Inhibition of Src and p38 MAP kinases suppresses the change of claudin expression induced on dedifferentiation of primary cultured parotid acinar cells

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Submitted 8 October 2007; accepted in final form 26 January 2008

Fujita-Yoshigaki J, Matsuki-Fukushima M, Sugiya H. Inhibition of Src and p38 MAP kinases suppresses the change of claudin expression induced on dedifferentiation of primary cultured parotid acinar cells. Am J Physiol Cell Physiol 294:C774–C785, 2008. First published January 30, 2008; doi:10.1152/ajpcell.00472.2007.—Sjögren’s syndrome and therapeutic radiation for head and neck cancers result in irreversible changes in the parenchyma of salivary glands, loss of acinar cells, prominence of duct cells, and fibrosis. To clarify mechanisms of salivary gland dysfunction, we identified a signaling pathway involved in the dedifferentiation of primary cultures of parotid acinar cells. We reported previously that the expression pattern of claudins changes during culture, is related to the three-dimensional organization of the cells, and reflects their ability to function as acinar cells. In this study, we found that this change of claudin expression is a process of dedifferentiation, because expression of other differentiation markers also changes during culture. The expression levels of claudins-4 and -6, cytokeratin 14, and vimentin are increased, and those of claudin-10, aquaporin 5, and amylase are decreased. Inhibitors of Src and p38 MAP kinases suppress these changes and increase the expression of acinar marker proteins. Differences in extracellular matrix components have no effect. Activation of p38 MAP kinase occurs during cell isolation from the parotid glands and is retained up to 6 h after the isolation. In contrast, activation of Src kinases does not increase during the cell isolation. The Src inhibitor PP1 suppresses the activation of p38 MAP kinase. Therefore, cellular stresses induced during cell isolation cause dedifferentiation and transition to duct-like cells through activation of p38 MAP kinase and constitutively active Src kinases.

salivary gland; exocrine gland; epithelium; tight junction

SALIVARY GLAND ATROPHY is an important problem in clinical dentistry because salivary glands produce and secrete saliva that creates and regulates the environment of the oral cavity. Hyposcretion of saliva and consequent xerostomia (dry mouth) lead to severe dental caries, periodontal disease, and mucosal infections (5, 48). Sjögren’s syndrome and therapeutic radiation for head and neck cancers result in irreversible changes in glandular parenchyma, the loss of acinar cells, prominence of duct cells, and fibrosis. In contrast to such severe symptoms, salivary glands generally have a capacity for functional recovery after infective sialadenitis, transoral sialolithectomy, or low-dosage irradiation. To investigate the mechanism of atrophy and regeneration of acinar cells, studies of duct ligation and deligation have been performed (7, 39, 44, 49, 53). Ligation of excretory ducts leads to acinar atrophy and numerous duct-like structures, which are considered to be derived from surviving atrophic acini (7, 49). Failure of secretion and damage to the corda tympani on the excretory duct lead to a reduction of stored granules and consequent acinar and glandular shrinkage. The loss of acini is due to shrinkage to duct-like small cells in addition to apoptosis (44, 53).

When the obstruction is removed, the synthesis and secretion can be re-established and acinar cells revert to their normal volume. Salivary glands can regenerate following severe atrophy such as cells with a flattened and undifferentiated appearance (39). The existence of stem cells in salivary glands has been postulated, and newly formed acinar cells are believed to differentiate from the rapidly proliferating intercalated duct cells (14, 34). On the other hand, other studies have shown that acinar cells can proliferate and are potential progenitor cells, although they are highly differentiated (24, 52).

Re-engineering salivary glands could provide many benefits to patients with xerostomia. Because acinar cells can proliferate, it should be possible to regenerate salivary glands by transplantation of cultured acinar cells. Nevertheless, when submandibular gland cells are cultured in vitro and are then injected into atrophic submandibular glands, the transplanted cells become involved in ductal regeneration, but not in myoepithelial or acinar differentiation (51). Because ducts are water impermeable and thus are not able to make saliva (56), regeneration of ducts is not sufficient to recover saliva secretion. In mammary glands, it was reported that a single stem cell generated an entire functional gland (46), whereas similar results have not been obtained for salivary glands. The loss of function of acinar cells is one of the major problems for reengineering salivary glands. Salivary acinar cells appear to have an intrinsic difficulty in maintaining their functions.

To yield feedback for studies on the regeneration of salivary glands, we established a system to generate primary cultures of parotid acinar cells and focused on analyzing time-dependent changes in their functions and their morphology (17). During that analysis, we found two types of cellular organization in the cultures: monolayers and semispherical clusters (41). Electron microscopy revealed tight junctions (TJs) near the apical regions of the lateral membranes between cells in the monolayers. Cells in the interior of each cluster also had TJs and were organized around central lumens. TJs function as selective...
barriers that allow the passage of some ions and solutes through the paracellular pathway, which is an essential function for epithelial tissues.

The claudin family consists of at least 24 members, and the combinations of different claudins expressed determine the overall barrier properties of the TJs (18, 20). Which claudin members are expressed is important for epithelial tissues. Although claudin-4 was not detected in cells immediately after isolation from the glands, its expression increased with time in culture. Immunofluorescence microscopy revealed that claudin-4 was synthesized in the monolayers, but not inside the clusters. Only claudin-3, which was expressed in the original acinar cells following their isolation and in the intact gland, was detected inside the clusters. These interior cells retained more secretory granules than the monolayer cells, suggesting that they maintained their original characteristics as acinar cells. These results suggest that differences in claudin expression are related to the three-dimensional organizations of the cells and reflect their ability to function as acinar cells (41).

