Immunohistochemical and immunoblotting identification of protein phosphatase 1γ1 in rat salivary glands

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Abstract **We have analyzed the distribution of the γ1 isotype of rat protein phosphatase type 1 catalytic subunit in rat salivary glands. Formaldehyde-fixed paraffin sections were reacted with the PP1γ1 antibody using an immunohistochemical method. Positive staining occurred in striated ducts of parotid gland. However, the staining reaction was less intense in submandibular gland. Proteins were also prepared from rat salivary glands and subjected to SDS-PAGE, followed by Western blotting analysis with the PP1γ1 antibody. The antibody interacted with protein corresponding to an estimated molecular mass of 36 kDa present in the parotid gland. The staining reaction was considerably weaker with the proteins from submandibular gland.**

Key words: Protein phosphatase; Salivary gland; Parotid gland; Submandibular gland

1. Introduction

Protein phosphorylation and dephosphorylation have been recognized as a key mechanism in the regulation of cellular metabolism and functions in various tissues [1,2]. Protein phosphatase type 1 (PP1) is a family of serine/threonine protein phosphatases (PPs) that are sensitive to heat-stable inhibitory proteins unlike the other known PPs, PP2A, PP2B, and PP2C [3]. The cDNAs encoding the catalytic subunit of PP1 were isolated from yeast, rabbit, and mouse [4]. Four distinct cDNA clones for rat PP1 catalytic subunits have been isolated from rat tissues and designated as PP1α, PP1γ1, PP1γ2, and PP1δ [5]. The antisera were developed in rabbits against peptides from the carboxy-termini of the four types of PP1 catalytic subunits. Characterization of the specificity of these antisera has recently been described [6]. Although the expression of PP1s has been investigated at the mRNA and protein levels [6], the distribution of PP1s in histological localization at the protein level is still unclear. Immunohistochemical studies have been performed in rat cerebellum [7] and testis [8] with PP1γ1 and PP1γ2, respectively. In the present study, PP1γ1 antibody was used to detect proteins interacting with the PP1γ1 antibody in rat salivary glands.

2. Material and methods

2.1. Antisera

The antisera used were those reported previously [6]. Briefly, antibody was raised against oligopeptide corresponding to the predicted amino acid sequence of the carboxy-terminal domain (CTPPRTGMITQAKK) of PP1γ1. The antibodies against PP1α and PP1δ were those reported previously [6].

2.2. Preparation of tissue proteins

Young male Wistar rats were anesthetized with diethyl ether and perfused with phosphate-buffered saline (PBS) via the vena cava. The salivary glands (parotid gland and submandibular/sublingual gland) were excised and placed in cold PBS. After washing several times with PBS, the tissue was transferred to the lysate buffer containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 2 μg/ml aprotinin and 5 mM EGTA in PBS, and minced with scissors. The tissue fragments were homogenized with 30 strokes using a glass homogenizer and a Teflon pestle, all placed in ice. The homogenate was centrifuged at $10000 \times g$ for 15 min and the supernatant was collected.

2.3. SDS-PAGE and immunoblot assay of proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the basic procedure of Laemmli [9]. The proteins were denatured in a sample buffer containing 2% SDS, 20% glycerol, 1.54% DTT, and 62.5 mM Tris-HCl buffer (pH 6.8). The mixtures were heated in boiling water for 5 min. Stacking and separating gels contained 4 and 10% acrylamide, respectively. After electrophoresis, proteins and prestained molecular weight markers (Gibco, BRL, Gaithersburg, MD, USA) were transferred from the gels to polyvinylidene difluoride (PVDF) transfer membrane sheets (Millipore, Bedford, MA, USA) according to the basic procedure of Towbin et al. [10]. The membrane sheets were incubated on a rotary shaker for 2 h at 20–22°C in blocking solution containing 4% BSA and 5% normal goat serum (NGS) in PBS. The sheets were rinsed briefly in PBS and incubated overnight at 4°C in blocking solution containing the antisera diluted 1:200. The washed sheets were incubated in blocking solution containing 1:1000 diluted horseradish peroxidase conjugated affinity-purified $F(ab')_2$ fragment of goat anti-rabbit IgG (Cappel Lab., Malvern, PA, USA) for 2 h at 20–22°C. Bound peroxidase was detected by developing the sheets with 4-chloro-1-naphthol and hydrogen peroxide according to the method of Hawkes et al. [11].

