Filopodia formation via a specific Eph family member and PI3K in immortalized cholangiocytes

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Fukushima, Koji, Yoshiyuki Ueno, Jun Inoue, Noriatsu Kanno, and Tooru Shimosegawa. Filopodia formation via a specific Eph family member and PI3K in immortalized cholangiocytes. Am J Physiol Gastrointest Liver Physiol 291: G812–G819, 2006. First published June 15, 2006; doi:10.1152/ajpgi.00250.2005.—Biliary ducts are lined with epithelial cells, which consist of at least two types of cholangiocytes, small and large. In contrast to large cholangiocytes, which are involved in secretion, the role of small cholangiocytes has not been elucidated. To address this question, we analyzed the proliferation and migration of small cholangiocytes. Interestingly, dibutyryl cAMP induced marked filopodia formation and cdc42 activation in the normal mouse cholangiocyte (NMC)-small cell line compared with the NMC-large cell line. Analysis of members of the ephrin (Eph)A family of guidance molecules revealed a distinct subcellular distribution of EphA5 and EphA8 members: EphA8 was equally expressed by both cell types and localized subcellularly in peripheral cell membranes, whereas EphA5 was expressed predominantly in NMC-S and localized to filopodia. Moreover, cAMP-inducible filopodia formation in these cells was abrogated using EphA5 short interfering RNA. Finally, we found that the Rho family GTPase cdc42 was activated in a manner dependent on EphA5. Wortmannin, a specific inhibitor of phosphotidylinositol 3-kinase (PI3K), abolished the activation of cdc42 dependent on EphA5, suggesting the involvement of PI3K in the EphA5-cdc42 pathway. Together, our findings suggest a cAMP-EphA5-cdc42-dependent regulation of small cholangiocyte migration, which are anticipated to facilitate the understanding of the nature of cholangiocytes and to explain certain general aspects of cAMP-cdc42 activation signaling with regard to cell morphogenesis.

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appears to contribute to the retraction of lamellipodial processes (28). Interestingly, overexpression of EphA2 caused malignant transformation associated with the formation of filopodia (31) as well as contributing to the invasiveness of mammalian epithelial cells (8). These findings suggest dual potential roles of Eph members in cell morphogenesis, namely, both the retraction and formation of cellular processes. The Eph receptor family constitutes the largest class of membrane-bound receptor tyrosine kinases, with 14 members identified to date (21). Eph receptors are subdivided into two groups, EphA and EphB receptors, which bind glycosylphosphatidylinositol ephrin-A ligands and transmembrane ephrin-B ligands, respectively, based on their structural properties and ligand-binding preferences (16). The role of cAMP in cholangiocytes has been intensively studied regarding cellular proliferation, vectorial water movement, and ion transport via cholangiocytes (2, 12, 29). Here, we also describe a role for cAMP, EphA, and cdc42 activation specifically in NMC-S cells, consistent with an important role for migration in small cholangiocytes intrahepatic repopulation. These data are an important step toward a better understanding of how bile ducts are formed and maintained in normal and diseased livers.

**EXPERIMENTAL PROCEDURES**

**Materials.** EphA5 rabbit anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA), EphA8 goat anti-mouse antibody (Sigma, St. Louis, MO), rabbit anti-c-Myc epitope tag (Affinity BioReagents, Golden, CO), rabbit polyclonal antibody against bovine keratin (DAKO, Glostrup, Denmark), Alexa 546 goat anti-rabbit whole IgG (Molecular Probes, Eugene, OR), Alexa 488 donkey anti-goat whole IgG (Molecular Probes), TOPRO-3, a membrane-impermeable fluorescent marker. Images were captured by confocal microscopy (Nikon) with EZ-C1 software after the PBS washing and embedding steps. For immunohistochemistry, the fresh frozen section was stained with a combination of anti-EphA5 antibody and the corresponding biotin-labeled secondary antibody and subsequently visualized by avidin-horseradish peroxidase (HRP) complex and diaminobenzidine. As the experimental control, PBS was substituted for the primary antibody. Each primary antibody was replaced by PBS for the control. To stain the proliferating biliary epithelium of BALB/c mice fed ANIT, we employed a specific antibody against cholangiocyte keratin (15) as noted in Materials.

