p38 MAPK Is Involved in Activin A- and Hepatocyte Growth Factor-mediated Expression of Pro-endocrine Gene Neurogenin 3 in AR42J-B13 Cells*

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Neurogenin3 (ngn3) is a transcription factor that is essential for the differentiation of pancreatic endocrine cells. To investigate the signaling pathway that regulates ngn3 expression, we used AR42J-B13 cells as a model of the differentiation of pancreatic islets. In these cells, treatment with activin A and hepatocyte growth factor (HGF) induced the expression of ngn3. Reporter gene analysis using human ngn3 gene (NEUROG3) promoter fragments of various lengths identified the region between −402 and −327 bp of NEUROG3 as an activin A- and HGF-responsive DNA sequence. This DNA sequence normally functions as a repressor in AR42J-B13 cells, but treatment with activin A and HGF negates the repressor activity. Interestingly, function of the activin A- and HGF-responsive sequence was not influenced by the overexpression of the Smad inhibitory factor, Smad7. Instead, activin A and HGF activation was inhibited by overexpression of a dominant-negative mutant of transforming growth factor-β-activated kinase 1 (TAK1), or mitogen-activated protein kinase kinase 3 (MKK3), or by treatment with a p38 MAPK-specific inhibitor, SB203580. Activin A and HGF function through the TAK1-MKK3-p38 MAPK pathway to relieve transcription repressors located between −402 and −326 bp on the NEUROG3 promoter, and consequently activate ngn3 expression and endocrine differentiation of AR42J-B13 cells.

During development, formation of the pancreas and its subsequent differentiation into various types of exocrine and endocrine cells are controlled by the activation and extinction of a large number of genes. The expression of these genes is regulated by a well-organized cascade of transcription factors (1, 2), and the basic helix-loop-helix factor neurogenin 3 (ngn3)1 is one of the essential transcription factors expressed early in the differentiation of islet cells (3–5).

In mice, the pancreas forms from evaginated buds generated by fusion of the dorsal and ventral gut endoderm at the foregut/midgut junction on embryonic days 9.5–10.5. At this stage, most of the cells located in the buds have the potential to differentiate into any of the mature pancreatic cell types (6). The signal pathway from Notch and notch ligand delta-like 1 participates in determination of the fate of cells in the exocrine pancreas (4). It is believed that cells eluding these signals start to express ngn3 and become endocrine cells. However, expression of ngn3 is not observed after the cells begin to express pancreatic hormones like glucagon or insulin (5, 6). Thus, expression of ngn3 is restricted to immature endocrine cells and is considered to be a marker of endocrine progenitor cells during development of the pancreas.

Interestingly, ngn3 is not just a marker of endocrine progenitor cells. When targeted disruption of ngn3 was performed in mice, the animals failed to generate pancreatic endocrine cells and did not express most of the transcription factors that are essential for endocrine differentiation (7). Conversely, the overexpression of ngn3 in pancreatic bud cells promoted differentiation into endocrine cells (4, 5). These findings indicate that ngn3 is an essential and sufficient factor for the differentiation of endocrine cells. Thus, investigation of the mechanisms regulating the expression of ngn3 is important to better understand the process of endocrine cell differentiation and probably also to understand the process of β cell regeneration.

In addition to the Notch-delta signaling pathway, several studies have suggested the importance of activins, which are members of the transforming growth factor-β (TGF-β) family, in signaling for development of the pancreas (8–10). Activins have diverse actions in a variety of mammalian tissues, including the pancreas, at many stages during development. It was recently reported that activin receptor type II (ActRII) A′′/B−/− mice showed a severe reduction of insulin and glucagon expression, but only a slight reduction of carboxypepti-

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3 The abbreviations used are: ngn3, neurogenin 3; TGF-β, transforming growth factor-β; TAK1, TGF-β-activated kinase 1; ERK, extracellular signaling-regulated kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; JNK, c-Jun N-terminal kinase; ActRI, activin receptor type I; ActRII, activin receptor type II; STAT, signal transducer and activator of transcription; RE1, repressor element 1; NBSE, neuron restrictive silencer element; PAI-1, plasminogen activator inhibitor-1; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco’s modified Eagle’s medium; PDVF, polyvinylidene difluoride.
dase A (a marker of exocrine pancreas), as early as embryonic day 13.5 (8). These results suggest that activin signals might be involved in determining the fate of cells in the pancreatic primordium, but the signaling pathways downstream of the activin receptor that are involved in the differentiation of endocrine cells have not been investigated.