In the present study, we investigated mechanisms that cause acinar cells to lose their functions to establish conditions under which they maintain their properties as acinar cells. The correlation between expression of claudin-4 and the loss of secretory granules raises the possibility that inhibition of cellular signaling that induces expression of claudin-4 will suppress the reduction of amylase synthesis. As candidates for signaling mediators that induce changes of claudin expression, we focused on Src family kinases (SFKs) and MAP kinases (MAPKs). Interactions between cells and the extracellular matrix (ECM) may affect gene expression and change the properties of the cells. Submandibular cell lines were reported to differentiate via interactions with ECMs (21, 25, 29), probably via integrins. SFKs and MAPKs are known to mediate integrin signaling and to regulate the spreading of cells (33, 36, 38, 50). Therefore, we assume that the signaling via SFKs and MAPKs might affect the properties of those cells.

MATERIALS AND METHODS

Preparation and culture of isolated acinar cells. Parotid glands were taken from male Sprague-Dawley rats (150–200 g each) anesthetized with pentobarbital sodium (Dainippon Pharmaceutical, Osaka, Japan). The experiment conforms with institutional guidelines for the use of experimental animals and was approved by the Experimental Animal Ethical Committee of Nihon University School of Dentistry at Matsudo. Acinar cells were isolated by digestion with collagenase A and hyaluronidase in isolating buffer (Hanks’ balanced salt solution containing 20 mM HEPES/NaOH, pH 7.4) as described previously (16). The cells were >90% viable, as determined by Trypan blue exclusion. Cells were diluted to 0.3 mg/ml with Waymouth’s medium containing 10% rat serum, ITS-X supplement, 10% fetal bovine serum, 50 μg/ml gentamicin, and 20 mM HEPES/NaOH, pH 7.4 containing 1× complete protease inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE and were transferred to Hybond-P membranes (Amersham). The same amounts of protein were applied to each lane of SDS-PAGE. The membranes were blocked at room temperature for 50 min in blocking agent (Amerham) and were blotted with antibodies. Immunoreactivity was determined by using an EC Western blotting kit (Amersham). The same images were acquired with a FluorImager 595SS (Molecular Dynamics). Intensity of immunoreactivities was quantified with ImageQuant software (Molecular Dynamics).

RNA preparation and real time RT-PCR analysis of mRNA expression. Total RNA was isolated from parotid acinar cells immediately after their isolation or after 1, 2, or 3 days in culture by using the Trizol reagent (Invitrogen). After treatment with DNase I, RNA was purified with RNeasy MinElute cleanup kits (Qiagen). Amounts of RNA were quantified by measuring the absorbance at 260 nm. Expression levels were determined with the QuantiTect SYBR RT-PCR kit (Qiagen) using the DNA Engine Opticon system (MJ Research, Waltham, MA). Primer pairs for amplification of rat GAPDH and occludin were designed according to previous studies (27, 32). Primer pairs for the amplification of rat claudin-4 and claudin-6 were designed according to a previous study (41). Primer pairs for the amplification of rat claudin-10 were 5′-GTC AGG TCT GTG TTC CAT G-3′ (sense) and 5′-TGA CAC CGC CAA TGA TGC-3′ (antisense) with a predicted size of 155 bp. Primer pairs for the amplification of rat E-cadherin (cdh1) were 5′-GTC AGG TCT GTG TTC CAT G-3′ (sense) and 5′-TGA CAC CGC CAA TGA TGC-3′ (antisense) with a predicted size of 136 bp. Primer pairs for the amplification of rat claudin-3 were obtained from Qiagen. PCR products were evaluated by melting curve analysis according to the manufacturer’s instructions and by examining the size of the PCR products separated on 2.0% agarose gels. Relative RNA equivalents for each sample were obtained by normalizing to GAPDH levels. Each sample was run in duplicate to determine sample reproducibility, and the average relative RNA equivalents per sample pair were used for further analysis.

Measurement of DNA concentrations by PicoGreen assay. Cells were lysed with Tris-EDTA buffer with 1% Triton X-100 on ice for 60 min and were then diluted tenfold with Tris-EDTA buffer without concentration of 10 μM. Inhibitors for p38 MAPK were dissolved in DMSO at a concentration of 5 mM and were diluted to 1:250 with the culture medium (final 0.4%). Inhibitors for p38 MAPK were dissolved in DMSO and the same volumes were added to the culture medium. Stock solution of U-0126 was freshly prepared just before experiments. When culture medium was changed, the inhibitors were added to the new medium unless indicated.

