MINI-REVIEW

Salivary gland development : its mediation by a subtilisin-like proprotein convertase, PACE4

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Abstract : The submandibular gland (SMG) develops under the epithelial-mesenchymal interaction. Its process is regulated by various growth/differentiation factors, which are synthesized as inactive precursors and activated *via* the limited proteolysis at their multi basic amino acid site(s) such as Arg-X-Lys/Arg-Arg. Although many of these processing steps are elucidated to be catalyzed by subtilisin-like proprotein convertases (SPCs), little is known about the role of SPCs in the SMG development. Here, we focused upon the physiological role of PACE4 (SPC4), a member of SPC family, in the SMG development. In the organ culture system of rat embryonic SMG (E15), Dec-RVKR-CMK, a potent inhibitor for SPCs, inhibited the salivary branching and the expression of an exocrine gland type water channel, AQP5. However, other peptidyl-CMKs and inhibitors for trypsin-like serine proteases including leupeptin did not affect the salivary branching and AQP5 expression. Dec-RVKR-CMK also suppressed the expression of PACE4, but not furin, another member of the family. The specific antibody for the catalytic domain of PACE4 suppressed the salivary branching and AQP5 expression similarly. These inhibitory effects of Dec-RVKR-CMK were partially rescued by the addition of recombinant BMP2 whose precursor is a candidate for the physiological substrates of PACE4. Further, the transcriptional silencing of PACE4 by its specific siRNAs caused the suppression of both the salivary branching and AQP5 expression in the present organ culture system. These observations strongly support the idea that PACE4 mediates the SMG development. J. Med. Invest. 56 Suppl. : 241-246, December, 2009

Keywords **:** *subtilisin-like proprotein convertase, PACE4, branching morphogenesis, water channel, AQP5, SMG, development*

INTRODUCTION

Many glandular tissues including salivary gland, lung, pancreas, and prostate are formed *via* the branching morphogenesis (BM), which is regulated under the epithelial-mesenchymal interaction (1, 2). Many investigators have analyzed the molecular mechanism of such salivary BM, and reported the involvement of various growth/differentiation factors and extracellular matrix (ECM) in this developmental process (3). The roles of EGF/FGF signaling have been investigated well in the organ culture system using mouse submandibular gland (SMG) rudiments, however, other growth/differentiation factors including activin, BMP, IGF, PDGF, and TGFβ have also been shown to play important roles in the

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salivary gland development and/or salivary acinar differentiation (Table 1). TGFβ-related growth/differentiation factors are synthesized as inactive precursors and are converted to their active forms by the limited proteolysis at multiple basic amino acid sites such as Arg-X-X-Arg and/or Arg-X-Lys/Arg-Arg (4). Additionally, some ECM proteins including collagen families also need the proteolytic removal of their propeptides at least at the similar site for compatible fibrillogenesis (Table 1). In general, such processing is catalyzed by subtilisin-like proprotein convertase (SPC) family proteases (5-7), however, little is known about the role of SPCs in the salivary gland development. In this review, we will describe the physiological role of PACE4 (SPC4), which is one of the member of SPC family, in the salivary gland development.

Table 1 Activation site of pro-growth/differentiation factors and other pro-proteins

pro-growth/ differentiation factor	P6	P4	P2	P1 ↓	P1'	P2'
Mouse βNGF	T H R	S K R			S	S
human TGF β1	S S R	H R R			A	L
human TGF β2	N R R	K R R			A	L
human TGF β3	G Q R	K R R			A	L
mouse BMP2	H K R	E K R			Q	A
rat BMP4	R R R	A K R			S	P
human Activin A	H R R	R R R			Q	A
other pro-protein						
human IGF receptor	P E R	K R R			D	V
human Integrin α3-chain	P Q R	R R R			Q	L
mouse E-cadherin	L R R	Q K R			D	W
human fibrillin	R G R	K R R			S	T
human α1(V) Collagen	A S R	T R R			N	I
mouse/rat α1(XI) Collagen	P K K	T R R			H	T
human BMP1	R S R	S R R			A	A

Arrow between the position 1 (P1) and position 1' (P1') indicates the cleavage site, and the important basic amino acids at P1, P2, P4, and P6 on the processing of each precursor are underlined.