AR42J cells were originally derived from a chemically induced pancreatic tumor and possess both exocrine and neuroendocrine properties. Upon treatment with activin A, AR42J cells stop growing and start to extend neurites. In the presence of betacellulin or hepatocyte growth factor (HGF) plus activin A, the cells differentiate into insulin-expressing cells (11). Thus, AR42J cells provide a model system for studying the molecular mechanisms involved in cell differentiation. Probably reflecting the physiological differentiation process, activin A and HGF can also provoke the expression of ngn3 (12).

In the present study, we investigated the mechanism by which activin A and HGF induce the expression of ngn3 using AR42J-B13 cells as a model for the differentiation of pancreatic islets. Our data revealed that the TGF-β-activated kinase 1 (TAK1)-mitogen-activated protein kinase kinase 3 (MKK3)-p38 mitogen-activated protein kinase (p38 MAPK) pathway regulates the function of a critical region on the human ngn3 gene (NEUROG3) promoter and changes the binding of various transcription factors to the region.

**EXPERIMENTAL PROCEDURES**

**Reporter Gene Constructs and Expression Plasmids**—To generate reporter plasmids, fragments of the 5′ region of NEUROG3 obtained by restriction digestion or PCR using the pFOX Luc1 NEUROG3–5.7kb plasmid (13) as a template were ligated upstream of the luciferase gene.
in pFOX Luc1 plasmids or upstream of the thymidine kinase (TK) minimal promoter in pFOX LucTK plasmids (14). Mutagenesis of the reporter gene constructs was performed using a Quick Change site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). All constructs were confirmed by sequencing. An expression plasmid for dominant-negative MKK6 (S169A-MKK6AA) was kindly provided by Dr. E. Nishida (Kyoto University, Kyoto, Japan). Expression plasmids for dominant-negative MKK3 (pCS2+DN MKK3) and dominant-negative MKK4 (pCS2+DN MKK4) were kindly provided by Dr. K. Sugiyama (Nippon Boehringer Ingelheim Co., Kawasaki, Japan) and Dr. M. Hibi (Center for Developmental Biology, RIKEN, Kobe, Japan). Expression plasmids for dominant-negative TAK1 (pCMV-HA-TAK1) and dominant-negative MKK3 (pCS2+HA-TAK1) and dominant-negative MKK4 (pCS2+HA-TAK1(K63W)) were kindly provided by Dr. K. Matsumoto (Nagoya University, Nagoya, Japan).

**Cell Culture and Transfection**—AR42J-B13 cells were cultured in low glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 20 mM HEPES, and 5 mM NaHCO3. To cause cell death, wild-type or mutant pFOX Luc1 ngn3 →461 bp plasmids were transfected into AR42J-B13 cells, with (solid bars) or without exposure to activin A and HGF (open bars). Relative luciferase activity was calculated by taking as 1 the activity of cells transfected with the pFOX Luc1 plasmid without exposure to activin A and HGF. All data are shown as the mean ± S.E.

