Co-localization of Rab4 with endocytosis-related proteins in the rat parotid glands

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Summary. Small GTP-binding proteins have been implicated in the regulation of vesicular traffic. We investigated the localization of Rab4 in the rat parotid glands by Western blotting and light-microscopic immunohistochemistry. Rab4 was localized mainly on the intracellular membranes in the subapical-actin terminal web, but was not present in the basolateral region both in acinar and ductal cells. Actin, α-adaptin, Rab5A and aquaporin5 were detected in the Rab4-containing intracellular membrane fraction prepared using anti-Rab4 antibody covalently coupled to magnetic beads. Detection of actin indicated that the Rab4-containing intracellular membranes were attached to the actin filaments. Although α-adaptin was immunohistochemically distributed along the plasma membrane, this protein coexisted with Rab4 only at the apical region. Rab5A immunoreactivity was distributed all around the cytoplasm. These findings suggested that Rab4 participates in endocytosis at the apical membrane of parotid glands.

Introduction

We previously investigated expression of the genes encoding Rab proteins in the rat parotid glands and detected mRNAs of Rab3D, Rab26, and Rab4. Rab26 is localized on the secretory granules and participates in regulated exocytosis (Yoshie et al., 2000) as well as Rab3D (Raffaniello et al., 1999). On the other hand, neither the localization nor the roles of Rab4 have been investigated in parotid glands.

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Several observations have suggested that Rab4 is a ubiquitous protein and is involved in the endocytotic pathway (review: Martínez and Goud, 1998). Rab4 is associated with vesicles containing glucose transporter 4 (GLUT4) and mediates the insulin-induced translocation of GLUT4 to the plasma membrane in rat muscle cells (Aledo et al., 1995; Sherman et al., 1996) and adipocytes (Shibata et al., 1996; Vollenweider et al., 1997). In pancreatic acini, which morphologically resemble parotid acini, Rab4 is localized in the subapical region (Valentijn et al., 1997). The functions of Rab4 in the pancreatic acini have been reported: Rab4 controls regulated exocytosis (Ohnishi et al., 1999), and is related to endocytosis, moving through the supranuclear-recycling compartment (Valentijn et al., 2000).

Endocytosis is triggered by the interaction with the endocytic clathrin adaptor AP-2 (see review by Kirchhausen et al., 1997). The internalized clathrin-coated vesicles are uncoated and transported to early endosomes. In the early endosomes, molecules destined for degradation are sorted towards the late endosome and molecules destined recycling are sorted to the plasma membranes via recycling compartment (Gruenberg and Maxfield, 1993). Rab5A is reported to be a common component of endocytic machinery, and is known as an early endosome marker (Bucci et al., 1994; Juvet et al., 1997). Aquaporin5, which functions as a water channel, is reported to be localized at the apical membranes of parotid acinar cells (Matsuzaki et al., 1999). It is considered that aquaporin5 is recruited from intracellular membranes to the apical plasma membrane (Ishikawa et al., 1998). The cytoskeleton is required for multiple cellular events including endocytosis. It has been reported that actin filaments are localized immediately under the apical membrane in the polarized cells (Gottlieb et al., 1993). Actin plays a role in internalization at cell surfaces (Gottlieb et al., 1993; Apodaca, 2001).

We hypothesized that Rab4 might play a role in intracellular transport including endocytosis in parotid glands. As the first step in the study of the role of Rab4 in the
rat parotid glands, we performed Western blotting and immunohistochemistry to investigate the localization of Rab4 and other proteins related to endocytosis: α-adaptin a subunit of AP-2, Rab5A, aquaporin5 and actin.

**Materials and Methods**

**Antibodies**

Rabbit polyclonal antibodies against rat Rab4 and Rab5A were purchased from Santa Cruz Biotechnology Inc. (USA). Mouse monoclonal antibody against actin (C4), rabbit polyclonal antibody against rat aquaporin5, Cy3-labeled anti-mouse IgG and Cy5-labeled anti-rabbit IgG were from Chemicon International Inc. (USA). Mouse monoclonal antibodies against α-adaptin (clone AC1-M11) and γ-adaptin (clone 88) were from Affinity Bioreagents Inc. (USA) and Transduction Laboratories (USA), respectively. Goat anti-mouse IgG and goat anti-rabbit IgG were from Sigma (USA).

