Developmental Biology 325 (2009) 434–443

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

Inhibition and transcriptional silencing of a subtilisin-like proprotein convertase, PACE4/SPC4, reduces the branching morphogenesis of and AQP5 expression in rat embryonic submandibular gland

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ARTICLE INFO

Article history:
Received for publication 18 December 2007
Revised 3 October 2008
Accepted 3 October 2008
Available online 25 October 2008

Keywords:
Subtilisin-like proprotein convertase
PACE4
Branching morphogenesis
Water channel
AQP5
Submandibular gland
siRNA

ABSTRACT

The submandibular gland (SMG) develops through the epithelial–mesenchymal interaction mediated by many growth/differentiation factors including activin and BMPs, which are synthesized as inactive precursors and activated by subtilisin-like proprotein convertases (SPC) following cleavage at their R-X-K/R-R site. Here, we found that Dec-RVKR-CMK, a potent inhibitor of SPC, inhibited the branching morphogenesis of the rat embryonic SMG, and caused low expression of a water channel AQP5, in an organ culture system. Dec-RVKR-CMK also decreased the expression of PACE4, a SPC member, but not furin, another SPC member, suggesting the involvement of PACE4 in the SMG development. Heparin, which is known to translocate PACE4 in the extracellular matrix into the medium, and an antibody specific for the catalytic domain of PACE4, both reduced the branching morphogenesis and AQP5 expression in the SMG. The inhibitory effects of Dec-RVKR-CMK were partially rescued by the addition of recombinant BMP2, whose precursor is one of the candidate substrates for PACE4 in vivo. Further, the suppression of PACE4 expression by siRNAs resulted in decreased expression of AQP5 and inhibition of the branching morphogenesis in the present organ culture system. These observations suggest that PACE4 regulates the SMG development via the activation of some growth/differentiation factors.

Introduction

Branching morphogenesis is one of the fundamental developmental processes seen in many glandular tissues during organogenesis, and it occurs as a result of epithelial–mesenchymal interactions (Davies, 2002; Hu and Rosenblum, 2003). Thus, the mature salivary gland is completed according to the following developmental scheme: the oral epithelium down-grows into the underlying mesenchyme to form the salivary bud, which then continually develops by epithelial cord growth and repeated dichotomous branching of the distal ends of the epithelial buds (Denny et al., 1997; Klein, 2002; Melnick and Jaskoll, 2000; Patel et al., 2006; Tucker, 2007). During this process, the elongated branches differentiate into the ducts, while the terminal epithelial buds become the acini. Many investigators have analyzed the molecular mechanism of such salivary branching morphogenesis, and reported the involvement of various growth/differentiation factors in this developmental process (Patel et al., 2006). Especially, the roles of EGF and FGF family molecules have been investigated well in the organ culture system using mouse submandibular gland (SMG) rudiments (De Moerlooze et al., 2000; Hoffman et al., 2002; Jaskoll and Melnick, 1999; Jaskoll et al., 2002; Jaskoll et al., 2004; Jaskoll et al., 2005; Kashimata and Gresik, 1997; Kashimata et al., 2000; Koyama et al., 2003; Melnick et al., 2001; Miyazaki et al., 2004; Morita and Nogawa, 1999; Noguchi et al., 2006; Steinberg et al., 2005; Umeda et al., 2001). Besides EGF/FGF families, other growth/differentiation factors including activin, BMP, IGF, PDGF, and TGFβ have also been shown to play important roles in the development of the SMG (Ball and Risbridger, 2001; Orr-Urtreger and Lonai, 1992; Roberts and Barth, 1994; Bläuer et al., 1996; Jaskoll and Melnick, 1999; Jaskoll et al., 2002; Werner and Katz, 2004). Furue et al. (2001) reported that activin A may regulate the salivary acinar differentiation via induction of Sel-1l, which is a negative regulator of the Notch signaling. Using BMP7-null mice, Jaskoll et al. (2002) showed the existence of disorganized mesenchyme and decreased epithelial branches with fewer lumina in the E17 SMG of this mutant mice compared with those in its wild-type counterpart. This finding suggests the importance of the BMP7 signaling during embryonic development of the SMG. These TGFβ-related growth/differentiation factors are synthesized as inactive precursors and are converted to their mature forms by limited proteolysis at multiple basic amino acid sites such as Arg-X-X-Arg and/or Arg-X-Lys/Arg-Arg to express their biological activities (Constam and Robertson, 1999; Constam and Robertson, 2000; Cui et al., 1998; Khalil, 1999). It was

