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Glycolytic enzyme Pgk1 is strongly expressed in the developing tooth germ of the mouse lower first molar

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Summary. This study examined detailed in situ expression patterns and possible functional roles of phosphoglycerate kinase 1 (Pgk1) gene in the developing tooth germ of the mouse lower first molar. The strong expression of Pgk1 mRNA was seen in the odontogenic epithelial cells and surrounding mesenchymal cells of the tooth germ from embryonic day 10.5 (E10.5) to E18.0. Western blotting analysis demonstrated that Pgk1 protein formed 84-kDa protein complex in these embryonic organs. The results of immunoprecipitation-western blotting also suggested this complex to be formed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Moreover, the immunofluorescence expression of those proteins was shown to overlap each other in the tooth germ at E15.0. A strong immunofluorescence expression of both Pgk1 and GAPDH also corresponded to the in situ expression of those mRNAs. These results suggested that Pgk1 plays some functional roles in the development of tooth germ and other embryonic organs by forming protein complex with GAPDH.

Key words Tooth germ, Pgk1, GAPDH, *In situ* hybridization, Protein complex

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Introduction

Vertebrate organs are typically composed of epithelial and mesenchymal tissues. The signaling between these two tissues governs many aspects of organogenesis, from the initiation of organ development to the terminal differentiation of organ specific cell types (Thesleff et al., 1995). Similar to many other embryonic organs, the development and differentiation of the mouse tooth germ depend on such inductive interactions (Thesleff et al., 1996).

In past studies, a large number of genes have been proven to be related to tooth morphogenesis (Maas and Bei, 1997; Thesleff and Åberg, 1999; Thesleff, 2003; Pispa and Thesleff, 2003; Kobayashi et al., 2006). However, the precise molecular mechanism of tooth germ development is still unclear. Some other odontgenesis-related genes may therefore exist that have not yet been identified. Based on this view point, we previously performed cDNA subtraction between the embryonic day 10.5 (E10.5) and E12.0 mouse mandibles (Yamaza et al., 2001a). We found several genes to be differentially expressed in the early developmental course of the mandible and the expression of some genes were apparently associated with the developing tooth germ (Yamaza et al., 2001a,b; Wada et al., 2002; Akhter et al., 2005). Phosphoglycerate kinase 1 (Pgk1) was one of the genes that were strongly expressed in the mandible at E10.5. Pgk is composed of two isozymes, somatic-type Pgk1 and testis-specific Pgk2 (Boer et al., 1987; Adra et al., 1987). Pgk1 is the sixth enzyme of the glycolytic pathway where it catalyzes the high energy phosphoryl transfer reaction from the acid anhydride bond of 1,3-bisphosphoglycerate to the ß-phosphate of

MgADP2-. The human enzyme has a molecular mass of 45-kDa, and it consists of a single polypeptide chain of 418 residues. Crystallographic studies of yeast (Banks et al., 1979; Watson et al., 1982), horse (Blake and Evans, 1974), and pig (Harlos et al., 1992; May et al., 1996; Szilagyi et al., 2001) enzymes showed the molecule to be composed of two domains of similar size, those corresponding to the N- and C-terminal halves of the chain separated by a hinge region. The human PGK1 spans 23-kb interrupted by 10 introns, and it is located at position Xq11-Xq13.

There have so far been no reports regarding the relation of Pgk1 to tooth germ development. We herein described the detailed expression patterns of mouse Pgk1 in the developing tooth germ by means of an in situ hybridization and immunofluorescence methods. In this study, we have found that Pgk1 formed a complex with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the tooth germ as well as in other embryonic organs. Pgk1 may exert some of its function for the development of tooth germ by forming protein complex with GAPDH.

Materials and methods

Animals

Embryos of BALB/c mice at 10.5, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0, and 18.0 days of age after gestation were used in this study. Adult BALB/c mice were obtained from Charles River Japan Incorporated (Yokohama, Japan). All animals were fed laboratory standard food pellets and water *ad libium* in an air-conditioned clean room. Female BALB/c mice were caged together with male mice. After 3 h, successful insemination was determined based on the presence of a postcopulatory plug in the vagina. The embryonic day was defined as E0 when such a plug was recognized. The experimental procedures were performed in accordance with the guidelines of the Animal Center of Kyushu University.

In situ hybridization

Three embryos of each gestational day were prepared to detect the gene expression by in situ hybridization. The embryos were removed from pregnant female mice of each gestational age under ether anesthesia, and fixed with 4% (w/v) paraformaldehyde in diethylpyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS, pH 7.4) for 12 h at 4°C. They were then embedded in OCT compound (Sakura, Tokyo, Japan). Frontal serial cryosections were cut at a thickness of 8 µm and mounted on silane-coated glass slides and air-dried. Murine Pgk1 cDNA was subcloned into the pGEM-3Z vector (Promega, Madison, WI) to synthesize both the antisense and sense probes (GenBank accession no. BC064681, nucleotides 23-1267). A template of an RNA probe for GAPDH was prepared using PCR amplification with primer pairs franking T7/SP6 RNA polymerase binding region (GenBank accession no. M15668, nucleotides 645-1096). These probes were labeled with digoxigenin (DIG)-UTP using the DIG RNA Labeling Kit (Roche, Mannheim, Germany). The labeling procedure was performed in the same way as our previous studies. The sequences of the primer pairs for RNA probes were as follows: Pgk1 forward primer 5'-ATG TCG CTT TCC AAC AAG-3', Pgk1 reverse primer 5'-CTA AAC ATT GCT GAG AGC ATC C-3'; GAPDH forward primer 5'-ACC ACA GTC CAT GCC ATC AC, GAPDH reverse primer 5'-TCC ACC ACC CTG TTG CTG TA. The detailed in situ hybridization procedure has been described in our previous studies (Yamaza et al., 2001b; Wada et al., 2002; Akhter et al., 2005).