2.4. Histochemistry

Young rats were anesthetized with diethyl ether and perfused with PBS via the vena cava, then fixed with 3.5% formaldehyde. Parotid and submandibular/sublingual glands were extirpated and fixed overnight in the same fixative. Selected portions of tissues were embedded in paraffin and sectioned at 6 μm by standard histological procedures. Sections were deparaffinized, washed with PBS, and preincubated with 0.3% hydrogen peroxide in methanol to inactivate endogenous peroxidase, followed by blocking with 4% BSA and 5% NGS in PBS at 20–22°C for 20 min in a humidified chamber. After rinsing with PBS, the sections were incubated for 20 min at 20–22°C with the antisera diluted 1:200 with PBS containing 4% BSA, and washed extensively with PBS. They were incubated with biotinylated goat anti-rabbit IgG for 20 min, washed as described above, and then incubated with horseradish peroxidase-labeled streptoavidin (LSAB kit, Dako Lab., Kyoto, Japan) for 20 min. The slides were washed as described above, visualized with 3-amino-9-ethylcarbazole, *N,N*-diethyl formamide (AEC, Merck, Darmstadt, Germany) and counterstained with Mayer's haematoxylin. As a negative control, each section was incubated with normal rabbit serum.

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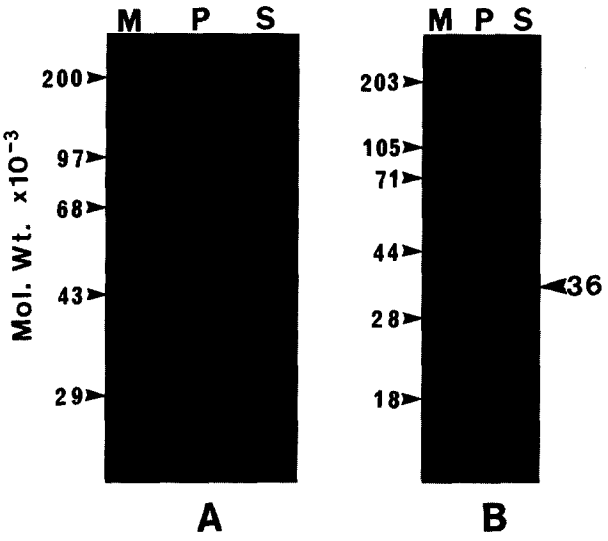

Fig. 1. Identification of PP1γl protein in rat salivary glands. (A) Rat parotid (P), submandibular/sublingual (S) proteins and molecular weight marker proteins (M) were run on a 10% gel and stained with Coomasie blue. (B) Western analysis of a parallel gel to that shown in (A) and interacted with the PP1γl antibody. In this case prestained molecular weight marker proteins were used.

3. Results

Fig. 1A shows the Coomassie blue staining pattern of the proteins obtained from rat parotid (P) and submandibular/sublingual (S) glands separated by SDS-PAGE. The Coomassie blue staining SDS-PAGE pattern of proteins from the parotid gland was quite different from that of the submandibular/sublingual gland. The major band corresponding to α-amylase is prominent in proteins of the parotid gland. Half of each protein sample was subjected to SDS-PAGE followed by immunoblotting. Fig. 1B shows the reaction between the PP1γl antibody and proteins obtained from parotid and submandibular/sublingual glands. The antibody interacted with a major band corresponding to an estimated molecular mass of 36 kDa present in the parotid gland. Two minor proteins of parotid gland with estimated molecular masses of 70 and 58 kDa were also stained. The 70 and 58 kDa bands were detected on membranes treated with normal rabbit serum, indicating the non-specific reaction of proteins. Very weak interaction was observed with proteins obtained from the submandibular/sublingual gland (Fig. 1B). The immunoreaction was eliminated by preincubating the antibody with the peptide used as the antigen.