SALIVARY GLAND DEVELOPMENT

In mammalians, there are three major salivary glands ; parotid, submandibular, and sublingual glands. The parotid glands develop from near the corners of the stomodeum. The SMG develops from the side of the root of the tongue, and the sublingual gland develops from the lateral part of the submandibular primordia. All salivary glands originate from the ectodermal epithelium as a result of interacting with the neural crest-derived mesenchyme (8, 9). The thickening of the oral epithelium, which is an initial sign of salivary gland formation, is observed around E12 in case of the formation of the rat SMG. Then, the oral epithelium downgrows into the underlying mesenchyme, and continually develops by the repeated dichotomous branching of the distal ends of the epithelial buds, generally called "branching morphogenesis (BM)". During this process, the elongated branches differentiate into the ducts, while the terminal epithelial buds differentiate into the acini. The epithelial-mesenchymal interaction and BM, which is the important and fundamental developmental process seen in many organogenesis of glandular tissues, is a cascade consisting of various signaling molecules including growth/differentiation factors, receptors, cell adhesion molecules, and ECM proteins (3, 10).

SUBTILISIN-LIKE PROPROTEIN CONVERTASE (SPC) FAMILY PROTEASE

Mammalian subtilisin-like proprotein convertases (SPCs), which have the catalytic domain similar to bacterial subtilisin (subtilisin-like catalytic domain ; SCD), have been identified and characterized as processing proteases catalyzing the limited proteolysis at the multiple basic amino acid sites (5-7). This processing is significant to generate and/or activate various signaling molecules including peptide hormones, growth/differentiation factors, cell adhesion molecules, receptors, and ECM proteins. SPCs are classified into 3 subgroups. The first group is secretory granule convertases consisting of PC2/SPC2, PC1/PC3/SPC3, and PC4/SPC5. They are localized in secretory granules and involved in the processing of peptide hormones. The second group is membrane-bound convertases consisting of furin/PACE/SPC1 and LPC/PC7/PC8/SPC7. These members are localized at trans-Golgi network and cell membrane *via* their C-terminal transmembrane domain, and activate various proproteins. The third group consists of PACE4/SPC4 and PC5/PC6/SPC6. These convertases have been shown to bind heparin, an experimental indicator of the physiological interaction with heparan sulfate proteoglycans in the ECM (11). Therefore these members are classified as ECM-bound convertases. Previous studies have been shown that PACE4, furin, and PC6 are candidate SPCs responsible for the

activation of growth/differentiation factors including TGFβ-related molecules (12-14), which also play important roles in the BM of various glandular tissues (1, 2).

EXPRESSION AND LOCALIZATION OF SPCs IN THE SALIVARY GLAND DEVELOPMENT

Farhadi, *et al.* first reported the expression and localization of furin in the mouse SMG during postnatal development ; *i.e.*, furin was widely expressed in the SMG, but especially in the duct cells, and colocalized with NGF in the granular convoluted tubule cells (15). They also revealed that PC2 mRNA was expressed in the parasympathetic neurons of intralobular ganglia, but not in any other cell type, within the SMG (15).

Previously, we showed the expression and localization of PACE4 in the rat SMG through development (16). PACE4 was intensely expressed in the submandibular epithelium-derived cells including proacinar, terminal tubular, and presumptive ductal cells in the prenatal SMG. By means of RT-PCR and Northern blot analyses, its expression was intense during the stages in which the prominent BM progresses (Fig. 1A). After birth, PACE4 was expressed still intensely in the terminal portion of the SMG containing proacinar and terminal tubular cells, whereas its expression in the ductal cells was obviously decreased. Additionally, acinar cells that do not express PACE4 appeared and their numbers increased following postnatal development, suggesting the apparent association of PACE4 with the proliferation, differentiation, and establishment of functional salivary acinar cells.