To confirm the specificity of the in situ probe for Pgk1 and GAPDH, the membrane hybridization of in situ probes was performed (Akhter et al., 2005). The dose-dependent decrease in the binding activity of antisense probe to RNAs was demonstrated (data not shown). In using the antisense RNA probe for Pgk1 or GAPDH, the in situ expression of Pgk1 or GAPDH mRNA was clearly demonstrated in the tooth germ at E18.0 (Fig. 1A,B,C). No hybridization signals were detected in the control tissue specimens to which a sense probe was applied at E18.0 (Fig. 1 D, E).

Western Blotting

A Western blot analysis for the Pgk1 was performed from the homogenate of tooth germ and the other embryonic organs at E15.0. The small tissue specimens were lysed in RIPA buffer containing 2% (w/v) protease inhibitor cocktail, 0.1% (w/v) lactacystin, 25 mM ß-glycerophosphate and 1 mM sodium orthovanadate (Na_3VO_4). For an immunoblotting analysis, 30-µg of total protein per sample was fractionated using standard SDS-PAGE, and transferred to Immun-Blot® PVDF Membrane (Bio-Rad, CA). The membrane was blocked with 5% non-fat milk powder in Tris-buffered saline (TBS). The membrane was incubated with primary antibody in TBS with Tween 20 for 1 h at room temperature (RT). We used polyclonal goat anti-Pgk 1 (diluted 1:100, Y-12, Santa Cruz, CA) or polyclonal goat anti-GAPDH (diluted 1:100, V-18, Santa Cruz) as a primary antibody. The membrane was incubated with horseradish peroxidase conjugated rabbit anti-goat IgG (diluted 1:5000, Santa Cruz) for 1 h at RT. Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL) system (Amersham). Emitted light was detected using a cooled CCD-camera (LAS-1000, Fujifilm, Tokyo, Japan).

Immunoprecipitation

Immunoprecipitation was performed with the protein G immunoprecipitation Kit (SIGMA-ALDRICH, MO). The sample was extracted from the mouse tooth germs at

E15.0. For one reaction, the extract (400 µg) was incubated with either anti-Pgk1 (diluted 1:250, Y-12, Santa Cruz) or anti-GAPDH (diluted 1:250, V-18, Santa Cruz) antibody in immunoprecipitation buffer (SIGMA-ALDRICH). All procedures were performed according to the manufacturer's protocol. For an analysis of the eluted proteins binding to Pgk1 or GAPDH, the proteins were fractionated using standard SDS-PAGE and then were immunoblotted with anti-Pgk1 or anti-GAPDH antibody, respectively.

Immunofluorescence expression of Pgk1 and GAPDH

Cryosections for the immunohistochemical assays were prepared in the same way as mentioned for the ISH method. The sections were incubated with anti-Pgk1 (diluted 1:200, Y-12, Santa Cruz) or anti-GAPDH (diluted 1:200, V-18, Santa Cruz) overnight at RT after blocking 10% normal donkey or bovine serum for 1 h in PBS. The sections were washed 4 times with PBS for 10 min each, and incubated with Alexa Fluor® 568-labeled donkey anti-goat IgG (diluted 1:200, Molecular Probes, OR) or fluorescein isothiocyanate (FITC)-labeled bovine anti-goat IgG (diluted 1:200, Santa Cruz) for 1 h at RT, respectively. The sections were stained with 4',6-diamino-2-phenylindole (DAPI, 0.5 mg/ml) (Wako, Osaka, Japan) for 10 min at RT. A negative control was done by omitting the incubation with primary antibodies. No immunoreactive products were demonstrated in these sections (data not shown). These sections were examined under a fluorescence microscopy Olympus IX71 (OLYMPUS, Tokyo, Japan) and immunofluorescent images were acquired using an Olympus DP-70 camera (OLYMPUS).

Results

Pgk1 mRNA expression in the tooth development

Pgk1 is a glycolytic enzyme, and therefore the expression of this gene is considered to be commonly demonstrated in all living cells. Indeed, a prolonged hybridization time resulted in the ubiquitous expression of in situ signals of Pgk1 mRNA in all epithelial and mesenchymal cells of the mandible (data not shown). However, when an adequate reaction time was employed for in situ hybridization, a different expression intensity was found within the same tissue section, thus resulting in the site-specific strong expression of Pgk1 mRNA in the developing tooth germ (Fig. 1B, Fig. 2A, a-i, Fig. 3A-D). Therefore, terms of "strong" and "weak" were used for the relative evaluation of the signal intensity in the same tissue section. The morphological signs of tooth germ formation were seen in the epithelial layer at E10.5. At this initiation stage of E10.5, the strong expression of Pgk1 mRNA was observed in the oral epithelium and the underlying mesenchyme at the site of the presumptive molar tooth germ (Fig. 2A, a). At E12.0, Pgk1 in situ signals were detected in the thickening of the epithelial layer and also the underlying mesenchyme (Fig. 2A, b). At E13.0 and E14.0, the tooth bud exhibited a strong expression of Pgk1 in both the epithelial and adjacent mesenchymal cells (Fig. 2A, c-d). Especially strong signals of Pgk1 were also observed at the tip of the tooth bud at E13.0. At E15.0 and E16.0, Pgk1 was strongly expressed in the enamel organ (Fig. 2A, e-f). Pgk1 signals of the primary enamel knot (PEK) were weakly positive in comparison with those of the inner enamel epithelium. The underlying mesenchymal cells were also strongly positive for Pgk1 mRNA. However, outer enamel epithelium at E16.0 showed no signals of Pgk1. At E17.0, strong signals were restricted to the cells of inner enamel epithelium and the stratum intermedium (Fig. 2A, g). No signal of Pgk1 was found in the outer enamel epithelium or cervical loops. Pgk1 strong signals were more restricted to the inner enamel epithelium at E18.0 (Fig. 2A, g-h). Pgk1-positive signals were observed in the inner enamel epithelium of presumptive buccal cusp site, and cervical loop and the adjacent inner/outer enamel epithelia of the lingual side. Strong signals of Pgk1 were seen in the mesenchyme of both buccal and lingual cusp sites, and also in the stratum intermedium. Positive signals in the inner enamel epithelium of the presumptive buccal cusp site were stronger than those of the presumptive lingual cusp site at E18.0. Especially, strong signals of Pgk1 were noted within the cytoplasm of the inner enamel epithelium and stratum intermedium (Fig. 2A, i). Pgk1 positive signals in the cervical loop and the adjacent inner/outer enamel epithelia of the lingual side were stronger than those of the buccal side.