Antibodies and immunoblot analysis. Mouse monoclonal anti-E-cadherin, anti-vimentin, and anti-p38 MAPK antibodies were purchased from BD Biosciences. Mouse monoclonal anti-occludin and anti-claudin-4 and rabbit polyclonal anti-claudin-3 antibodies were purchased from Zymed. Goat polyclonal anti-claudin-6 antibody was from Santa Cruz Biotechnology. Rabbit polyclonal anti-aquaporin 5 (AQPS) antibody and mouse monoclonal anti-cytokeratin 14 (CK14) antibody were purchased from Chemicon. Rabbit polyclonal antiamylase antibody was purchased from Sigma-Aldrich. Mouse monoclonal anti-smooth muscle actin (SMA) antibody was from Progen, and rabbit polyclonal anti-phospho-p38 MAPK (T180/Y182) antibody was from R&D Systems. Rabbit polyclonal anti-ERK1/2 antibody was from Cell Signaling, and rabbit polyclonal anti-phospho-ERK1/2 (T185/Y187) antibody was from Biosource. Cells were harvested and lysed in homogenizing buffer (in mM: 150 NaCl, 1 EDTA, 1 EGTA, 1 PMSF, 10 NaF, 1 Na3VO4, 5 μg/ml benzamidine, and 20 HEPES/NaOH, pH 7.4) containing 1× complete protease inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE and were transferred to Hybond-P membranes (Amersham). The same amounts of protein were applied to each lane of SDS-PAGE. The membranes were blocked at room temperature for 50 min in blocking agent (Amersham) and were blotted with antibodies. Immunoreactivity was determined by using an ECF Western blotting kit (Amersham), and the images were acquired with a FluorImager 595SS (Molecular Dynamics). Intensity of immunoreactivities was quantified with ImageQuant software (Molecular Dynamics).

RNA preparation and real time RT-PCR analysis of mRNA expression. Total RNA was isolated from parotid acinar cells immediately after their isolation or after 1, 2, or 3 days in culture by using the Trizol reagent (Invitrogen). After treatment with DNase I, RNA was purified with RNeasy MinElute cleanup kits (Qiagen). Amounts of RNA were quantified by measuring the absorbance at 260 nm. Expression levels were determined with the QuantiTect SYBR RT-PCR kit (Qiagen) using the DNA Engine Opticon system (MJ Research, Waltham, MA). Primer pairs for amplification of rat GAPDH and occludin were designed according to previous studies (27, 32). Primer pairs for the amplification of rat claudin-4 and claudin-6 were designed according to a previous study (41). Primer pairs for the amplification of rat claudin-10 were 5′-GTC AGG TCT GTG TTC CAT G-3′ (sense) and 5′-TGA CAC CGC CAA TGA TGC-3′ (antisense) with a predicted size of 155 bp. Primer pairs for the amplification of rat E-cadherin (cdh1) were 5′-GTC AGG TCT GTG TTC CAT G-3′ (sense) and 5′-TGA CAC CGC CAA TGA TGC-3′ (antisense) with a predicted size of 136 bp. Primer pairs for the amplification of rat claudin-3 were obtained from Qiagen. PCR products were evaluated by melting curve analysis according to the manufacturer’s instructions and by examining the size of the PCR products separated on 2.0% agarose gels. Relative RNA equivalents for each sample were obtained by normalizing to GAPDH levels. Each sample was run in duplicate to determine sample reproducibility, and the average relative RNA equivalents per sample pair were used for further analysis.
Triton X-100. DNA concentrations in the samples were measured with Quanti-iT PicoGreen reagent (Invitrogen).

Amylase assay. The activity of amylase was measured by the method reported previously (6), modified for incubation at 30°C for 5 min. In this method, one unit of amylase is defined as the quantity of enzyme that liberates 1 mg maltose/min at 30°C.

Immunoprecipitation analysis. Homogenates and cell lysates were suspended in homogenizing buffer containing 1% Triton X-100 over-night. After centrifugation at 20,000 g for 15 min, the supernatants were obtained as the total extracts. Total extracts (500 µg each) were suspended in homogenizing buffer containing 1 × complete protease inhibitor cocktail. Samples were added to protein A-Sepharose 4FF conjugated with normal rabbit IgG and were incubated at 4°C for 60 min. After centrifugation, the supernatants were recovered and incubated with rabbit polyclonal anti-Src (Abcam) or anti-Yes (Santa Cruz Biotechnology) antibodies conjugated with protein A-Sepharose 4FF at 4°C for 2 h. Following this, protein A-Sepharose was collected by centrifugation and was boiled in sample buffer for SDS-PAGE. After transfer to Hybond-P membranes, the membranes were blocked at room temperature for 50 min in blocking agent (Amersham) and were then blotted with rabbit polyclonal anti-Tyr418-phosphorylated Src (anti-PY418) or anti-Tyr529-phosphorylated Src (anti-PY529) antibodies, which were purchased from Biosource, or with mouse monoclonal anti-Src (Biosource) or anti-Yes (BD Biosciences) antibodies.

Statistics. All values are reported as means ± SE. Statistical analyses of the differences in the means of experimental pairs were evaluated by t-test. The P values obtained are indicated in the figure legends when statistically significant. Studies with more than two groups were evaluated by ANOVA. The ANOVA included a Bonferroni/Dunn multiple-comparison test. P < 0.05 was considered significant.