The reaction of the PP1α and PP1δ with rat parotid gland

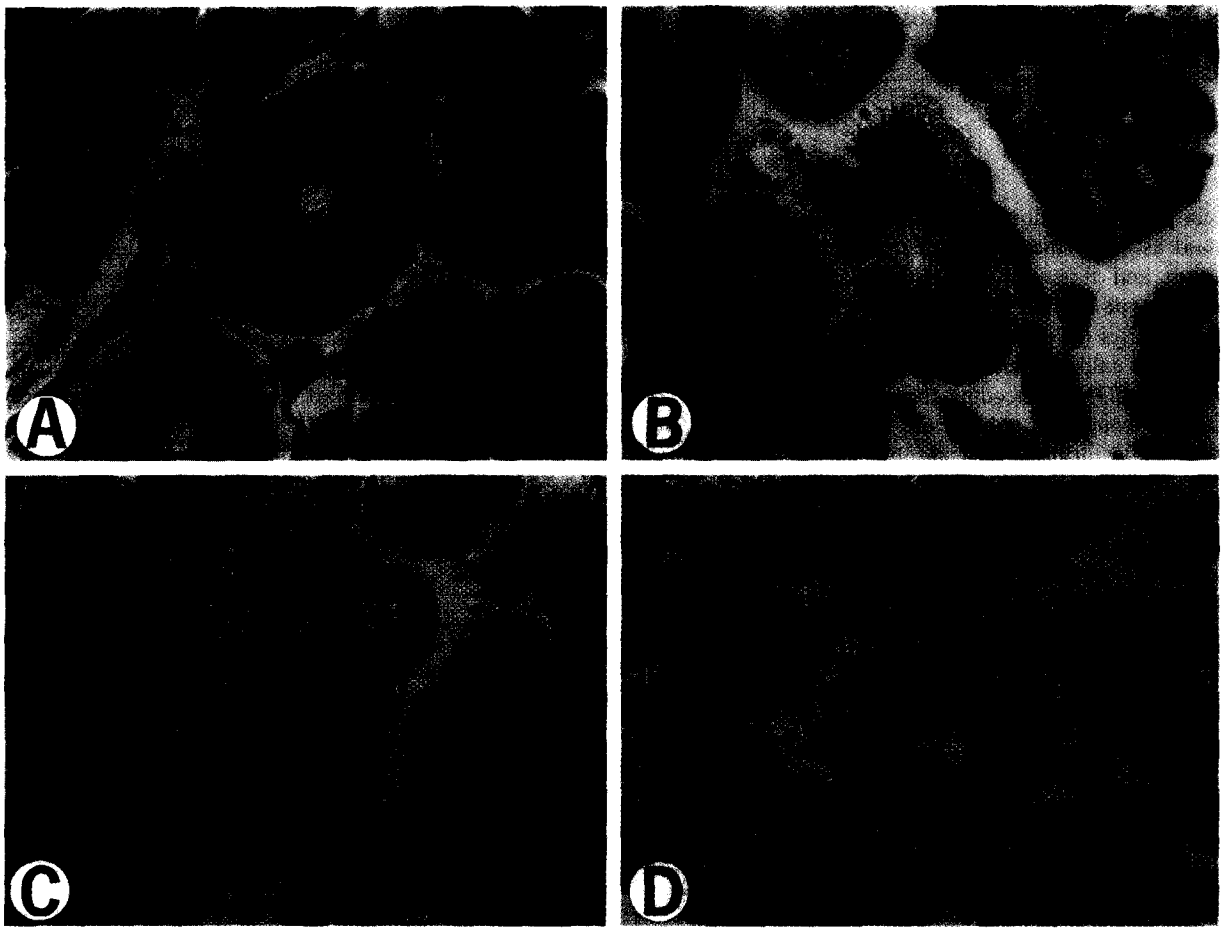

Fig. 2. Immunohistochemical identification of PP1γl protein in rat salivary glands. The staining method is described in the text. (A) Immunohistochemical staining of PP1γl protein in rat parotid gland. Intense staining is present in the basal area of a striated duct. (B) A control section of rat parotid gland treated with normal rabbit serum. A striated duct is in the center of the micrograph. (C) Immunohistochemical staining of PP1γl protein in rat submandibular gland. The staining reaction is less apparent in the basal area of the striated duct compared to those in the parotid gland. (D) A control section of rat submandibular gland treated with normal rabbit serum. A striated duct is in the center of the micrograph. Bar=10 μm.

was greatly reduced compared with the PP1γl antibody. Although a few faint bands were observed in both parotid and submandibular/sublingual gland proteins, the corresponding bands were also detected on membranes treated with normal rabbit serum (data not shown).

The cellular localization of the immunoreactive PP1γl protein in rat salivary glands was examined by an indirect immunohistochemical method using the PP1γl antibody. Fig. 2 shows the immunohistochemical staining of formaldehyde-fixed paraffin sections of rat parotid gland. Intense staining occurred in the cytoplasm of striated ducts of parotid gland (Fig. 2A). Weak staining was also observed in the secretory endpieces of the parotid gland. However, the staining reaction was less intense at both sites in submandibular gland (Fig. 2C). When the sections were stained with normal rabbit serum at the same dilution, no staining reaction was observed in parotid gland (Fig. 2B) and submandibular gland (Fig. 2D).