In situ expression of Pgk1 in embryonic organs

A Pgk1-positive signal was observed in the development of the cranio-facial organs (Fig. 3A-F). At E10.5 and E12.0, Pgk1-positive cells were seen in the forebrain, oral mucosa, mesenchyme of maxilla and mandible, and retina of eye (Fig. 3A,B). At E15.0 and E18.0, strong signals of Pgk1 were found in not only the forebrain, the mesenchymes of the masseter muscle and tongue and the retina, but also lingual and nasal epithelia (Fig. 3C,D). Strong signals of Pgk1 were also detected in Meckel's cartilage primordial cells (Fig. 3E), and epithelial cells of lower and upper first molar germs (Fig.3F). In the embryonic trunk at E15.0, strong signals of Pgk1 were observed in the cardiac mesenchyme (Fig. 3G), the bronchial epithelium (Fig. 3H), the collecting tubules of kidney (Fig. 3I), the intestinal epithelium (Fig. 3J) and the parenchymal cells of liver (Fig. 3K).

These results indicated that the site-specific strong expression of Pgk1 was observed in the developing organs and tissues.

Western blotting (WB) analysis of Pgk1 in the embryonic and adult organs

Since site-specific strong expression was detected in

the developing tooth germ and embryonic organs, WB analysis was performed to confirm the existence of Pgk1 protein in these organs. As shown in Fig. 4A, bands at 49-kDa indicating Pgk1 were demonstrated in both embryonic and adult organs. Interestingly, the bands at 84-kDa were observed in the embryonic organs. The