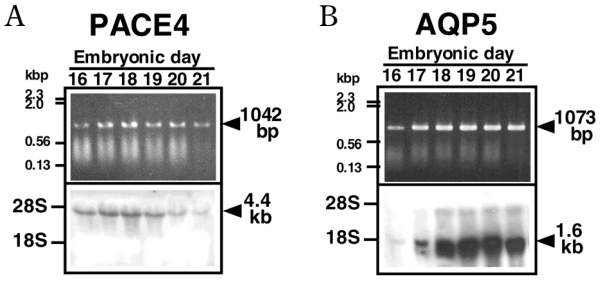

Fig. 1 Expression of PACE4 (A) and AQP5 (B) mRNAs in the prenatal rat SMG.
Expression of PACE4 and AQP5 mRNAs was analyzed by RT-PCR (upper) and Northern blotting (lower). One-microgram and ten-microgram samples of total RNA from rat embryonic SMG were used for RT-PCR and Northern blotting, respectively. Each transcript was detected by using each specific DIG-labeled antisense riboprobe. The arrowheads with their correct size indicate the position of each RT-PCR product or transcript. The size markers from a λDNA-*Hind* III digest and the position of ribosomal RNAs are shown on the left. Modified from Akamatsu, *et al.* (20, 21).

EXPRESSION AND LOCALIZATION OF AN EXOCRINE TYPE WATER CHANNEL, AQUAPORIN 5 (AQP5) IN THE SALIVARY GLAND DEVELOPMENT

One of the important physiological functions of salivary glands is saliva secretion. As the key molecules, which are responsible for water movement across the cell membrane in secretory and absorptive cells, aquaporin family transmembrane proteins have been identified. In the salivary gland, AQP5 was first identified to be localized at the apical membranes of the salivary acinar cells (17). Involvement of this water channel in saliva secretion was revealed by the fact that AQP5 KO mice and AQP5 mutant rats show decreased and viscous hypertonic saliva secretion (18, 19). Previously, we reported the expression and localization of AQPs (AQP1-AQP5) in the course of development of rat SMG (20). Interestingly, AQP5 expression is dramatically increased in the prenatal SMG in good accordance with the acinar differentiation (Fig. 1B), although its molecular mechanism is still unclear.

ROLE OF PACE4 IN THE SALIVARY GLAND DEVELOPMENT

To elucidate the physiological role of PACE4 in the salivary gland development, we investigated the effects by inhibition and transcriptional silencing of PACE4 in the organ culture system of rat embryonic SMG rudiments (21). We first analyzed the effects of protease inhibitors in the organ culture system. Based on the above mentioned amino acid sequence at the cleavage site of various proproteins including growth/differentiation factors, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-CMK) is generally used as a potent and useful inhibitor for SPC family. As expected, the SMG rudiments cultured in the presence of Dec-RVKR-CMK clearly showed the inhibition of the BM of SMG in the organ culture system as compared with the control culture (Fig. 2A, B), and the finding is well coincided with the report by Uchida, *et al.* showing the