**Detection of mRNA.** mRNA was extracted from NHC-S and NHC-L cells with a RNaseasy mini kit (Qiagen) and was subjected to the following genomic DNA digestion step. RT-PCR was performed to detect mRNAs of EphA5 and EphA8 with a random hexamer primer and the following specific primer pairs: 5'-GCTGGACGAAAGATGCAGAAC-3' and 5'-CCGTTTACCATCTGACCTTT-3' was used for amplifying mouse EphA5 and 5'-TCACTCTCATGGGTTCACCGAGG-3' and 5'-GCCCGAGGTCTAGAGAGAACACG-3' was used for EphA8. For the control PCR, the primer pairs for 18S rRNA were 5'-TCAAGAACGAAAGTCGGAGG-3' and 5'-GCCCGAGGTCTAGAGAGAACACG-3'. The specificity of RT-PCR with all of the primer pairs was evaluated by direct sequencing with a Big Dye terminator and an ABI 3100 sequencer (Applied Biosystems, Foster, CA).

**Transfection of siRNA and plasmid vectors.** siRNAs were designed for targeting the EphA5-specific sequence 5'-AAGAGAUUAU-AUAAUCAAGUAC-3'. Random RNA duplex was used as the control for specific RNA interference. Either siRNA or the random RNA duplex at a concentration of 100 nM was transfected into NMC-S cells at 80% confluence according to the manufacturer's protocol. After 2 days of incubation, the medium was changed, and the amount of EphA5 expression was evaluated by densitometric analysis of immunoblots after an additional 5 days as well as by the average of total fluorescence intensity of the whole pixels in three fields under confocal microscopy with EZ-C1 software. For transient transfection of the constitutively active form of cdc42 or the dominant negative form of cdc42 in NMC-S cells, vectors were introduced into NMC-S cells cultured at 80% confluence by lipofectin. Expressed cdc42 with the c-Myc tag was visualized by fluorescence. For the evaluation of transfection efficiency, the c-Myc tag was visualized by the combination of the specific antibody and fluorescence-labeled secondary antibody. The fluorescence was evaluated by fluorescent microscopy in three independent view fields with a magnification of ×100. The value of the average ratio of number of cells with fluorescence against the total number of cells was calculated individually for the c-Myc vector-transfected cells and mock-transfected cells. The subtracted value of the ratio was defined as the transfection efficiency.

**Immunoblot and activated cdc42 pulldown assay.** For immunoblot analysis, whole cell lysates were subjected to electrophoresis and subsequently transferred to polyvinylidene difluoride membranes. For the activated cdc42 assay, activated cdc42 in cell lysates was pulled down with glutathione S-transferase-p21-activated kinase 1 (Pak 1) containing the p21 binding domain and subjected to immunoblot

**Cell culture and morphological analysis.** NMC-S and NMC-L cells were cultured in MEM with 10% FBS and antibiotics. For photorecording, NMC-S and NMC-L cells were cultured at 30% confluence in 96-well clear-bottom glass dishes and then digitally recorded at the 1-, 2-, 5-, 15-, 30-, 40-, and 50-min time points during the incubation at 37°C on the heating block of a phase contrast microscope. The numbers of filopodia in the photographs were counted. The numbers of filopodia newly formed or extended were denoted as the number of formations and the numbers of filopodia retracted or diminished were denoted as the number of repulsions, as evaluated using the sequential digital images. The numbers of formations and repulsions in three random microscopic fields at a magnification of ×200 at the 15-min time point were statistically analyzed with Mann-Whitney U-test.