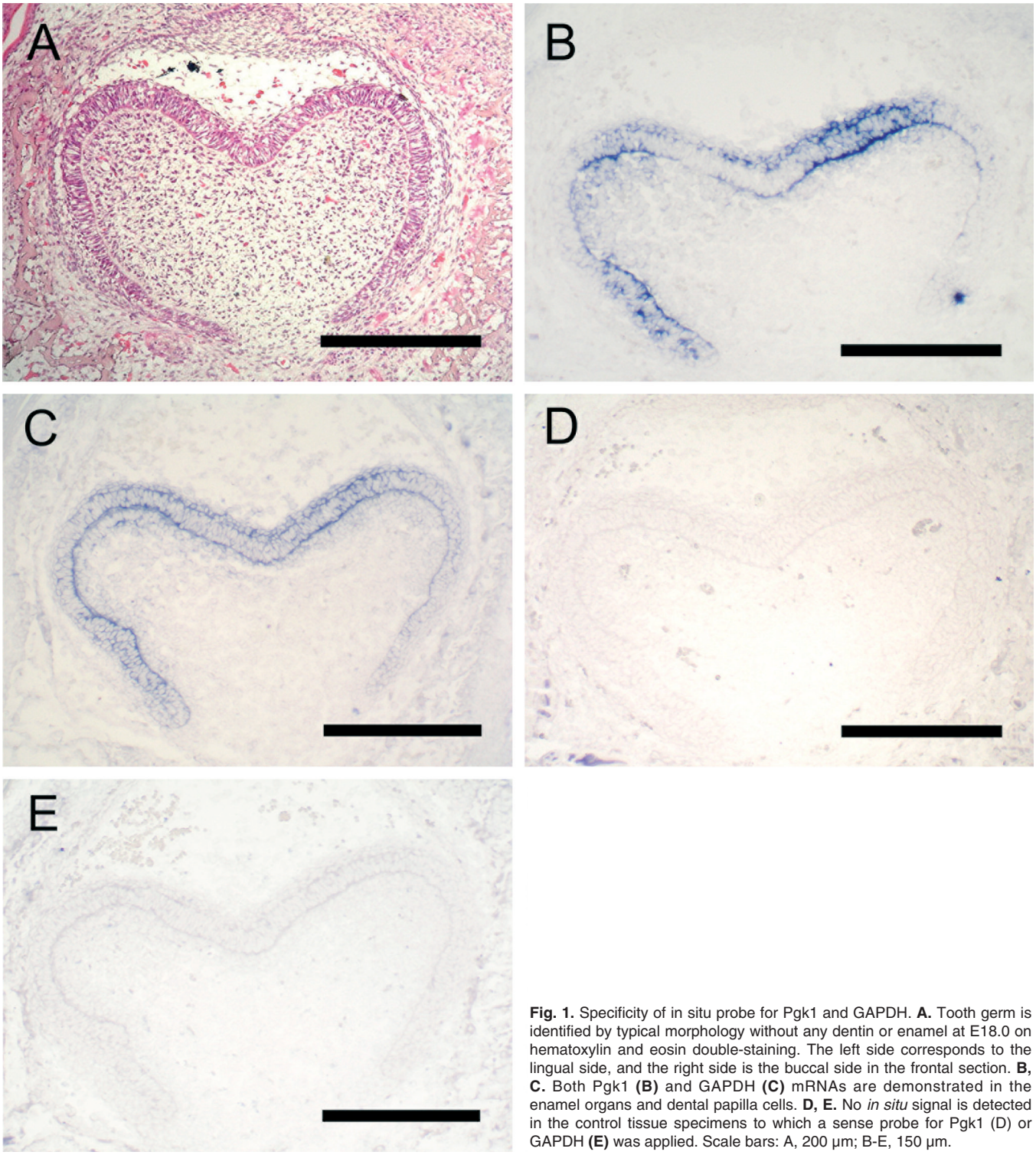

Fig. 1. Specificity of in situ probe for Pgk1 and GAPDH. **A.** Tooth germ is identified by typical morphology without any dentin or enamel at E18.0 on hematoxylin and eosin double-staining. The left side corresponds to the lingual side, and the right side is the buccal side in the frontal section. **B, C.** Both Pgk1 **(B)** and GAPDH **(C)** mRNAs are demonstrated in the enamel organs and dental papilla cells. **D, E.** No *in situ* signal is detected in the control tissue specimens to which a sense probe for Pgk1 (D) or GAPDH **(E)** was applied. Scale bars: A, 200 μm; B-E, 150 μm.