RESULTS

Src inhibitors suppress the changes in claudin expression. Although acinar cells in parotid glands do not express claudin-4 (40), claudin-4 begins to be expressed after their isolation, and its level of expression increases during culture over time as reported previously (41). To determine the signaling pathway that induces the expression of claudin-4, we examined the effects of inhibitors for SFKs. We added 10 µM PP1 to the culture medium and harvested cells after culture for 1–3 days. Levels of claudin-4 expressed in cells cultured in the absence of PP1 were similar 1 day after the isolation (Fig. 1A). After culture for 2–3 days, claudin-4 expression remained constant in the presence of PP1 but was markedly increased in the absence of PP1. In a previous study, we reported that levels of claudin-6 increased, claudin-3 slightly decreased, and occludin did not change during culture (41). Levels of E-cadherin remained constant over time [Fig. 1B; compare the homogenate (H) and lysate of isolated cells (0) to the control (cont) at 3 days]. We then compared the effects of two Src inhibitors, PP1 and PP2, with PP3 (a negative control for PP2) on the expression of E-cadherin and TJ proteins. Levels of E-cadherin, occludin, and claudin-3 were not changed by the addition of these inhibitors (Fig. 1B), which indicates that intercellular junctions were maintained despite the addition of Src inhibitors. We confirmed the maintenance of TJs by immunofluorescence microscopy using anti-occludin and anti-zonula occludens-1 antibodies. The signal intensity of intercellular junctions in PP1-treated cells is very similar to that of non-treated cells (data not shown). In contrast, levels of claudin-4 and claudin-6 decreased following the addition of PP1 or PP2. PP3 did not affect the expression of any claudins. Claudin-4 is expressed by ductal cells in parotid glands, and claudin-4 and claudin-6 were detected in ducts during organ development (19, 40). Cultured cells seem to dedifferentiate, and the Src inhibitors suppress these changes. mRNA expression levels of E-cadherin (cdh1), occludin (ocln), and claudins-3 (cldn3), -6 (cldn6), -10 (cldn10) at 3 days measured by real-time RT-PCR. Values are shown as relative to GAPDH expression levels. (*P < 0.001; **P < 0.0001; ***P < 0.001; ****P < 0.05; n = 5).

Fig. 1. Src inhibitors suppress expression of claudin-4. A: effect of PP1 on expression of claudin-4. Lysates of isolated cells (0) and cells cultured for 1, 2, and 3 days in absence (control) or presence (+PP1) of 10 µM PP1 were harvested. Same amounts of protein (1 µg) were used for immunoblot analysis with anti-claudin-4 antibody. Results are representative of 3 independent experiments. Graph shows band intensities acquired by FluorImager 595S and evaluated by densitometry using NIH Image analysis software. Legend shows levels of claudin-4 and claudin-6, claudin-10 (cldn10), occludin (ocln), and claudins-3, -4, and -6. *P < 0.05; **P < 0.001; ***P < 0.0001; ****P < 0.05; n = 5).
intact acinar cells, and its expression decreases during culture over time (41). PP1 suppressed that decrease of claudin-10 expression. Therefore, it seems that primary cultured parotid acinar cells become dedifferentiated to the embryonic stage and that Src inhibitors suppress that transition from the differentiated to the dedifferentiated condition.

**PP1 suppresses the change in expression of differentiation markers.** Tumorigenesis and fibrosis are accompanied by changes in gene expression. CK14 and vimentin have been reported to be expressed in some salivary gland tumors (12, 31), and their expression patterns are used as markers for classifying salivary tumors (13, 45). AQP5 is specifically expressed in acinar cells of salivary glands (35) and is used as a marker for the differentiation of acinar cells (59). Because claudin-4 and claudin-6 are expressed (Fig. 1, A and C), primary cultured parotid acinar cells seem to be dedifferentiated. To identify what alterations occur in the cultured cells, we examined changes in the expression of those other markers during culture (Fig. 2A). Expression levels of AQP5 were high in homogenates of the whole glands and in cell lysates after isolation of acinar cells. Levels of AQP5 decreased rapidly over time, and the immunoreactive band became very faint at 3 days. Neither CK14 nor vimentin was detected in homogenates or in cell lysates just after isolation but began to be detected during culture. Whereas vimentin is negative in normal salivary glands (12), CK14 is expressed in myoepithelial cells and basal cells in striated ducts (12, 31). A small number of myoepithelial cells contaminate the isolated cells, as reported previously (17). However, that population does not change after isolation, which was confirmed by immunofluorescence microscopy using an anti-SMA antibody. SMA, a marker of myoepithelial cells, was detected in the homogenate and in cell lysates after the isolation, but its levels did not change during culture (Fig. 2A). These results confirm that the population of myoepithelial cells did not change. Therefore, the increase in expression of vimentin and CK14 is not due to an increase of myoepithelial cells but rather to other cells in the culture.

The effects of Src inhibitors on the expression of the markers at 3 days were then examined (Fig. 2B). In the presence of PP1 or PP2, levels of residual AQP5 increased. The expression of vimentin and CK14 were suppressed compared with the control culture. The amount of SMA was not changed by the addition of Src inhibitors. As another marker of acinar cells, amylase activity was measured. The relative amylase activity per milligram of protein at 1 day after isolation was similar to that just after isolation but then rapidly decreased over time in culture (Fig. 2C). In the presence of PP1 or PP2, the rate of its decrease was slower than in the absence of inhibitors, which indicates that cells retained more amylase. Relative amylase activities at 3 days in the PP1- or PP2-treated cells were 2.9- and 2.6-fold higher, respectively, than in the untreated cells. The increase of amylase was confirmed by immunoblot analysis with an anti-amylase antibody (Fig. 2C, inset). Because parotid acinar cells secrete amylase in the resting state without secretagogues, it is possible that Src inhibitors suppress the secretion of amylase from acinar cells, which would result in increases of amylase activity. To verify that the Src inhibitors increased the expression of the amylase gene, its mRNA expression level was determined by real-time RT-PCR (Fig. 2D). In the presence of PP1 or PP2, amylase mRNA levels increased to 4.4- and 4.3-fold higher than in the absence of inhibitors, respectively. These results indicate that inhibition of SFKs suppresses the changes in gene expression and the characteristics of cultured parotid acinar cells.