**Preparation of subcellular fractions from the rat parotid glands**

All procedures were approved by the animal care committee of our university. Male Wistar rats (9 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and killed by cardiac puncture. Subcellular fractions were prepared as described previously with some modifications (Cormont et al., 1993). The dissected parotid glands were sliced, minced and homogenized with a glass homogenizer and Teflon pestle in 20 vol of homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 250 mM sucrose, pH 7.4, 100 units/ml aprotinin, and 1 mM amidinophenylmethylsulfonyl fluoride). The homogenate was first centrifuged at 30 g for 10 min at 4°C to eliminate nuclei and then the supernatant was centrifuged at 18,000 g for 15 min at 4°C. The 18,000 g supernatant was recentlyfuged at 240,000 g for 75 min at 4°C. The resultant precipitate was used as the intracellular membrane (ICM) fraction. The precipitate obtained by centrifugation at 18,000 g was resuspended in homogenization buffer and centrifuged with 40% Percoll at 20,000 g for 20 min at 4°C. The upper phase was collected and centrifuged with homogenization buffer at 240,000 g for 45 min at 4°C. The resultant precipitate (plasma membranes, PM) was suspended in PM suspension buffer (5 mM HEPES buffer, pH 7.4, 50 mM mannitol, 0.25 mM MgCl2).

**Preparation of Rab4-containing intracellular membranes**

Covalent coupling of anti-Rab4-antibody to magnetic beads (Dynabeads M-280 sheep anti-rabbit IgG; Dynal, Norway) was performed according to the manufacturer's instructions with slight modifications. Briefly, anti-Rab4 antibody (6 µg) or the IgG fraction of rabbit serum was incubated with 1.7 mg of magnetic beads in 100 µl of wash solution (phosphate-buffered saline, pH 7.4, 0.1% BSA) overnight at 4°C with gentle mixing. After washing four times in the same solution with bidirectional mixing for 30 min each time, beads were resuspended in 1 ml of cross-linking buffer solution (0.2 M triethanolamine, pH 8.2) and were washed two more times with the same solution. Dimethyl pimelimidate (DMP, 20 mM) in 10 ml of cross-linking buffer solution was added to the beads and incubated for 45 min at room temperature with bidirectional mixing. After incubation in 10 ml of cross-linking buffer without DMP for a further 2 hr, beads were resuspended and incubated in 10 ml of 1% Nonidet P-40 (NP-40)-containing cross-linking buffer solution for further 10 min to completely eliminate noncovalently bound IgG. The beads were washed in 1 ml of wash buffer three times and were used in the fractionation step. The ICM fraction (1 mg) resuspended in wash buffer (0.5 ml) was added to the beads and incubated for 5 h at 4°C with gentle mixing. After washing in 1 ml of 0.5 M NaCl following three times in 1 ml of wash buffer, the bound proteins were eluted with 100 µl of Laemmli sample buffer and were boiled for 5 min or incubated at 20°C for 3 h for the detection of aquaporin5.

**Immunoblot analysis**

Fractions solubilized in Laemmli sample buffer were subjected to 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE). After PAGE, the separated proteins were electrotransferred from the gel on a PVDF membrane (Immun-Blot, Bio-Rad Laboratories, USA). The blots were probed with the primary antibodies. The immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Biosciences UK Ltd, UK).

**Light-microscopic immunocytochemistry**

Parotid glands were excised from anesthetized animals and rapidly frozen in precooled isopentane. Frozen sections were cut at a thickness of 8 µm on a cryostat, mounted on gelatin-coated glass slides and immediately immersed in 4% paraformaldehyde in 0.07 M phosphate buffer (pH 7.3) for 30 min. After fixation, the slides were rinsed in phosphate-buffered saline (pH 7.3) and processed for the following procedures.

Immunohistochemistry was performed using the indirect fluorescence method. Individual sections were incubated overnight at 4°C with one of the polyclonal antisera against Rab4 (dilutions: 1:400–1:1600), Rab5A (1:100–1:400) and aquaporin5 (1:200–1:400) or monoclonal antibodies against α-adaptin (1:100–1:400) and γ-adaptin (1:400–1:400).
After incubation, these sections were exposed to Cy3-labeled anti-rabbit IgG (1:200) or to Cy3-labeled antimouse IgG (1:100) for 2 h at room temperature. Some sections immunostained were further stained with Alexa 488-labeled phalloidin (Molecular Probes, USA), which binds specifically to F-actin. The stained sections were observed under a fluorescence microscope (Axioplan, Zeiss, Germany) and/or confocal laser scanning microscope (LSM 510, Zeiss).

The specificity of immunoreactivity was checked using the following negative controls: omission of the primary antiserum, incubation of tissue sections with normal rabbit or mouse serum in place of each primary antiserum.