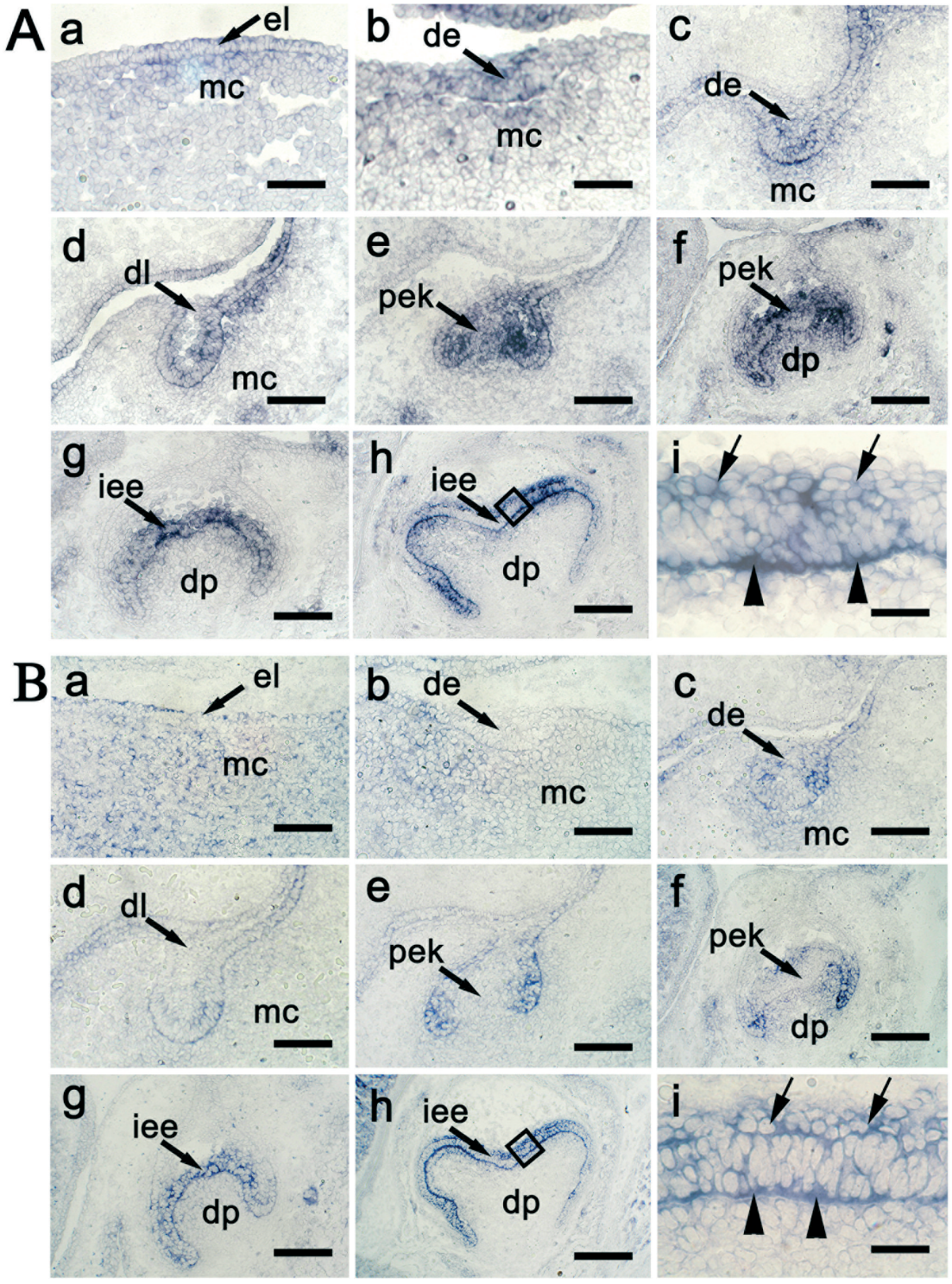

Fig. 2. *In situ* expression of Pgk1 **(A)** and GAPDH **(B)** in the developing tooth germ of the mouse lower first molar. **Aa.** Strong signals of Pgk1 at E10.5 are observed in the oral epithelial and mesenchymal cells. **Ab.** A thickening of the epithelial layer becomes evident at E12.0, and strong signals of Pgk1 are found in the thickened epithelial layer and the underlying mesenchymal cells. **Ac,d.** At E13.0 and E14.0, strong signals of Pgk1 are observed in both the epithelial cells of tooth bud and the underlying mesenchymal cells. Strong signals are noted at the tip of tooth bud at E13.0. **Ae,f.** At E15.0 and E16.0, Pgk1 mRNA is strongly expressed in the enamel organ and dental papilla. The cells of PEK are weakly positive for Pgk1 mRNA. However, outer enamel epithelium at E16.0 shows no signals of Pgk1. **Ag.** At E17.0, strong signals of Pgk1 are seen in the inner enamel epithelium and the stratum intermedium. The outer enamel epithelium and cervical loop show no Pgk1 positivity. **Ah.** At E18.0, Pgk1 strong signals are restricted to the inner enamel epithelium of presumptive buccal cusp site, cervical loop and the adjacent inner/outer enamel epithelia of the lingual side. Strong signals of Pgk1 are seen in the mesenchyme of both buccal and lingual cusps sites, and also in the stratum intermedium. The positivity in the inner enamel epithelium of presumptive buccal cusp site is stronger than that of the presumptive lingual cusp site. **Ai.** The boxed area of Fig. 2A, h is shown at higher magnification in Fig. 2A, i. Strong signals of Pgk1 are noted within the cytoplasm of inner enamel epithelium (arrowheads) and stratum intermedium (arrows). Pgk1 positivity in the cervical loop and the adjacent inner/outer enamel epithelia of the lingual side is stronger than that of the buccal side. **Ba.** At E10.5, signals of GAPDH are evenly observed in the cells of the mandibular arch containing epithelial and mesenchymal cells. **Bb.** At E12.0, strong signals of GAPDH are found in the mesenchymal cells facing a thickened epithelium, and no signal is observed in the thickened epithelial layer. **Bc.** At E13.0, strong signals of GAPDH are found in the tooth bud, and weak signals of GAPDH are also observed in the underlying mesenchymal cells. Strong signals of GAPDH are noted at the tip of tooth bud. **Bd.** At E14.0, the tooth bud exhibits strong signals of GAPDH. The underlying mesechymal cells show weaker signals. **Be,f.** At E15.0 and E16.0, GAPDH mRNA is strongly expressed in the enamel organ except for PEK. No in situ signal is found in the underlying mesenchymal cells. **Bg.** At E17.0, strong signals of GAPDH are observed in the inner enamel epithelium, stratum intermedium and cervical loop. The outer enamel epithelium shows no GAPDH positivity. **Bh.** At E18.0, strong expression of GAPDH is observed in the inner enamel epithelium except for buccal site. Strong signals of GAPDH are noted in the inner enamel epithelium of presumptive buccal cusp site and the adjacent site of lingual cervical loop. Strong signals of GAPDH are also seen in the stratum intermedium and the underlying mesenchyme. **Bi.** The enclosed area of Fig. 2B, h is shown at higher magnification in Fig. 2B, i. Strong signals of GAPDH are noted within the cytoplasm of inner enamel epithelium (arrowheads) and stratum intermedium (arrows). The left side in Fig. 2A, B corresponds to the lingual side, and the right side is the buccal side. el: epithelial layer, mc: mesenchymal cells, de: dental epithelium, dl: dental lamina, pek: primary enamel knot, dp: dental papilla, iee: internal enamel epithelium. Scale bars: Aa,b, Ba,b, 40 μm; Ac-e, Bc-e, 80 μm; Af-h, Bf-h, 150 μm; Ai, Bi, 15 μm.