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doi:10.1016/j.ydbio.2008.10.015

previously revealed that such processing is catalyzed by proteases of the subtilisin-like proprotein convertase (SPC) family (Seidah et al., 1994; Rouille et al., 1995), although little is known about the role of SPCs in SMG development. Recently, we reported that one of SPC family members, PACE4, is intensely expressed in the prenatal rat submandibular primordia, although its expression decreases post-natally (Akamatsu et al., 2007). Disruption of the PACE4 gene reportedly causes cyclopia, one of the phenotypes of the serious condition called holoprosencephaly, which shows defects and/or malformation in craniofacial development including that of the submaxillary region (Constam and Robertson, 2000). However, the physiological significance of this convertase in SMG development is still unclear.

On the other hand, saliva secretion is one of the important physiological functions of salivary glands, and the water channel AQP5 is known to be involved in this secretion process; i.e., AQP5-null mice and naturally occurring AQP5 mutant rats show decreased and viscous hypertonic saliva secretion compared with their wild-type counterparts (Ma et al., 1999, Murdiastuti et al., 2006). Previously, we reported that the expression of AQP5 is dramatically increased in the prenatal SMG in good accordance with the differentiation of the acini (Akamatsu et al., 2003).

In the present study, therefore, we focused upon the branching morphogenesis and expression of the water channel AQP5 as differentiation markers, and analyzed the effects of various protease inhibitors, antibodies, and siRNAs on rat embryonic SMG rudiments in the organ culture system to explore the physiological role of PACE4 in the developing salivary gland.

Materials and methods

Reagents

Monoclonal anti-bone morphogenetic protein-2 and TRI reagent were purchased from Sigma-Aldrich, Inc. (Saint Louis, Missouri, USA). Recombinant human BMP2 was procured from PeproTech EC (London, UK). Decanoyl-Arg-Val-Lys-Arg-chloromethylketone was from Alexis Corporation (Lansen, Switzerland); and Decanoyl-Arg-Val-Arg-Lys-chloromethylketone, H-D-Phe-Phe-Arg-chloromethylketone, and H-D-Phe-Pro-Arg-chloromethylketone were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Leupeptin was from Peptide Institute, Inc. (Osaka, Japan), and soybean trypsin inhibitor and heparin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclopore™ Track Etched Membrane (0.4, 0.6 μm polycarbonate clear) was obtained from Whatman (Maidstone, England). Superscript™/Superscript™ III One-Step RT-PCR System, BGJb medium, and Oligofectamine were from Invitrogen Corporation (Carlsbad, CA). Hybond-N+ was purchased from GE Healthcare Bio-Sciences Corp. (NJ, USA); and DIG Luminescent Detection Kit, from Roche Diagnostics (Tokyo, Japan). A Historesin Plus kit purchased from Leica Microsystems (Heidelberg, Germany) was used.

Animals

Pregnant Sprague–Dawley rats were purchased from Japan SLC (Hamamatsu, Japan). Animals were kept in our animal facility and provided water and laboratory chow ad libitum. Lighting of the animal house was periodically controlled so that it was illuminated during the period of 0600–1800. The Institutional Review Board of the Animal Committee of the University of Tokushima approved the protocol applied for the present animal experiments.