Although PP1 and PP2 are often used as specific inhibitors for SFKs, they were reported to have nonspecific inhibitory effects on other kinases (3). To confirm that SFKs are involved in these changes in gene expression, another type of inhibitor for SFKs, Src kinase inhibitor 1, was used. Addition of Src kinase inhibitor 1 increased the expression of amylase gene, its mRNA expression level was determined by real-time RT-PCR (Fig. 2D). In the presence of PP1 or PP2, amylase mRNA levels increased to 4.4- and 4.3-fold higher than in the absence of inhibitors, respectively. These results indicate that inhibition of SFKs suppresses the changes in gene expression and the characteristics of cultured parotid acinar cells.
kinase inhibitor 1 increased residual amylase (Fig. 3A) and suppressed the expression of claudin-4 (Fig. 3B) like PP1. The effect of Src kinase inhibitor 1 was a little smaller than PP1. These results suggest that SFKs mediate the signaling for dedifferentiation.

**PP1 affects the three-dimensional structure of culture organization but not cell attachment to the dish surface or cell growth.** Because PP1 affected the cell-differentiation stage, we hypothesized that the cell morphology and growth are influenced by inhibition of SFKs. During culture, parotid acinar cells initially gather and form spherical clusters and then attach to the dish surface. After attachment, cells spread on the surface and the clusters gradually become flat (17). After 3 days in culture, most cell clusters became flatter than those at 1 day (Fig. 4A). In the presence of PP1, although cells spread on the dish surface as they did in the absence of PP1, the clusters remained semispherical (Fig. 4B). The structures of these clusters were observed by thin sections cut vertically to the dish surface. The clusters in PP1-treated culture were more spherical than those of control cells (Fig. 4, C and D). We previously showed that cells within the clusters retained more granules and did not express claudin-4, whereas the cells in monolayers lost their granules and expressed claudin-4 (41). It is possible that the expression of claudin-4 participates in the alteration of the three-dimensional structure of the cell culture, although changes in the expression of many other genes should be considered. Expression patterns of intermediate filaments such as CK14 and vimentin may affect the culture organization because they influence the cell morphology.

To examine whether PP1 affects the cell viability and/or cell attachment to the dish surface, cell numbers should be counted, but that is difficult because they form clusters. Therefore, we measured DNA concentrations in the cell lysates by using the PicoGreen assay to compare cell numbers between cultures in the absence or presence of PP1. The amount of DNA in the cell lysate at 1 day indicates the efficiency of cell attachment, and the increase in DNA at 2 or 3 days indicates the cell growth rate (Fig. 4E). At each time point measured, the DNA concentration in the PP1-treated or untreated cells was not significantly different. Therefore, PP1 affects the three-dimensional structure of tissue organization but not cell attachment or cell growth.

**Interaction with the ECM is not a major cause of dedifferentiation.** Interaction with the ECM is an important factor for the differentiation of epithelial cells (4, 8, 26). In particular, submandibular cell lines have been reported to differentiate by interaction with the ECM (21, 25, 29). Thus claudin-4 expression might be expected to be altered by differences in the ECM. To examine the effects of interaction between integrin and the ECM, we used several different types of ECM-coated culture dishes. We usually use collagen I coated dishes, and we compared them to noncoated, collagen IV-, laminin-, or fibronectin-coated dishes. After culture for 3 days, cells were harvested and the levels of claudin-4 and amylase were examined by immunoblot analysis. Unexpectedly, different ECM coatings of the culture dishes did not influence claudin-4 expression (Fig. 5). Addition of echistatin (an inhibitor of integrin-β1 and -β3) or GoH3 (a function-blocking antibody to integrin-α6) did not affect claudin-4 expression either. The amount of residual amylase was not affected by different ECMs or by the addition of echistatin or GoH3. Therefore, it is unlikely that interaction with the ECM plays a role in the induction of dedifferentiation.

**Inhibition of p38 MAPK suppresses the dedifferentiation of parotid acinar cells.** The MAPK family includes key mediators of many signal-transduction pathways, such as cell growth, apoptosis, and differentiation. In particular, stresses induce many changes in cell functions via p38 MAPK activation. To identify the mediators of Src signaling, we used three inhibitors of the MAPK family: the p38 MAPK inhibitor SB-203580, the JNK inhibitor SP-600125, and U-0126, which is an inhibitor of the ERK1/2 (p44/p42 MAPK) pathway. After culture for 3 days in the presence of these inhibitors, their effects on gene expression were investigated. The expression of claudin-4 was suppressed in the presence of SB-203580, although the other MAPK inhibitors had no effect (Fig. 6A). All inhibitors, particularly SP-600125, decreased the level of CK14, SB-203580 or SP-600125 decreased vimentin, but U-0126 had no effect on that protein. The level of SMA was not affected by SB-203580 but was increased in the presence of SP-600125 or U-0126. With the use of real-time RT-PCR, SB-203580 was confirmed to suppress claudin-4 and claudin-6 mRNA levels and to increase claudin-10 mRNA like the Src inhibitors (Fig. 6B). Relative amylase activity retained in the cells was increased by SB-203580 or U-0126 (Fig. 6C). Levels of amylase mRNA were also increased by SB-203580 or U-0126 treatment (Fig. 6D), although the increase by U-0126 was not statistically
significant. The effects of SB-203580 are very similar to those elicited by the Src inhibitors. In contrast, the effects of SP-600125 or U-0126 are quite different from those of SB-203580 and the Src inhibitors. These inhibitors enhanced some changes and suppressed other changes of cultured acinar cells. Because MAPKs are well known to be involved in cell proliferation, survival, or apoptosis, the effect on cell number was measured by PicoGreen assay. Cell attachment and cell growth in the presence of SB-203580 were similar to that of the control (data not shown). SP-600125 seems to somewhat suppress the cell attachment at 1 day, and U-0126 reduced the cell number at 3 days compared with the control, but neither difference was significant. The signaling and responses mediated by JNK and ERK1/2 seem to be more complicated, and further detailed analysis would be necessary to clarify the roles of these kinases.