Results

Western blotting with anti-Rab4 antibody

Figure 1 shows that Rab4 antibody recognizes a single band of the expected size in the rat parotid homogenate. We performed subcellular fractionation of the rat parotid glands and obtained two membrane fractions, a plasma membrane fraction (PM) and an intracellular membrane fraction (ICM), as well as the supernatant fraction (cytosol). We then performed Western blotting of these subcellular fractions with anti-Rab4 antibody (Fig. 2). Rab4 was primarily present in the ICM fraction, less abundant in the PM fraction, and was detected only faintly in the cytosolic fraction. Further, the endocytosis-related proteins were examined by Western blotting. Actin was present in all the fractions except the PM fraction. The ICM fraction also contained Rab5A, α-adaptin, aquaporin5, and γ-adaptin. γ-Adaptin is a subunit of clathrin adaptor AP-1, and is contained in the coated buds or vesicles derived from the TGN (review: Hirsh and Robinson, 1998). Aquaporin5 was reported to be localized at the apical region of acinar cells but not seen in the intercalated ducts (Matsuzaki et al., 1999). It was suggested that the ICM fraction was of microsomal origin and contained endosomes from the acinar cells (Pol et al., 1997).

Presence of actin and endocytosis-related proteins in the Rab4-immunoisolated fraction

The Rab4-immunoisolated fraction was obtained from the intracellular membrane (ICM) fraction with magnetic beads covalently coupled to anti-Rab4 antibody. Figure 3 shows immunoblots of the fraction with antibodies against α- and

Fig. 1. Immunoblotting analysis of Rab4 in the homogenate of the rat parotid gland. Electrophoresis of the homogenate (10 μg) on a 12% polyacrylamide gel showed a single band of 25 kDa of Rab4 immunoreactivity. The bars indicate the positions of the molecular weight markers.

![Image of immunoblot analysis](image_url)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight</th>
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<tr>
<td>Rab4</td>
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<tr>
<td>Rab5A</td>
<td>25 kDa</td>
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<tr>
<td>Actin</td>
<td>48 kDa</td>
</tr>
<tr>
<td>AQP5</td>
<td>25 kDa</td>
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<tr>
<td>α-Adaptin</td>
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<td>γ-Adaptin</td>
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![Image of immunoblots](image_url)

Fig. 2. Immunoblotting analysis of Rab4 and intracellular vesicle-related proteins in the subcellular fractions from the rat parotid gland. Aliquots (10 μg) of protein from each fraction were analyzed except in the case for aquaporin5, for which aliquots of 50 μg of protein were analyzed. For Rab4 and aquaporin5, 12% polyacrylamide gels were used, and 10% polyacrylamide gels were used for the other proteins. Cyto: cytosol, PM: plasma membrane, ICM: intracellular membrane; AQP5: aquaporin5.
\( \gamma \)-adapts, actin, Rab5A, and aquaporin5. Clear immunoreactive bands for actin and aquaporin5 were detected in this fraction. This suggested that Rab4, aquaporin5 and actin colocalized on the same intracellular membranes. \( \alpha \)-Adaptn was detected in the immunolocalized fraction, but \( \gamma \)-adaptn was not. Rab5A was also detected in the Rab4-immunolocalized fraction.

![Figure 3](image)

**Fig. 3.** Immunobots of Rab4, Rab5, actin, aquaporin5, and \( \alpha \)- and \( \gamma \)-adapts in the Rab4-containing intracellular membrane fraction. The Rab4-containing intracellular membrane fraction (100 \( \mu \)l in Laemml sample buffer) was isolated from the ICM fraction (1 mg) using magnetic beads covalently bound with the anti-Rab4 antibody as described in “Materials and Methods”. For aquaporin5, 50 \( \mu \)l of protein from ICM fraction and 5 \( \mu \)l of the Rab4-containing intracellular membrane fraction were analyzed. For the other proteins, 10 \( \mu \)g of protein from the ICM fraction and 1 \( \mu \)l of the Rab4-containing intracellular membrane fraction were analyzed. Polyacrylamide gel concentrations were 12% for Rab4, Rab5A, actin and aquaporin5, and was 10% for \( \alpha \)- and \( \gamma \)-adapts. In the figure, the left lane indicates the immunobots of the Rab4-containing intracellular membrane fraction, and the right lane indicates the immunobots of the control fraction obtained using the magnetic beads covalently bound with the rabbit IgG. AQP5: aquaporin5.

**Immunohistochemical localization of Rab4 and the endocytosis-relating proteins**

We examined the cytoplasmic distribution of Rab4 in the rat parotid gland by immunofluorescence microscopy (Fig. 4). Rab4 was localized in the subapical region of acinar and ductal cells (Fig. 4a). The actin terminal web stained with fluorescent phalloidin was detected in the region surrounding the lumen (Fig. 4b). The merged image (Fig. 4c) showed that the localization of Rab4 immunoreactivity overlapped the actin terminal web in acinar and ductal cells. This supported the Western blotting findings that Rab4 and actin co-localized in the same membrane fraction. The immunolocalization of Rab4 around the apical plasma membrane of the acinar cells was confirmed by confocal laser scanning microscopy as follows. Rab4 was detected at the apex and in the region of the actin terminal web in acinar cells (Fig. 5). Immunostaining was negative in the absence of anti-Rab4 antibody (data not shown).