**Results**

Mapping the Activin A- and HGF-responsive DNA Sequence in the NEUROG3 Promoter.—To analyze the activin A-responsive cis-element of the NEUROG3 promoter, we constructed a series of progressive 5’ deletions of the promoter (each extending +261 bp on the 3’-end) linked to the firefly luciferase gene and transfected these constructs into AR42J-B13 cells. It has been shown already that activin A plus HGF can induce the expression of ngn3 in this cell line (12). Although HGF alone, but not activin A, induced a modest increase of NEUROG3 promoter activity, the addition of activin A plus HGF induces a marked increase of its activity (Fig. 1A). Similar activation was observed with the reporter gene constructs containing −2.6 kb, −461 bp, and −402 bp NEUROG3 promoter fragments (Fig. 1B). However, deletion of the fragment between −402 and −327 bp caused an increase in basal NEUROG3 promoter activity and a loss of responsiveness to activin A and HGF. These findings demonstrated that the region between −402 and −327 bp contained the activin A-responsive cis-element.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts from AR42J-B13 cells seeded in 10-cm dishes were prepared according to the procedure described by Sadowski and Gilman (16). Single-stranded oligonucleotides corresponding to the sequences in the NEUROG3 promoter were 5’-end-labeled with 32P-ATP using T4 polynucleotide kinase. The labeled oligonucleotide was purified on a column and annealed to an excess of the complementary strand. The EMSA buffers and electrophoresis conditions have been described previously (17). One microliter of in vitro reaction mixture or 1 μg of nuclear extract was used for each 10-μl binding reaction. The mixture was incubated for 15 min at room temperature before polyacrylamide gel electrophoresis, and radioactive bands were visualized using Fuji BAS2500 (Fujifilm, Tokyo, Japan). The oligonucleotides used in these experiments are as follows. Region 1, 5’-CAA GTT CCC CTC CAG GAC AGA TGC TAA GGA AAG-3’; region 2, 5’-CTA ATT TTC CCC ATG TGT AAT AAC GTG CAG GCA TTG-3’. Western Blot Analysis—AR42J-B13 cells seeded in 10-cm dishes were starved in serum-free medium with or without SB203580 (Calbiochem, San Diego, CA) for 1 h, followed by incubation with or without activin A and HGF stimulation for different periods of time. Then 30 μg of total cell extract prepared from these cells was separated on SDS-polyacrylamide gel, transferred to a PVDF membrane (Millipore, Tokyo, Japan), and incubated overnight at 4 °C with the specific antibody (Erk1/2, p38 MAPK, phospho-p38 MAPK, phospho-MKK3/6, JNK, and phospho-JNK (Cell Signaling, Tokyo, Japan)). Bound primary antibodies were detected with peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies, and immunoreactivity was visualized by chemiluminescence (Western Lightning, PerkinElmer Life Sciences, Tokyo, Japan) according to the manufacturer’s instructions.

**Fig. 3.** Mutations of the sequence within the activin A- and HGF-responsive site and effects on the transcriptional activity of the NEUROG3 promoter. A, the sequence of the activin A- and HGF-responsive region in the NEUROG3 promoter is shown. Potential binding sites for transcription factors are underlined. The locations of mutations introduced into the promoter are shown as large letters. C-to-T or G-to-A conversions were introduced into the potential cis-elements. B, wild-type or mutant pFOX Luc1 ngn3 →461 bp plasmids were transfected into AR42J-B13 cells, with (solid bars) or without exposure to activin A and HGF (open bars). Relative luciferase activity was calculated by taking as 1 the activity of cells transfected with the pFOX Luc1 plasmid without exposure to activin A and HGF. All data are shown as the mean ± S.E.
and −327 bp functions as a repressor and that activin A and HGF can negate its repressor effect.

To investigate whether the DNA sequence between −402 and −327 bp was sufficient to function as a repressor and an activin A- and HGF-responsive element, we investigated the promoter activity of reporter constructs containing the DNA fragment upstream of a heterologous promoter, the TK minimal promoter (Fig. 2). In every cell line that we tested, this fragment acted as a repressor, but activin A- and HGF-responsive transactivation was not observed. These data suggest that this fragment alone was sufficient to function as a repressor, but the basal promoter sequence of \( \text{NEUROG3} \) seems to be essential for activity as an activin A- and HGF-responsive cis-element.