To confirm the role of p38 MAPK, other inhibitors for p38 MAPK (PD-169316 and SB-202190) were used. Both inhibitors increased the amount of amylase (Fig. 7A) and decreased expression of claudin-4 (Fig. 7B) similarly to SB-203580, suggesting that p38 MAPK is involved in the change of gene expression.

**Activation of p38 MAPK is inhibited by the Src inhibitors.** Because the effects of inhibitors for p38 MAPK and SFKs are similar, we hypothesized that SFKs are necessary for the activation of p38 MAPK or vice versa. The amount of activated p38, which is phosphorylated at Thr180/Tyr182, was measured first. As shown in Fig. 8A, p38 MAPK had already been phosphorylated immediately after the isolation, whereas it was not phosphorylated in the homogenate. p38 MAPK can be activated by many stresses, such as tissue injury or superoxide production (11, 22, 23, 57). The enzymatic dispersion of acinar cells may induce such a stress signal. The activation of p38 MAPK remained up to 6 h after the start of culture (Fig. 8, A and B). The phosphorylation of p38 MAPK decreased to the control level at 24 h after the isolation and increased again at
48 and 72 h. Several studies have shown that SFKs are involved in the activation of p38 MAPK (55, 58, 61). To examine the effect of PP1 on p38 MAPK activation, PP1 was added to the medium after the isolation. The phosphorylation of p38 MAPK disappeared within 2 h after the addition of PP1, and the second activation at 48 or 72 h was not observed. Therefore, SFK activity is necessary for the activation of p38 MAPK. We also examined the activation of ERK1/2 (p44/p42 MAPK) because inhibition of the ERK1/2 pathway also suppressed the decrease of AQP5 and amylase (Fig. 6, A and C). ERK1/2 were also activated during the isolation, but PP1 did not decrease the level of phosphorylated ERK1/2, suggesting that a different signal pathway induced the activation of ERK1/2 (Fig. 8C).

We examined the effect of inhibitor treatment during cell isolation. PP1 or U-0126 was added to the isolating buffer, and cells were harvested just after the isolation. PP1 inhibited p38 MAPK activation but not ERK1/2 activation, similarly to its treatment during the culture (Fig. 8D). U-0126 specifically suppressed ERK1/2 activation. Src kinase inhibitor 1 and SU-6656 also suppressed the activation of p38 MAPK (Fig. 8E). PP1 suppressed p38 MAPK activation more efficiently than Src kinase inhibitor 1 or SU-6656, which may be due to the direct inhibition of p38 MAPK by PP1 (3). However, Src kinase inhibitor 1 or SU-6656 does not inhibit p38 MAPK directly (3). Thus SFK activity is necessary for the induction and maintenance of p38 MAPK activation.

SFKs are constitutively active in parotid glands and do not appear to be further activated during isolation of acinar cells. The results presented above showed that p38 MAPK is activated during isolation and that SFK is involved in the activation of p38 MAPK. Those results suggested to us that SFKs are activated during the isolation before p38 MAPK activation. SFK members share two tyrosine phosphorylation sites, Tyr418 in the activation loop (whose autophosphorylation is required for activity). The phosphorylation sites are located within the kinase domain, and their autophosphorylation is required for full activation of SFKs.

**Fig. 5.** Neither difference in extracellular matrix (ECM) components nor inhibition of integrins affects expression of claudin-4 or amylase. Left: cells cultured in uncoated dishes (non) or in collagen I- (col1), collagen IV- (col4), laminin- (LN), or fibronectin-coated dishes (FN). Right: cells cultured in absence (cont) or presence of 100 nM echistatin (ech) or 10 μg/ml GoH3. After 3-day culture, cells were harvested and analyzed with antibodies to amylase or claudin-4. Results are representative of 2 independent experiments.

