only one exclusion: water-soluble transducin in photoreceptor cells [16].) Moreover, when purified under mild conditions only a single 45 kDa polypeptide was isolated while the known G-proteins have a three-subunit structure (α-, β- and γ-subunits) [17]. So, the function of isolated protein still remains unknown.

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