**Immunohistochemistry of EphA5, EphA8, keratin, and c-Myc tag.** For immunofluorescence, NMC-S and NMC-L cells were immediately fixed with pure methanol and dried for membrane permeabilization. Anti-EphA5, EphA8, and c-Myc antibodies were used as primary antibodies at a concentration equivalent to a 100-fold dilution. After cells were washed three times with PBS for 5 min, fluorescence-labeled secondary antibodies, corresponding to each primary antibody, were added. The nuclear staining was performed with TO-PRO-3, a membrane-impermeable fluorescent marker. Images were captured by confocal microscopy (Nikon) with EZ-C1 software after the PBS washing and embedding steps. For immunohistochemistry, the fresh frozen section was stained with a combination of anti-EphA5 antibody and the corresponding biotin-labeled secondary antibody and subsequently visualized by avidin-horseradish peroxidase (HRP) complex and diaminobenzidine. As the experimental control, PBS was substituted for the primary antibody. Each primary antibody was replaced by PBS for the control. To stain the proliferating biliary epithelium of BALB/c mice fed ANIT, we employed a specific antibody against cholangiocyte keratin (15) as noted in Materials.
procedures. β-Actin in the whole cell lysate before the pulldown assay was also evaluated as the input protein control. Samples transferred to the membranes were blotted with the corresponding specific primary antibody and detected by the combination of HRP-labeled secondary antibodies and chemiluminescence. Densitometry was performed with the Las-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

RESULTS

Distinct expressions and subcellular localizations of Eph members in immortalized cell lines NMC-S and NMC-L. Our preliminary studies have revealed distinct cAMP-dependent morphological changes in our immortalized NMC-S and

Fig. 1. a: Expressions of ephrin (Eph)A5 and EphA8 were detected by RT-PCR. Left, normal mouse cholangiocyte (NMC)-small (S) cells and NMC-large (L) cells with and without (−) the reverse transcription step as a control as well as 18S rRNA (Cont). Right, immunoblots (Cont, β-actin).

b–h: Confocal microscopic images of EphA5 (red) and EphA8 (green) in NMC-S and NMC-L cells. Characteristic subcellular distributions of EphA5 to filopodia or meshwork structures in NMC-S cells are indicated by arrows. The lowest magnification is shown b, a higher magnification is shown in c, and the highest magnification is shown in e and f in NMC-S cells. Much less expression of EphA5 is seen in NMC-L cells (d). EphA8 was shown to be localized to lamellipodia or sheetlike structures at the cell periphery in both NMC-S (g) and NMC-L cells (h). Images without the primary antibodies are shown as experimental controls in g and h, insets. The nucleolar blue fluorescence was derived from TOPRO-3 staining. i and j: Proliferating biliary epithelial cells from livers of BALB/c mice fed α-naphthylisothiocyanate (ANIT) with keratin (1 h, red) were labeled with EphA5 (i).
NMC-L cell lines. dcAMP induced filopodia formation predominantly to NMC-S cells, resulting in a stellate cell shape (e.g., see Fig. 5, a and b, as representative images). To clarify the mechanism underlying these phenomena, we reviewed our previous microarray data with regard to the differences in expressed molecules between these cell lines. We noted the expression of Eph receptor tyrosine kinases EphA5 and EphA8 in cholangiocytes. These molecules are candidates for an involvement in morphogenetic changes due to the differences in their expression levels between these cell lines and their potential for modulating cellular morphogenesis. Confirmation of the expression of these genes was performed using nonquantitative RT-PCR in NMC-S and NMC-L cells (Fig. 1, left). Immunoblot analysis (Fig. 1a, right) and confocal microscopic evaluation (Fig. 1, b–d) revealed much greater EphA5 expression in NMC-S cells than in NMC-L cells. EphA5 localized to the peripheral membrane and especially in filopodia (the red spindle structures indicated by the arrows in Fig. 1, b, c, e, and Fig. 2. The repulsion of filopodia, the cellular processes, by the Eph ligand ephrin-A5 proved the functional expression of Eph receptors in NMC-S cells (shown by arrows). A and B: phase contrast images; C: quantified repulsion ratio. The repulsion ratio is the number of repulsed filopodia to the initial number of total filopodia. Before this experiment, ephrin-A5 was preclustered with anti-IgG Fc antibody (28). Anti-IgG Fc antibody alone was used as a control.