**Fig. 6.** Effects of MAPK inhibitors on expressions of differentiation marker of parotid acinar cells. A: immunoreactivities of claudin-4, AQP5, CK14, vimentin, and SMA in cell lysates after culture for 3 days in absence (control) or presence of 20 μM SB-203580 (SB), 10 μM SP-600125 (SP) or 10 μM U-0126. Results are representative of 4 independent experiments. B: mRNA expression levels of occludin (ocln) and claudins-4 (cldn4), -6 (cldn6), and -10 (cldn10). Addition of SB-203580 suppresses expression of claudins-4 and -6 and increases that of claudin-10 (*P < 0.01; **P < 0.05; n = 4). C: relative amylase activities in lysates of cells cultured for 3 days without (control) or with inhibitors. SB-203580 and U-0126 increased residual amylase activity (*P < 0.05 vs. control; n = 4). D: mRNA expression level of amylase (amy1) becomes higher in presence of SB-203580. SP-600125 or U-0126 did not significantly affect amylase expression (*P < 0.05 vs. control; n = 4).
for Src kinase activity) and Tyr529 in the carboxy-terminal tail (that binds to the Src homology 2 domain of the molecule in its inactive form). We used an antibody that specifically recognizes Src phosphorylated at Tyr418 (anti-PY418) to examine the activation of Src kinases. Tyr418 phosphorylation was detected in homogenates, and its level was increased slightly until 72 h after the isolation (Fig. 9A). No significant difference was detected between the homogenate and the cell lysate at 0 h. The Src kinase family has many members, and anti-PY418 reacts with other members in addition to the original Src because the phosphorylation sites are so similar. Which SFK members are expressed in salivary glands has not been reported. We found that Src and Yes are expressed in parotid acinar cells. Both Src and Yes increase somewhat during the culture (Fig. 9A). Fyn, Lyn, Hck, and Lck were not detected in cell lysates just after isolation (data not shown). To determine the activation of each SFK individually, each kinase was immunoprecipitated and the precipitates were tested for reactivity with anti-PY418. Tyr418 phosphorylation of Src or Yes did not change during cell isolation (Fig. 9B). The phosphorylation of Tyr529 (which indicates the inactive SFK) did not increase either, suggesting that both Src and Yes are constitutively active and are not activated during isolation, although the possibility remains that other SFKs that were not detected or determined were activated. In human neutrophils, TNF-induced β2-integrin activation requires SFK activity, but activation of SFKs was not observed (10). In addition to the constitutive activation of SFKs, a certain signal may be involved in the activation of p38 MAPK. The phosphorylation of Src was increased at 72 h (Fig. 9C), suggesting that the increase of the anti-PY418-immunoreactive band intensity during the culture (shown in Fig. 9A) is due to Src activation.

Inhibition of SFK activity for the first 24 h is sufficient for the suppression of acinar dedifferentiation. The activation of p38 MAPK was maintained up to 6 h after the isolation, and PP1 suppressed the maintenance of p38 MAPK activation. We assumed that the activation of p38 MAPK only for 1 day was sufficient to induce the dedifferentiation of parotid acinar cells. The isolated cells were cultured in the presence of PP1 for 1 day and then were cultured without PP1 for another 2 days. Following that, the expression of claudins-4 and -6 and levels of residual amylase activity were determined. The expression of claudins-4 and -6 were clearly suppressed by incubation with PP1 for 1 day (Fig. 10A). The relative amylase activity was 2.8 times higher than in cells cultured without PP1 (Fig. 10B). These results suggest that the inhibition of SFKs and p38 MAPK for only 24 h after the cell isolation is sufficient to suppress the dedifferentiation. After culture for 1 day, only a small population of cells had directly attached to the dish surface, but most cells had not (17). Our current results show that the signaling for dedifferentiation was already finished at 1 day. The fact that the signaling is an early event supports the conclusion that interaction with the ECM is not a major cause of the dedifferentiation.

**DISCUSSION**

This study showed that changes of parotid acinar cells during isolation and subsequent culture are suppressed by inhibition of SFKs and p38 MAPK. The changes in cultured cells can be interpreted as dedifferentiation because: 1) expression levels of amylase and AQP5, marker proteins of acinar cells, decreased, 2) the expression pattern of claudins changed, and 3) levels of CK14 and vimentin increased. In salivary glands, claudin-4 is expressed only in duct cells and not in acinar cells. Claudin-6 is expressed in ducts of the embryonic organ but disappears after birth (19). In contrast, claudin-10 is detected only in acinar cells. Differentiation to acinar cells may correlate with suppression of claudins-4 and -6 and induction of claudin-10. CK14 is strongly detected in many salivary tumors. Although CK14 is detected in basal cells or myoepithelial cells in normal glands, Burns et al. (12) reported that the expression pattern of cytokeratins in salivary tumors is determined by differentiation degree rather than by the lineage of the cell. In general, different keratin isoforms are differentially expressed during development and differentiation and vary in different types of epithelia (15, 37, 43). CK14 is stratification-related keratin and is abundant in mitotic cells. Its expression is considered to depend on the degree of anaplasia (43). From these facts, the expression of CK14 can be used as an index of dedifferentiation. Vimentin is considered to be a marker of epithelial-mesenchymal transition (EMT). The term EMT describes a series of events during which epithelial cells lose their epithelial characteristics and take on properties that are typical of mesenchymal cells (30, 42, 54). EMT plays an important role in the development of tissues during embryogenesis and also in tissue repair following epithelial injury of adult organs.
Similar changes in morphology and gene-expression patterns are observed during pathological processes, such as fibrosis and tumorigenesis, suggesting that EMT play central roles in those processes (30). The change of parotid acinar cells studied here is not an EMT, because neither E-cadherin nor occludin expression decreased. On the contrary, we found that TJ formed at the intercellular junctions (41), which are characteristic of polarized epithelium. Nevertheless, the change partially resembles an EMT, because vimentin levels increased (Fig. 2A) and some cells formed filopodia (17).

Dedifferentiation of epithelium is generally considered to be a loss of polarity, such as the decrease of occludin and claudin expression.
expression. On the other hand, it has been reported that the expression patterns of claudins change during organ development (1, 9, 19, 28), which suggests that claudin expression is related to the differentiation stage. It is interesting that the dedifferentiation of acinar cells is accompanied with changes but not the loss of claudin expression. This study provides new information with regard to the regulation of claudin expression.