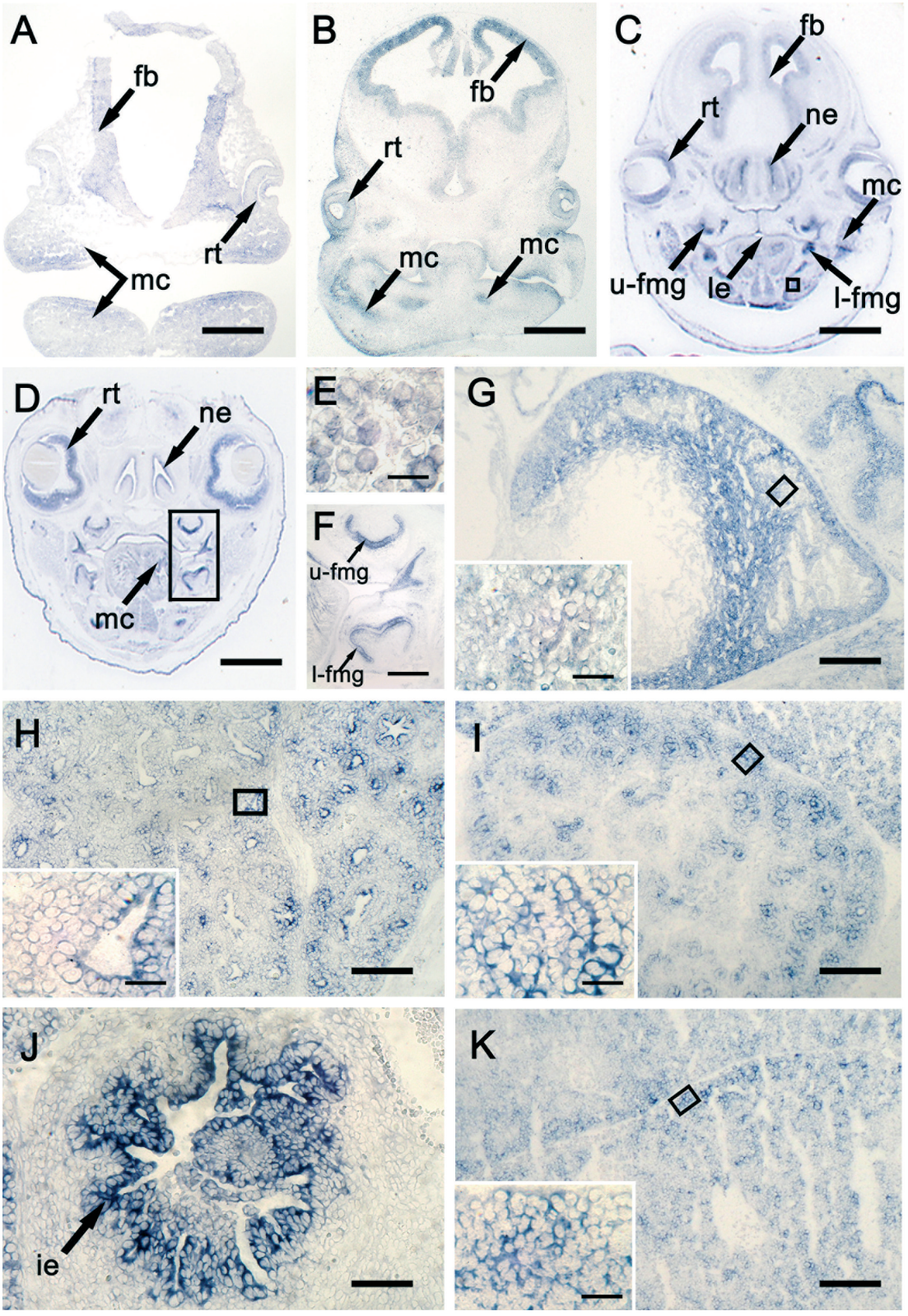

Fig. 3. Site-specific *in situ* expression of Pgk1 in the embryonic cranio-facial **(A-F)** and truncal **(G-K)** organs.
A. At E10.5, strong signals of Pgk1 are observed in the forebrain (fb), oral mucosa, the mesenchyme (mc) of maxilla and mandible, and the retina (rt) of eye. **B.** At E12.0, strong signals of Pgk1 are found in the fb, the mc of mandible, and rt. **C.** At E15.0, strong signals of Pgk1 are seen in the fb, mc of masseter and tongue muscles, lingual epithelium (le), rt and the nasal epithelium (ne). Strong signals of Pgk1 are also detected in the epithelial cells of lower (l-fmg) and upper (u-fmg) first molar germs. The boxed area indicates Meckel's cartilage primordium that is shown at a higher magnification in figure E. **D.** At E18.0, distribution of strong signals is similar to that of the signals at E15.0. The enclosed area shows the enamel epithelia of l-/u-fmg that are at higher magnification in figure F. **E.** Higher magnification of the boxed area in figure C. **F.** Higher magnification of the enclosed area in figure D. **G, H, I, J and K.** In the embryonic trunk at E15.0, strong signals of Pgk1 are observed in the cardiac mesenchyme (**insert of G**), the bronchial epithelium (**insert of H**), the collecting tubules of kidney (**insert of I**), the intestinal epithelium (ie) (**arrow of J**) and the parenchymal cells of liver (**insert of K**). The **insert of G, H, I** or **K** shows a higher magnification of the enclosed area in each figure. fb: forebrain, rt: retina, mc: mesenchyme, ne: nasal epithelium, le: lingual epithelium, l-fmg: lower first molar germ, u-fmg: upper first molar germ, ie: intestinal epithelium. Scale bars: A, 250 µm; B, 700 µm; C, 800 µm; D, 1200 µm; E, inserts of G, H, I, K, 15 µm; F, 120 µm; G, 180 µm; H, I, K, 60 µm; J, 45 µm.

bands of 84-kDa were also observed in the adult brain, tongue, heart, lung and liver. However, the signal intensity of these bands was markedly weaker than that in embryonic organs. The results of WB analysis suggested the high molecular protein to consist of both Pgk1 and a specifically binding partner protein.