A

B

C

Fig. 2. The repulsion of filopodia, the cellular processes, by the Eph ligand ephrin-A5 proved the functional expression of Eph receptors in NMC-S cells (shown by arrows). A and B: phase contrast images; C: quantified repulsion ratio. The repulsion ratio is the number of repulsed filopodia to the initial number of total filopodia. Before this experiment, ephrin-A5 was preclustered with anti-IgG Fc antibody (28). Anti-IgG Fc antibody alone was used as a control.

A

B

C

Fig. 3. cAMP increased filopodia by a mechanism involving both the induction of filopodia formation and the reduction of filopodia repulsion exclusively in NMC-S cells, i.e., not in NMC-L cells (A). The difference between NMC-S and NMC-L cells was evident in the formation-to-reduction ratio (F/R; B). Confocal microscopic evaluation (vertical arrows) and the repulsion of filopodia (horizontal arrows). S, NMC-S cells; L, NMC-L cells, C, cells treated with N6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate sodium (dcAMP); N, cells treated with PBS instead of dcAMP as controls. NS, not significant.
Intracellular EphA5 formed the meshwork structure, suggesting a colocalization with cytosolic filamentous structures in these cells. EphA8 was detected at an equivalent level in these cell lines, lining the peripheral membranes, mainly of lamellipodia, in both cell types (Fig. 1, g and h). Morphologically, the NMC-S cell line was characterized by a large number of filopodia on the cell surface in accordance with the abundant EphA5 expression. To confirm that EphA5 is associated with small cholangiocytes in vivo, we immunostained intrahepatic biliary ducts that had been induced to proliferate using the ANIT-based model previously described (1). As shown in Fig. 1i, biliary epithelial cells specifically stained for keratin prominently proliferated in the ANIT-fed BALB/c liver. Moreover, strong EphA5 expression was localized specifically to cell clusters within these small proliferating, small tubular structures, which are comprised primarily of small cholangiocytes, to interlobular bile ducts in the portal area in vivo (Fig. 1j). To test the physiological role of EphA5 expression, we treated NMC-S cells with ephrin-A5, a cognate ligand of the EphA family, and performed sequential photorecording. Figure 2A
shows that ephrin-A5 treatment induced rapid filopodia repulsion and also shrank target cells, leading to the rounded appearance of NMC-S cells (Fig. 2, B and C). The repulsive effect on filopodia of the ligand was obvious during the first 40-min period, especially 15 min after the initial stimulation. These data suggest a functional expression of Eph family members in these cells and a potential repulsive role for these molecules in vivo.

dcAMP induced filopodia formation in NMC-S cells but not in NMC-L cells via an EphA5 pathway. Proliferation of cholangiocytes has been proved to be relevant to intracellular cAMP. To test whether membrane-permeable cAMP has the ability for formation of filopodia, which is closely related to cell proliferation and migration, we analyzed the filopodia formation and filopodia repulsion under the effect of cAMP. In contrast to ephrin, which led primarily to filopodia repulsion (as shown in Fig. 2), dcAMP induced filopodia formation and reduced filopodia repulsion, leading to a significant change in the filopodia formation-to-repulsion ratio of NMC-S cells. No significant change was observed in NMC-L cells in regard to cell morphogenesis before vs. after the addition of dcAMP (Fig. 3), suggesting that the cAMP-mediated effect on NMC-S cells may specifically involve the mechanism based on the specific attribute of NMC-S cells. EphA5-mediated processes could be hypothetically candidates in terms of the pathway.

**cAMP effects on NMC-S cells is EphA5 dependent.** To test the role of EphA5 in mediating cAMP-induced changes in cell morphology, we synthesized siRNA oligonucleotides specifically against EphA5. Figure 4A shows that siRNA treatment knocked down EphA5 mRNA and protein levels by ~60% at 7 days after the transfection into NMC-S cells. We used these cells to measure cAMP-inducible filopodia formation and found that filopodia formation was reduced in accordance with the inhibition of EphA5 expression (Fig. 4B).