Several Transcription Factors Are Possibly Involved in the Activin A- and HGF-induced Transactivation of \( \text{ngn3} \)—The activin A- and HGF-responsive region that we mapped had the following elements: one E-box, one repressor element 1/neuron-restrictive silencer element (RE1/NRSE)-like sequence (18), and one AT-rich sequence (Fig. 3A). To identify the cis-elements required for activin A- and HGF-mediated transactivation of \( \text{ngn3} \), we engineered mutations within the possible cis-elements located in the activin A- and HGF-responsive region (−402 to 327 bp), to investigate the function of each DNA sequence as a cis-element. When mutations were introduced into either the E-box or the RE1/NRSE-like sequence, basal \( \text{NEUROG3} \) promoter activity was enhanced with reducing activin A and HGF responsiveness. On the other hand, mutations of the AT-rich sequence only had a modest effect on basal \( \text{NEUROG3} \) promoter activity or on activin A- and HGF-responsive promoter activity (Fig. 3B).

Next, to try to identify the factors mediating activin A- and HGF-responsive transactivation of \( \text{ngn3} \), we investigated the nuclear protein that binds to each mutation-sensitive cis-element before and various times after treatment with activin A and HGF using EMSA (Fig. 4). EMSA was able to identify factors that bound to each site and gradually decreased in binding affinity after treatment with activin A and HGF (C1 and C3 in Fig. 4) as well as factors (C2 and C4 in Fig. 4) that bound to each cis-element and gradually increased in affinity after treatment. Antiserum directed against RE1-silencing transcription factor/neuron-restrictive silencer factor antibody,
basic helix-loop-helix protein E47, and c-Myc antibody did not recognize any of these protein-DNA complexes (data not shown). These results suggested that several factors with positive or negative effect on NEUROG3 promoter activity might be involved in the activin A- and HGF-induced transactivation of ngn3.

Smad-dependent Signaling Is Not Required for Activin A- and HGF-induced Transactivation of ngn3—Activin A is a member of the TGF-β superfamily, and signaling for this family is mediated by a heteromeric complex of two types of transmembrane serine/threonine kinase receptors. Binding of a ligand to the receptor complex leads to the phosphorylation of type II receptor kinase and thereby activates type I receptor kinase (19). The activated type I receptor then phosphorylates receptor-activated Smads (R-Smads), such as Smad2 or Smad3, which bind to the related factor Smad4 and move into the nucleus. Once inside the nucleus, this Smad complex associates with other transcription factors to activate the transcription of target gene (19).

To investigate the involvement of a Smad-dependent pathway in activin A- and HGF-induced transactivation of ngn3, we transfected NEUROG3 promoter reporter plasmids along with a plasmid expressing the inhibitory-Smad (Smad7) (20) (Fig. 5). In AR42J-B13 cells, as in other TGF-β-responsive cells, treatment with activin A and HGF activated a reporter plasmid containing the Smad-binding site of the PAI-1 promoter (20), and this activation was inhibited by Smad7. However, we could not find any influence of Smad7 on activin A- and HGF-induced stimulation of NEUROG3 promoter activity. These findings demonstrated that Smad signaling is not required for the induction of ngn3 by activin A and HGF.

P38 MAPK Is Required for Activin A- and HGF-induced Transactivation of ngn3—In addition to Smad activation, members of the TGF-β family activate other signaling cascades, including MAPK pathways (19). TGF-β is known to activate the extracellular signaling-regulated kinase (Erk), c-Jun N-terminal kinase (JNK), and p38 MAPK pathways (19, 21). To investigate whether the MAPK pathway was involved in the signal transduction pathway for ngn3 induced by activin A and HGF, we tested for phosphorylation of Erk, JNK, and p38 MAPK after stimulation with activin A and HGF. As shown in Fig. 6, p38 MAPK and Erk were phosphorylated after treatment with...
activin A and HGF, although with different time courses. On the other hand, JNK was not phosphorylated.

Next, we focused on the involvement of p38 MAPK in this signal transduction pathway. SB203580 is a well known p38 mitogen-activated protein kinase (p38 MAPK)-specific inhibitor. As shown in Fig. 6, it inhibited the activation of p38 MAPK provoked by activin A and HGF, while showing no effect on the activity of Erk in this cell line. In this state, the typical morphological changes and promoter activity of *NEUROG3* provoked by activin A and HGF were disturbed (Fig. 7, A and B). Furthermore, whereas C2 and C4 showed no definite changes of binding affinity after the addition of SB203580 (data not shown), SB203580 inhibited the decrease of C1 and C3 binding affinity provoked by activin A and HGF (Fig. 7C). These results suggested that signaling through p38 MAPK regulate the binding affinity of at least two transcription factors and thus may play an important role in activin A- and HGF-induced differentiation of AR42J-B13 cells.