**Discussion**

Rab4 is a ubiquitous small GTP-binding protein, and the functional roles of Rab4 have been investigated mainly in relation to endosomal trafficking in adipocytes (Hashimoto and James, 2000), HeLa cells (McCaffrey et al., 2001).
and human embryonic kidney cells (Seachrist et al., 2000). In these cells, Rab4 is present in the endocytic vesicles recycling from early endosomes back to the plasma membrane.

In the present study of the rat parotid glands, Rab4 was mainly localized in the subapical region, where actin filaments are co-localized. Furthermore, actin was detected in the Rab4-immunostained fraction from the intracellular membrane fraction. Therefore, Rab4 was thought to be contained in certain vesicles attached to the actin filaments in the subapical region. The actin filaments are important during exocytosis in the polar cells including the parotid and pancreatic acini (Segawa et al., 1998; Valentijn et al., 1999a, b). It has been suggested that Rab4 plays a role in cytoskeletal rearrangement in adipocytes (Vollenweider et al., 1997). In parotid glands, Rab4 may also function in actin filament rearrangement in the subapical region especially in acinar cells, where exocytosis of the secretory granules drastically occurs (Segawa and Riva, 1996).

Rab5 and AP-2 are known to participate in endocytosis. In our immunohistochemical study, β-adaptin, a subunit of

Fig. 4. Rab4 immunoreactivity (a), phalloidin reaction (b), and overlay of a and b (c). The Rab4 immunoreactivity coincides with the phalloidin-reactive actin filament layer of the acinar cells (A) and intercalated duct cells (I). Fluorescence microscopy. Bar: 7 μm

Fig. 5. Closer view of Rab4 immunoreactivity by confocal laser scanning microscopy. The immunoreaction is localized along the apical membrane of acinar cells. Bar: 7 μm
AP-2, was localized both along the apical and the basolateral plasma membranes. The immunoblot of the anti-Rab4-immunoisolated fraction prepared from the ICM fraction in parotid glands showed a faint band with anti-α-adaptin antibody. These observations indicated that Rab4 participated in endocytosis with α-adaptin only at the apical region, while α-adaptin functioned in endocytosis in both the apical and the basolateral regions. Rab5A was detected in the anti-Rab4-immunoisolated fraction with immunoblotting, whereas the immunoreactivity of Rab5A was distributed throughout the cytoplasm of acinar and ductal cells. These observations suggested that Rab4 and Rab5A coincided only in a part of the endocytic pathway in parotid glands. It has also been reported in A431 cells that Rab5 expression coincided with that of Rab4 in part but not all the pathway of endocytosis (Sonnichsen et al., 2000).

Aquaporin5 is a component of the apical membrane in parotid acini (Matsuzaki et al., 1999). It has been reported that aquaporin5 is present in the apical membrane and in the intracellular vesicle fraction, and is transported to the apical membrane from the intracellular vesicles by muscarinic or α-adrenergic stimulation (Ishikawa et al., 1999). In the present study, aquaporin5 was contained in the anti-Rab4-immunoisolated fraction, therefore, this fraction contained the apical endosome of acinar cells and Rab4 might be related to the transporting pathway for aquaporin5. It is reasonable for the rapid transport between the apical plasma membrane and the intracellular vesicles that the Rab4-containing vesicles containing aquaporin5 are localized under the apical membrane of acinar cells.

In summary, Rab4 was localized mainly in the subapical region in the rat parotid glands, and was co-localized with the actin terminal web, partially with α-adaptin and Rab5A. Aquaporin5 was co-localized with Rab4 in the parotid acinar cells. The results of the present study suggested that Rab4 participates both in endocytosis at the apical membrane and in the transport of apical membrane components. Further studies are required to determine the function of Rab4 in parotid glands.

References


Figs. 6-9. Fluorescence microscopy. Bars: 10 μm
Fig. 6. α-Adaptin immunoreactivity (a) and merged with phalloidin reaction (b). The immunoreactivity is localized along the plasma membrane of the acinar cells.
Fig. 7. γ-Adaptin immunoreactivity (a) and merged with phalloidin reaction (b). The immunoreactivity is seen in the vicinity of nuclei in the acinar cells.
Fig. 8. Rab5A immunoreactivity (a) and merged with phalloidin reaction (b). The immunoreactivity is spread throughout the cytoplasm of acinar cells.
Fig. 9. Aquaporin5 immunoreactivity (a) and merged with phalloidin reaction (b). The immunoreactivity is localized along the apical plasma membrane of the acinar cells.