**Cdc42 activation by cAMP via EphA5 and PI3K pathways in NMC-S cells.** NMC-S cells are nonuniform in shape, exhibiting a range of filopodial numbers and degrees of extension among cells, as shown in Fig. 5, a, b, e, and f. Moreover, this range in morphological corresponds with varying levels of EphA5 expression. Because cdc42 is frequently associated with filopodia formation, we determined whether NMC-S cell morphology changes in response to cdc42 activity. To test this, we transfected NMC-S cells with a dominant negative cdc42 molecule under the experimental conditions at a rate of 21 ± 2.5% as the transfection efficiency and found that our cell population was now uniformly rounded with little to no filopodia (Fig. 5c). Moreover, cells transfected with a constitutively active cdc42 molecule exhibited extensive filopodia formation (Fig. 5d).

To test whether cdc42 acted downstream of the cAMP-EphA5-induced pathway, we quantified cdc42 activation in cells treated or untreated with cAMP and with or without EphA5 siRNA treatment. These data demonstrated that cAMP significantly stimulates cdc42 activity and that this stimulation was significantly reduced in EphA5 siRNA-treated cells (Fig. 6A). The involvement of EphA5 in cAMP-cdc42 activation appeared to be consistent with the subcellular localization of EphA5. This pathway was considered to be PI3K dependent by employing a specific inhibitor, wortmannin, which reduced the activity of cdc42 dependent on EphA5 expression. However, nocodazole, a microtubule-disrupting agent, reduced the whole activity of cdc42 independent of EphA5 expression (Fig. 6, B and C). Interestingly, the portion of cdc42 activation not occurring through EphA5 appeared to be dependent on microtubules.

**DISCUSSION**

Biliary trees are vulnerable under pathological conditions. Biliary epithelial cell damage is typically observed in primary biliary cirrhosis (PBC) as florid bile duct lesions in the early stage of the disease. With disease progression, proliferating bile ductules are frequently observed in the portal area. This type of biliary epithelial cell proliferation appears to be unregulated and to have relatively little compensatory ability in injured bile ducts. In the end-stage disease, bile ducts apparently disappear due to the failure of biliary tree regeneration. The cholestasis resulting from biliary duct damage directly induces apoptosis of hepatocytes and hepatic failure in the late stage of PBC. Therefore, functional regeneration of biliary ducts can be regarded as an alternative therapeutic strategy in addition to the substantial curative regime for this disease, which remains to be established. In this study, we hypothesized that there is a cAMP-inducible morphological reaction relevant to cell migration in cholangiocytes. Thus, the possibility of cAMP or modulators of cAMP serving as therapeutic agents remains. Because of the divergent reactions to extracellular...
stimuli (e.g., secretin) with regard to cAMP production between NMC-S and NMC-L cells, we used dcAMP, a membrane-permeable cAMP analog (6), for direct stimulation of cholangiocytes. Even in the in vitro condition, these cells maintained the basal level of intracellular cAMP (30). This cAMP analog caused characteristic morphological changes in cholangiocytes, namely, prominent filopodia formation was seen in small cholangiocytes. Furthermore, abundant expression of G protein-coupled receptor, a member of the G protein-coupled receptor family, was recognized in NMC-S cells (data not shown) and considered to be a candidate molecule for intracellular cAMP production in the physiological state that is presumed to activate internal adenylate cyclase directly (10). This filopodia formation occurs via filamentous actin, which is considered to form as a consequence of the activations of cdc42 and N-WASP (23). The activation of cAMP/PKA signaling has been shown to promote the activation of cdc42 in mast cells (11) and neurons (18). However, in contrast to Rho, which is the target for PKA, the exact mechanism of cdc42 activation by cAMP is not totally clear (14). The involvement of PI3K in the cAMP-cdc42 pathway in neuronal cells has been reported (18). As a result of our investigation of the mechanism underlying this phenomenon, particularly in NMC-S cells, the cAMP-inducible activation of cdc42 was found to be dependent on both EphA5 and PI3K. Moreover, the subcellular localization of activated cdc42 to filopodia, as recently documented (24), corresponds to the characteristic subcellular distribution of EphA5 in cholangiocytes. Furthermore, the morphological resemblance between cells with activated cdc42 and high EphA5 expression in the present study supports this result. It is noteworthy that the large cholangiocytes expressed much less EphA5, consistent with their lower morphological reactivity to cAMP compared with NMC-S cells. The protein sequence identity between EphA5 and EphA8 was 55%, showing similarity among Eph members. The structures shared by these two submembers include Eph ligand binding, fibronectin binding, tyrosine kinase, and the sterile a-motif domain. These structural similarities indicate a similar response to cognate ligands. Our present results show the specific properties of EphA5 with regard to filopodia formation in response to cAMP, rather than to ligand stimulation, based on the characteristic pattern of subcellular localization. We have not yet ascertained the actual function of EphA8 in our cholangiocytes. The difference in subcellular localization of these molecules might be related to structural differences (e.g., signaling peptides) or to functional differences between these molecules.