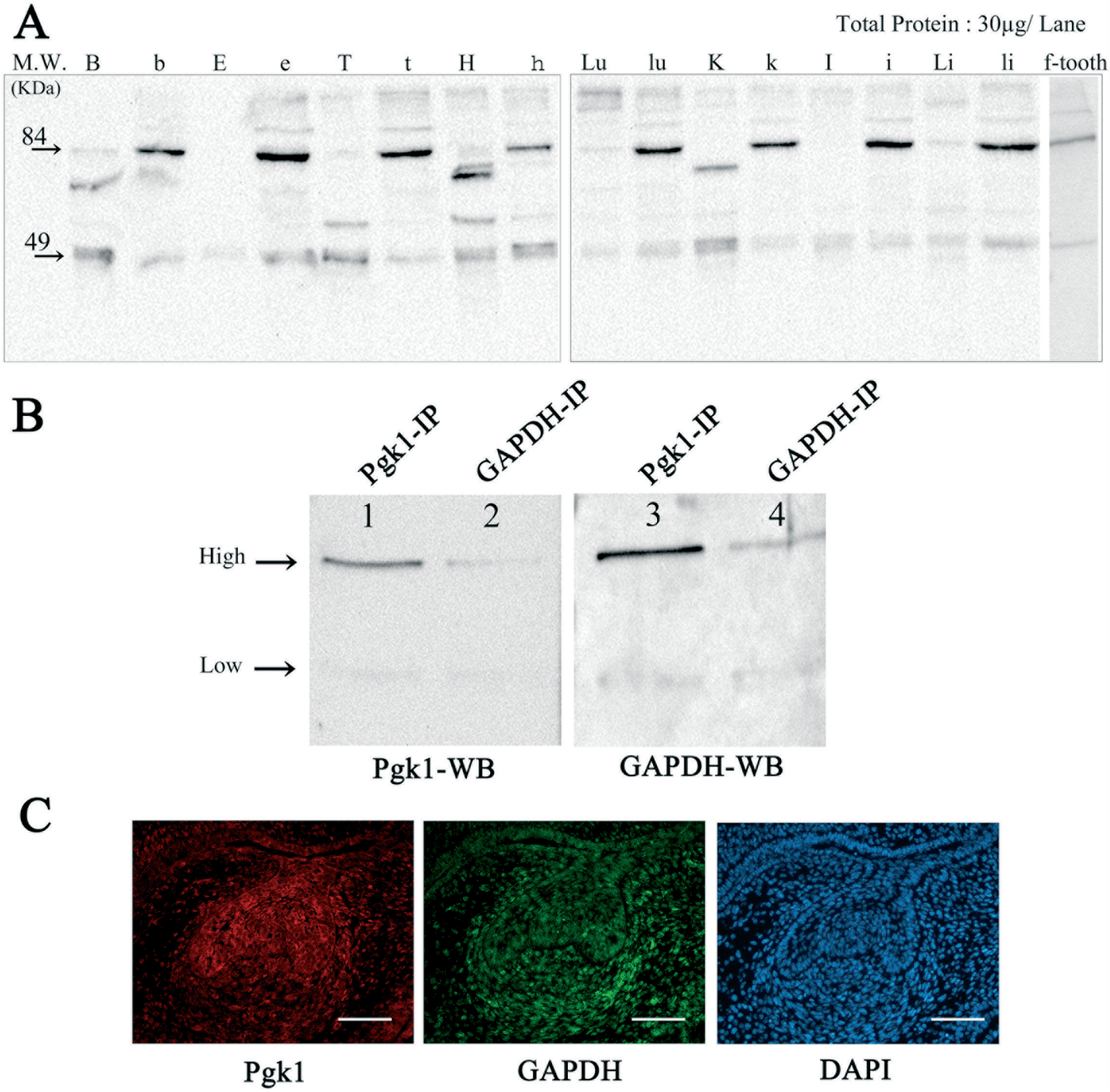

Fig. 4. WB analysis and immunofluorescence expression of Pgk1 and GAPDH. **A.** High molecular bands are observed at the lanes of tooth germ and the other embryonic organs. MW: molecular weight, B: brain, E: eye, T: tongue, H: heart, Lu: lung, K: kidney, I: intestine, Li: liver, f-tooth: fetal tooth germ. Large and small characters show adult and embryonic organs, respectively. **B.** Immunodetection for Pgk1 or GAPDH was performed by IP-WB analysis. Lanes 1, 3: IP using anti-Pgk1. Lanes 2, 4: IP using anti-GAPDH. Low molecular bands of lanes 2 and 3 are considered to be dissociation of Pgk1/GAPDH complex proteins. **C.** The immunofluorescence expression of Pgk1 protein overlaps with that of GAPDH in the tooth germ at E15.0. Strong expressions of Pgk1 and GAPDH are detected in the epithelium of the tooth germ. The nuclei were counterstained by DAPI. Scale bars: 150 μm.

Immunoprecipitation (IP)-WB analysis and immunofluorescence expression of Pgk1 and GAPDH

Previous studies reported that Pgk1 formed a protein complex with GAPDH under restricted conditions (Ashmarina et al., 1994; Fokina et al., 1997; Ikemoto et al., 2003), and therefore we considered that the high molecular protein observed in the embryonic organs might thus be Pgk1/GAPDH complex. Based on this viewpoint, we further performed IP-WB analysis. Fig. 4B showed that Pgk1 formed protein complex with GAPDH in the tooth germ at E15.0. A strong immunofluorescence expression of Pgk1 was found in the epithelial tissue of the tooth germ at E15.0 (Fig. 4C). Strong immunofluorescence expression of GAPDH was also demonstrated in the epithelial tissue of the tooth germ (Fig. 4C), and was shown to overlap the expression of Pgk1 in the tooth germ. Moreover, a strong immunofluorescence expression of both Pgk1 and GAPDH corresponded to the in situ expression of those mRNAs (Fig. 2A, e & B, e).

GAPDH mRNA expression in the tooth development

Since GAPDH is also a glycolytic enzyme, in situ signals of GAPDH mRNA could be exhibited in the ubiquitous epithelial and mesenchymal cells of the mandible. However, in situ hybridization for GAPDH mRNA in an adequate reaction time also resulted in the site-specific strong expression of GAPDH mRNA at E18.0 (Fig. 1C, 2B, a-i). At E10.5, signals of GAPDH were evenly observed in the cells of the mandibular arch containing epithelial and mesenchymal cells, thus resulting in no site-specific expression of GAPDH mRNA (Fig. 2B, a). At E12.0, a strong expression of GAPDH was found in the presumptive dental mesenchyme, however no signal was observed in the thickened dental epithelium (Fig. 2B, b). At E13.0 and E14.0, strong signals of GAPDH were found in the tooth bud, and the signals were especially prominent at the tip of the tooth bud (Fig. 2B, c-d). Relatively weak signals of GAPDH were also observed in the mesenchymal cells facing the tip of the tooth bud. At E15.0, GAPDH mRNA was strongly expressed in the enamel organ except for PEK (Fig. 2B, e). No in situ signal was found in the underlying mesenchymal cells. At E16.0, the signals of GAPDH were noted in the inner enamel epithelium and stratum intermedium. GAPDH-positive cells were consistently seen in the cervical loops and the adjacent inner/outer enamel epithelia (Fig. 2B, f). No in situ signal was seen in PEK and outer enamel epithelium, except for the adjacent outer enamel epithelium of the cervical loops. Relatively weak signals of GAPDH were also observed in the mesenchymal cells of future dental papilla. At E17.0, strong signals of GAPDH mRNA were observed in the inner epithelium and stratum intermediate and cervical loops (Fig. 2B, g). Relatively weak signals of GAPDH were found in the adjacent outer enamel epithelium of both buccal and lingual cervical loops, and the mesenchymal cells facing the inner enamel epithelium. At E18.0, the strong expression of GAPDH mRNA was restricted to the inner enamel epithelium and cervical loops and the adjacent outer enamel epithelium (Fig. 2B, h). The GAPDH signal in the inner enamel epithelium of presumptive buccal cusp was stronger than that of the lingual side. Especially, strong signals of GAPDH were noted within the cytoplasm of the inner enamel epithelium and stratum intermedium (Fig. 2B, i). The GAPDH-positive signals in the cervical loop and the adjacent inner enamel epithelia of the lingual side were stronger than those of the buccal side. The outer enamel epithelium, except for the adjacent site of the lingual cervical loop, showed no GAPDH positivity. Strong signals of GAPDH were also observed in the mesenchymal cells facing the inner enamel epithelium. The expression patterns of GAPDH closely corresponded to those of Pgk1 during the late stages (E15.0 – E18.0) (Fig. 2A, e-i, B, e-i).