In Madin-Darby canine kidney (MDCK) cells, EGF differentially regulates claudin expression and increases transepithelial resistance (47). Because EGF simultaneously induces cell proliferation, that study assumed that tissue injury in the renal epithelium promotes EGF receptor activation, leading to tissue repair and increased TJ barrier function. The acinar cells in our cultures maintained their epithelial characteristics but became similar to undifferentiated ductal cells and acquired some characteristics of mesenchymal cells. Ligation of excretory ducts in salivary glands induces the transition of acinar cells to duct-like cells for a short period and for longer periods increases connective tissue (39, 44). These results suggest that the change of claudin expression is an early response for the transition to duct-like cells. After severe atrophy, epithelial cells turned into fibroblast-like cells and grow actively, but in the early stage, enduring stress without EMT is efficient to restore the glands after the end of cellular stress.

It is difficult to identify the origin of the cultured cells. Salivary glands consist of various types of epithelial cells (such as acinar, duct, and myoepithelial cells) and connective tissues, as well as inflammatory cells (such as mast cells and macrophages). Although >90% of the cells were observed to have secretory granules, which suggests that most cells in the isolated fractions are acinar cells (17), it is possible that other types of cells contaminate the cultures. Because the isolated cells are not uniform, we cannot rule out the possibility that the changes in protein expression are due to some contaminating cells. We detected a 1–2% contamination of myoepithelial cells in the isolated cells, but they are unlikely to increase during culture because SMA levels did not increase. We have confirmed that the claudin-4-positive cells are derived from acinar cells by the observation of secretory granules (41), and hence we conclude that changes in claudin expression are due to changes in acinar cells and not to other types of cells. The cells in monolayers and those inside the clusters show different phenotypes, although both of them are derived from acinar cells. Thus the cells also become heterogeneous in culture, and changes of gene expression reported in this study do not necessarily occur in individual cells. The fates of the cells may be influenced by their environment such as their interaction with the ECM or with other cells.

What kind of signal induces the dedifferentiation of parotid acinar cells? Because p38 MAPK was already activated after cell isolation (Fig. 8), the signaling for dedifferentiation starts during the isolation process. The incubation with digestion enzymes probably induced inflammation responses such as the production of reactive oxygen species or inflammatory cytokines and led to the activation of p38 MAPK via phosphorylation reaction by SFKs. There are several reports that Src is necessary for the activation of p38 MAPK induced by cellular stress (55, 58, 61). Activation of SFKs during isolation was not detected in this study, whereas the activation of Src at 3 days was detected. Because the activation of p38 MAPK occurred during the isolation and could be suppressed by PP1, further activation of SFK is not necessary to activate p38 MAPK. Bouaquina et al. (10) reported that TNF-induced activation of β2-integrin involves SFKs and the redox-regulated activation of p38 MAPK, although neither TNF nor oxidants increased SFK activation. A similar pathway appears to be involved in our culture.

Protein kinase inhibitors often have nonspecific inhibitory effects on other kinases. The selectivity of kinase inhibitors was recently reported in detail (3). We should consider the contribution of such side effects of inhibitors. It is possible that inhibition of EGF signaling affected gene expression in parotid acinar cells as well as MDCK cells because Src inhibitors may have an inhibitory effect on EGF receptor. Src inhibitors (PP1 and Src kinase inhibitor 1) and p38 MAPK inhibitors (SB-203580, PD-169316, and SB-202190) were reported to inhibit Rip2 (2, 3, 60), which is one of the upstream factors to induce activation of p38 MAPK (60). We could not exclude the involvement of Rip2 in changes of gene expression by the experiments using inhibitors. However, Rip2 was not detected in the homogenates of parotid glands or lysates of the isolated cells, whereas it was detected in the lysates of cells cultured for 3 days (data not shown). It is unlikely that Rip2 mediates the signaling for dedifferentiation of parotid acinar cells. SP-600125 was also reported to inhibit some protein kinases such as protein kinase D and Aurora kinases in addition to JNK (3). Because expression levels of amylase and claudin-4 were not affected by SP-600125, such protein kinases do not contribute to these changes in gene expression.

The inhibition of SFKs and p38 MAPK for only 24 h after tissue injury is sufficient to suppress the dedifferentiation. The effects of the ECM are unexpectedly minor. We have already reported that differences of ECM hardly affect the cell morphology or amylase activity (17). It is possible that different ECM should affect the differentiation stage and gene-expression pattern after a longer culture. On the other hand, the induction of claudin-4 expression is an early response compared with other claudins and other marker proteins (41). We hypothesize that acinar cells are programmed to change gene expression for protection from tissue injury and for survival from stress, and it was previously proposed that acinar atrophy can be regarded as a defense mechanism that evolved to protect the acinar cells during sialadenitis (44). The program for
changes in gene expression is one of the reasons that acinar cells easily lose their functions. Specific inhibition of Src-p38 MAPK signaling suppressed the dedifferentiation without affecting cell viability. This study suggests the possibility that the dysfunction of salivary acinar cells could be rescued by inhibition of this signaling pathway.

ACKNOWLEDGMENTS

The expert technical assistance of M. Morino is gratefully acknowledged. Present addresses of M. Matsuki-Fukushima: Laboratory of Signal Transduction, National Institute of Environmental Health Sciences/NIH, Research Triangle Park, NC 27709.

GRANTS

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Culture, Sports and Technology (MEXT) of Japan (16591868), by a Niho University Multidisciplinary Research Grant for 2006 and 2007, and by a Grant-in-Aid for a 2003 Multidisciplinary Research Project from MEXT.

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