The difficulty in this research field might be attributable to the diversity of cell types that have been used in previous experiments. We examined the pathway using our own cholangiocyte lines, and we certainly acknowledge that questions remain to be addressed. For instance, how does cAMP activate PI3K via EphA5 or vice versa? To address this question, we postulated that there is a microtubule-dependent membrane trafficking mechanism for activation of these molecules. PI3K constitutes a lipid kinase family characterized by the ability to phosphorylate the inositol ring 3'-OH group in inositol phospholipids to make phosphatidylinositol (3,4,5)-trisphosphate from phosphatidylinositol bisphosphate beneath the cell membrane. Class I PI3K, the target of wortmannin, is further divided into subclass 1A, which is activated by receptors with protein tyrosine kinase activity, and subclass 1B, which is activated by receptors coupled with G proteins (13). Thus, the activation of receptor tyrosine kinase could initiate the PI3K cascade. For example, ErbB2 is a representative receptor tyrosine kinase, the overexpression of which causes constitutive activation of Akt via PI3K (13). As for Eph, regarding the interaction between Eph members and PI3K, PI3K has been shown to suppress the ability of EphA8 to bind fibronectin via binding to the juxtamembrane segment of EphA8, which recruits p110-γ of PI3K. Because Eph is a membrane bound type of receptor tyrosine kinase, kinases that interact with and phosphorylate each other, it is reasonable that EphA5 relocated to the cell surface membrane would be subject to phosphorylation by kin receptors and subsequently bound by PI3K with its SH2 domain. Although cAMP appeared to recruit EphA5 to the cell membrane in our experiment, nocodazole, a microtubule-disrupting agent, reduced overall activated cdc42 but did not alter the fraction of activated cdc42 dependent on EphA5. These strikingly contradictory results complicate the process of unraveling the details of this mechanism.

Several types of cellular spreading processes have been proposed for in vitro conditions. Our NMC-S cells showed a tendency to spread with filopodia, whereas NMC-L cells spread mainly in a sheetlike formation probably with lamellipodia, a round-type cellular protrusion. Although the signals of in vitro cells, namely, immortalized cells, may be altered or may not represent the natural physiological state of in vivo cell groups, the features distinguishing these cells could be considered to reflect their natural states. Taken together with our results, earlier observations support the notion that NMC-S cells may use intracellular EphA5 as a path-finding molecule, leading to cellular migration in response to cAMP elevation, or to termination of the migrating process when the cell comes across ephrins expressed by other cells in vivo. We defined the formation of filopodia as the extension or formation of filopodia, which has been previously utilized just for the expression of the de novo formation of filopodia (3). This discrepancy was noted by a reviewer and should be taken account for the interpretation of the results. Further studies regarding the regulation of these Eph family proteins would contribute to understanding the mechanism of cholangiocyte regeneration, which is known to be limited under pathological conditions, as clinically represented by ductopenia.

GRANTS

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