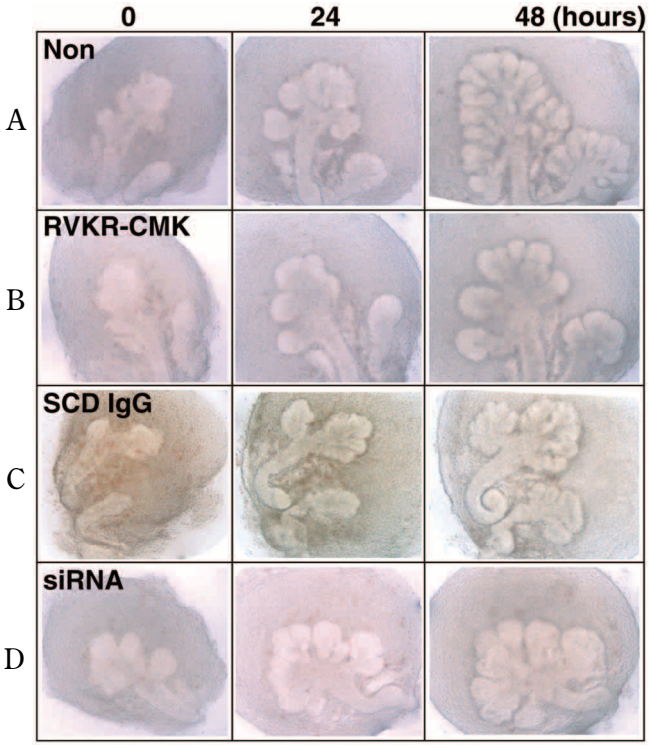

Fig. 2 Effects of Dec-RVKR-CMK, anti-PACE4 (SCD) IgG, and siRNA for PACE4 on the branching morphogenesis of cultured rat SMG rudiments. The SMG rudiments extracted from E15 rat embryos were placed on polycarbonate membrane floated on BGJb medium containing 10% FCS. Then they were cultured in the presence of nothing (A ; Non), Dec-RVKR-CMK (B ; RVKR-CMK, 10 μM), specific antibody for the subtilisin-like catalytic domain (SCD) of PACE4 (C ; SCD IgG, 0.36 mg/ml), and specific siRNA for PACE4 (D ; siRNA, 1 μM), respectively. Modified from Akamatsu, *et al.* (21).

inhibition of prostatic branching by Dec-RVKR-CMK (22). In these SMG rudiments, it was revealed that the expression level of AQP5 was not increased as compared with the control culture without the inhibitor, suggesting the retardation of the differentiation of the salivary cells. Interestingly, the expression level of PACE4, but not furin, was also suppressed similarly, suggesting the participation of PACE4 in the differentiation of the salivary cells. Based on the amino acid sequence, Dec-RVKR-CMK can probably inhibit various trypsin-like serine proteases, however, leupeptin and soybean trypsin inhibitor, which can inhibit general trypsin-like serine proteases, showed no inhibitory effects in the present organ culture system. Additionally, other peptidyl-CMK affected neither BM of SMG rudiments nor the expression levels of AQP5 and PACE4 in the same culture system. These results suggest the involvement of SPCs, especially PACE4, in the salivary gland development. To verify the possible involvement of PACE4 in regulating BM of and AQP5 expression in the SMG rudiments, we initially analyzed the effects of specific antibody for the catalytic domain of PACE4 in the present organ culture system. As a result, this antibody showed similar inhibitory effects caused by Dec-RVKR-CMK, suggesting the validity of PACE4 participation in the gland development (Fig. 2C). Further, the rescue experiment by using recombinant growth/differentiation factors revealed that BMP2 is a candidate important regulator to restore the Dec-RVKR-CMK-caused inhibition of BM and decrease in AQP5 expression, especially for the recovery of AQP5 expression. The significance of this BMP2 signaling in regulating both the BM and AQP5 expression was furthermore confirmed by the experiment in which the neutralizing monoclonal antibody for BMP2 suppressed the BM of and AQP5 expression in the SMG rudiments in the present organ culture system. These observations strongly suggest the validity of PACE4-mediated development of the salivary gland. We finally conducted RNAi experiment to corroborate aforementioned observations. The transcriptional silencing of PACE4 in the organ culture system was efficiently caused by plural siRNAs specific for various region of PACE4, and reliably reduced both the BM of and AQP5 expression in the SMG rudiments in the present organ culture system (Fig. 2D). In contrast, siRNAs for furin and PC6 did not show such inhibitory effects at present.

Among SPCs, some members frequently play redundant roles (12, 14, 23), however, the inhibition and transcriptional silencing of PACE4 undoubtedly causes the reduction of both the BM of and AQP5 expression in the SMG rudiments. This fact indicates that the physiological role of PACE4 is very critical for SMG development and neither furin nor PC6 could restore the lost function of PACE4 in the SMG development.

In conclusion, the SMG development is regulated by a subtilisin-like proprotein convertase PACE4 *via* the activation of proproteins involved in salivary branching and/or salivary acinar differentiation. The precursor of BMP2 is one of the candidate physiological substrates of PACE4, and BMP2 signaling may upregulate the expression of an exocrine type water channel AQP5 in the SMG development.

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