Discussion

In this study, we demonstrated detailed expression patterns of both mRNA and protein of Pgk1 in the developing tooth germ by means of in situ hybridization and immunofluorescence methods, and showed that Pgk1 might play a functional role in the initiation, development and differentiation of the tooth germ by forming a protein complex with GAPDH.

Pgk1 is the sixth enzyme of the glycolytic pathway, and it is commonly recognized to be related to ATP synthesis. Therefore, all living cells in the body contain this enzyme, and the mRNA of this gene is considered to be ubiquitously detected in all living cells. Indeed, a prolonged hybridization time caused the in situ signals of Pgk1 mRNA to be demonstrated in all epithelial and mesenchymal cells of the mandible at E10.5. However, the in situ expression intensity of Pgk1 mRNA apparently differed from site to site in the tissue section, and strong expression was detected in the odontogenic epithelial cells and surrounding mesenchyme of the developing tooth germ. Furthermore, a strong immunofluorescence expression of Pgk1 was observed in the tooth germ at E15.0. Interestingly, a different expression intensity was also observed in the other developing organs. Fundele et al. (1987) reported a pronounced decrease in the maternal Pgk1 activity to be observed in the inner cell mass as compared with the trophectoderm of mouse blastocytes in vitro. Therefore, it seems likely that the strong expression of Pgk1 mRNA might be related to the specific function of this enzyme during the morphogenesis of embryonic organs.

WB analysis of the Pgk1 isolated from embryonic organs at E15.0 showed two bands, namely 49-kDa low molecular and 84-kDa high molecular proteins. Pgk1 is a low molecular protein. An IP-WB analysis demonstrated that a higher molecular protein was considered to be a complex of Pgk1 and GAPDH. The amount of

Pgk1/GAPDH complex was markedly higher in embryonic organs than in adult organs by WB analysis. The immunofluorescence expression of both Pgk1 and GAPDH overlapped each other in the tooth germ at E15.0, thus suggesting co-localization of these proteins in the odontogenic epithelial cells. Pgk1 and GAPDH have already been reported to form a protein complex under restricted conditions (Ashmarina et al., 1994; Fokina et al., 1997; Ikemoto et al., 2003). Fokina et al. (1997) reported that the formation of Pgk1/GAPDH complex depended on the pH in the human erythrocyte, and this protein complex thus might play some roles in determining the fate of the 1,3-bisphosphoglycerate produced in the glycolytic pathway. Pgk1/GAPDH complex has also been found in the synaptic vesicles, and it is also considered to play a role in effective ATP synthesis, thereby ensuring maximal glutamate accumulation into the synaptic vesicles. In the growing process of tooth germ, glycogen has been observed to accumulate in the cytosol of ameloblasts. When dentin formation starts, the nutrition supplied to the dental organ remarkably decreases. Under such conditions, ameloblasts could thus maintain their activity to consume the accumulated glycogen (Ten Cate, 1998). Together with these reports, it seems that the strong expression of Pgk1 mRNA in the odontogenic epithelial cells and the surrounding mesenchyme thus plays a role in effective ATP synthesis regarding the growth and differentiation of these cells by forming Pgk1/GAPDH complex.

However, Ohshima et al. (1999) reported a distribution of glycogen deposits in the murine tooth germ by histochemical periodic acid-Schiff (PAS). Although glycogen is well known as a major energy source of ubiquitously metabolic processes, Ohshima et al. (1999) observed a complete absence of glycogen deposits during the active phase of tooth morophogenesis. In comparison with the strong signal pattern for Pgk1 from initiation to late bell stages, the lack of glycogen deposits was observed in the epithelium and mesenchyme facing each other, in which the strong signals of Pgk1 were noted through these stages. The strong signal pattern of Pgk1 in stratum intermedium was consistent with the distribution of glycogen deposits in the same intermediate cells during the bell stages (Ohshima et al., 1999). At the cap stage, glycogen deposits were completely absent in the inner enamel epithelium (Ohshima et al., 1999), although in this study Pgk1 protein was restricted to the same inner enamel epithelium. Therefore, the localizations of Pgk1 mRNA and glycogen were not necessarily coincident with each other. We have no idea regarding the difference of localizations of Pgk1 mRNA and glycogen at present. However, these paradoxical results may indicate that Pgk1 has also been shown to play a role not only in the glycolytic process, but also in another biological phenomena. Pgk1 has been reported to influence DNA replication and repair in the mammalian cell (Vishwanatha et al., 1992; Popanda et al., 1998) and to stimulate viral mRNA synthesis in the cytosol (Ogino et al., 1999). Moreover, Pgk1 participates in the angiogenetic process as a disulphide reductase (Lay et al., 2000, 2002). As a result, some still unknown function(s) of Pgk1 may therefore exist. Some unknown functions might occur by formation of protein complex with GAPDH because protein complex was observed in the developing tooth germ as well as other embryonic organs.

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