RT-PCR

Total RNA was isolated from the cultured SMG rudiments by using TRI reagent (Sigma) according to the manufacturer's protocol. RT-PCR was performed by using SuperScript™/SuperScript™ III One-Step RT-PCR System (Invitrogen) according to manufacturer's protocol and as described previously (Akamatsu et al., 2000, Akamatsu et al., 2003). The primers used in this study were 5′-CCCCAAGGCACCATGAAAAA-3′ (sense, 98–117) and 5′-TCACGAATCTCTGAGGTCTG-3′ (antisense, 1151–1170) for AQP5, 5′-CCCTCTGGAACCAAGTCTCAACTT-3′ (sense, 1658–1681) and 5′-TGAAGCCAGCTTTACATCTGCTGC-3′ (antisense, 2676–2699) for PACE4, 5′-TATGGCTACGGGCTGTTGGA-3′ (sense, 1713–1732) and 5′-CTCGCTGGTATTTTCAATCTCT-3′ (antisense, 2090–2111) for furin, and 5′-ACCCACACTGTGCCCATCTA-3′ (sense, 478–497) and 5′-CGGAACCGCTCATTGCC-3′ (antisense, 751–767) for β-actin.

Northern blot analysis

RNA samples isolated from the embryonic rat SMGs were analyzed by Northern blotting as described previously (Akamatsu et al., 2000; Akamatsu et al., 2003). A 10-μg aliquot of total RNA was resolved by electrophoresis in a 1% agarose gel containing 6.7% formaldehyde and transferred onto a nylon membrane, Hybond-N+ (GE Healthcare Bio-Sciences Corp.). The membrane was prehybridized at 68 °C for 4 h with hybridization buffer composed of 2×SSC (1×SSC is 15 mM sodium citrate containing 0.15 M NaCl, pH 7.2), 50% formamide, 10% dextran sulfate, and 10 mM dithiothreitol, and then hybridized with 100 ng/ml of DIG-labeled antisense riboprobe specific for rat PACE4A (Akamatsu et al., 2000; Akamatsu et al., 2007) at 68 °C for 17 h. The membranes were washed with 2×SSC containing 0.1% SDS at RT, and with 0.1×SSC containing 0.1% SDS at 65 °C. Riboprobe-hybridized PACE4 mRNA was immunologically detected by using a DIG Luminescent Detection Kit (Roche Diagnostics) according to the manufacturer's protocol. DIG-labeled mouse β-actin antisense cRNA probe (nucleotides 317–989) was used as a positive control.

Organ culture

Embryos were obtained on day 15.5 of gestation (E15) from their mother under ether anesthesia. The submandibular primordia were extracted from E15 embryos under a stereoscopic microscope. The paired rudiments extracted from an embryo were separately used; e.g. one for control culture, and the other for the inhibitor experiment. The SMG rudiments (5±1 rudiments/assay) were placed on Cyclopore membranes (Whatman) floated on BGJb medium (Invitrogen) containing 10% FCS; they were precultured for 1 h without any additive, and then further cultivated in the presence or absence of various reagents. Morphological changes that appeared were photographed at 24–48 h in culture. Total RNA was isolated from the cultured SMG rudiments, and the expression levels of PACE4, furin, AQP5, and β-actin were analyzed by RT-PCR as described above. Mann–Whitney *U*-test was used for the statistical analysis of the expression level of PACE4 and AQP5 (Tsumura et al. 2006, Li et al. 2008). For the histological analysis, the SMG rudiments treated with CMK were fixed for 2 h with ice-cold modified Bouin's fixative and were embedded in Historesin Plus as described previously (Akamatsu et al. 2007, Yao et al. 2006). Then the tissue sections were cut at 3-μm thickness and stained with 0.1% toluidine blue O.

RNAi experiment

Various siRNAs for PACE4 were designed and purchased from iGENE Therapeutics, Inc. (Ibaraki, Japan) and RNAi Co. Ltd. (Tokyo, Japan). After 1 h of preculture, SMG rudiments were transfected with siRNAs (0.5 or 1 μg) by using Oligofectamine (Invitrogen) according to the manufacturer's protocol and to the report of Sakai et al. (2003). The sequences of sense-strand RNAs for siRNAs used in this study were summarized in Fig. 7A.