4. Discussion

The present study showed that the PP1γl antibody raised against the synthetic polypeptide of the catalytic subunit of rat PP1γl localized in the striated ducts of rat parotid gland. In contrast, PP1γl-positive cells were not observed in the rat submandibular gland. Although the significance of PPs in rat parotid gland is obscure, protein phosphorylation and dephosphorylation may be involved in amylase secretion. Stimulation of parotid gland lobules with a β-adrenergic receptor agonist such as isoproterenol induces exocytotic release of amylase [12]. The β-receptor activation is accompanied by specific phosphorylation of three particulate proteins of 35, 26 and 22 kDa [13,14]. Protein phosphatase inhibitors, okadaic acid and calyculin A, induce hyperphosphorylation of proteins in the rat parotid gland and inhibit amylase exocytosis [15,16].

In addition to increased phosphorylation of specific proteins, increased dephosphorylation of other proteins can be observed [17,18]. These findings suggest that the degree of phosphorylation of a specific protein in the parotid gland might be affected by changes in the activities of protein phosphatases. Our present results suggest that PP1γl protein may not play a direct role in amylase secretion because this phosphatase localized in parotid striated ducts rather than in secretory endpieces. The 35 kDa phosphoprotein phosphorylated by protein kinase A and/or C was identified as the ribosomal protein S6, suggesting that the role of S6 protein may be to coordinate receptor-stimulated exocytosis with cellular protein synthesis [19,20]. The S6 phosphoprotein in rat parotid gland [21] and Swiss 3T3 cells [22] was dephosphorylated by protein phosphatase type 1. The relationship between the S6 protein and PP1γl is now being studied.

The present study also showed that the PP1γl antibody recognized a major protein with an estimated molecular mass of 36 kDa in the rat parotid gland, which is in good agreement with that calculated from the amino acid sequence of the rat PP1γl protein [5,6]. Two minor proteins with estimated molecular masses of 70 and 58 kDa were also recognized. The 70 and 58 kDa bands are non-specific reactions of a major band of salivary glands and amylase, respectively, because these bands were also detected on membranes treated with the control serum.

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References

[1] Nishizuka, Y. (1986) Science 233, 305–312.
[2] Shenolikar, S. and Nairn, A.C. (1991) Adv. Sec. Messenger Phosphoprotein Res. 23, 1–121.
[3] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[4] Cohen, P.T.W., Brewis, N.D., Hughes, V. and Mann, D.J. (1990) FEBS Lett. 268, 355–359.
[5] Sasaki, K., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T. and Nagao, M. (1990) Jap. J. Cancer Res. 81, 1272–1280.
[6] Shima, H., Hatano, Y., Chun, Y.S., Sugimura, T., Zang, Z., Lee, E.Y.C. and Nagao, M. (1993) Biochem. Biophys. Res. Commun. 192, 1289–1296.
[7] Hashikawa, T., Nakazawa, K., Mikawa, S., Shima, H. and Nagao, M. (1995) Neurosci. Res. 22, 133–136.
[8] Shima, H., Haneji, T., Hatano, Y., Kasugai, I., Sugimura, T. and Nagao, M. (1993) Biochem. Biophys. Res. Commun. 194, 930–937.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Towbin, H., Staelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[11] Hawkes, R.E., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[12] Butcher, F.R. and Putney, J.W., Jr. (1980) Adv. Cyclic Nucleotide Res. 13, 215–249.
[13] Jahn, R., Unger, C. and Söling, H.D. (1980) Eur. J. Biochem. 112, 345–352.
[14] Quissell, D.O. and Deisher, L.M. (1992) Arch. Oral Biol. 37, 289–295.
[15] Takuma, T. and Ichida, T. (1991) FEBS Lett. 285, 124–126.
[16] Takuma, T., Ichida, T., Okumura, K. and Kanazawa, M. (1993) FEBS Lett. 323, 145–150.
[17] Burnham, D.B., Munowitz, P., Hootman, S.R. and Williams, J.A. (1986) Biochem. J. 235, 125–131.
[18] Mieskes, G. and Söling, H.D. (1987) Eur. J. Biochem. 167, 377–382.
[19] Freedman, S.D. and Jamieson, J.D. (1982) J. Cell Biol. 95, 909–917.
[20] Jahn, R. and Söling, H.D. (1981) Proc. Natl. Acad. Sci. USA, 78, 6903–6906.
[21] Hara-Yokoyama, M., Sugiya, H., Furuyama, S., Wang, J.H. and Yokoyama, N. (1994) Biochem. Mol. Biol. Int. 34, 1177–1187.
[22] Oliver, A.R., Ballou, L.M. and Thomas, G. (1988) Proc. Natl. Acad. Sci. USA 85, 4720–4724.