The involvement of p38 MAPK in the signal transduction pathway suggested that TAK1 (22, 23) might be involved in the TGF-β signaling pathway. TAK1 is a member of the MAPK kinase kinase family. TAK1 activates several members of the MAPK kinase family, including MKK6, MKK3, MKK7, and MKK4. Among them, MKK6 and MKK3 activate p38 MAPK. To investigate the possible involvement of the TAK1 pathway in the process of differentiation into pancreatic β-cells, we examined the effect of overexpression of TAK1 and its dominant-negative mutants. Interestingly, co-transfection of TAK1 modestly augmented the activin A- and HGF-responsive transactivation of the *NEUROG3* promoter and co-transfection with dominant-negative mutants of TAK1 caused a reduction in activin A- and HGF-responsiveness (Fig. 8A).

Next, we investigated the effect of dominant-negative mutants of MKK6, MKK3, and MKK4 on this pathway. In agreement with other findings, MKK3 and/or MKK6 were phosphorylated after addition of activin A and HGF to cultures of these cells. The time course of the phosphorylation of MKK3 or MKK6 resembled that of p38 MAPK, suggesting that MKK3 or MKK6 could directly activate p38 MAPK (Fig. 8B). Co-transfection with dominant-negative mutants of MKK3 reduced the activin A- and HGF-responsive transactivation of the *NEUROG3* promoter (Fig. 8C), whereas co-transfection with dominant-negative mutants of MKK6 and MKK4 had no effect. These results suggested that induction of ngn3 expression by activin A and HGF involves the TAK1- MKK3-p38 MAPK signal transduction pathway.

**DISCUSSION**

Previous studies have revealed that signals from the notochord are essential for the differentiation of endocrine cells in the early stage of pancreatic development. In fact, removal of the notochord disrupts endocrine cell differentiation in the pancreas. Interestingly, treatment with activin A can induce endocrine cell differentiation even without the notochord (24).
Thus, the signals triggered by activin A play an important role in endocrine cell differentiation. In this study, we investigated the signal transduction pathway of activin A- and HGF-induced expression of ngn3 using AR42J-B13 cells as a model of endocrine cell differentiation.

Our 5' deletion analysis of the NEUROG3 promoter identified the region between -402 and -326 bp of the NEUROG3 as an activin A- and HGF-responsive DNA sequence. This region seems to function as a repressor in AR42J-B13 cells, whereas activin A and HGF relieve this repression. When this region was isolated from the NEUROG3 basal promoter sequence and ligated upstream of a heterologous promoter, it still repressed the transcription but was no longer responsive to activin A and HGF. These results suggested that some transcriptional repressors might bind directly to the DNA sequence within this region, but the response to activin A and HGF required the basal promoter of NEUROG3. The proximal NEUROG3 promoter may contain activin A- and HGF-responsive sequences that are required for relief of the distal repression activity. Alternatively, activin A and HGF signals may influence co-activators or co-repressors that interact with factors binding to both the proximal and distal sequences.

For transmembrane signaling, activin requires two types of receptors containing the serine/threonine kinase domain, activin receptor type I (ActRI) and type II (ActRII) (25). Activin directly binds to ActRII, and this complex associates with ActRI, resulting in hyperphosphorylation of ActRI by the kinase activity of ActRII (19, 26). The activated serine/threonine kinase of ActRI phosphorylates Smad proteins, especially Smad2 or Smad3, which activate transcription of the target genes of activin. In AR42J-B13 cells, Smad2 is far more abundant than Smad3; and the signals from activin A can activate Smad2 (27).

In our study, treatment with activin A and HGF also activated a control DNA sequence containing a TGF-β-responsive element, showing that the activin-ActR-Smad pathway is intact in AR42-JB13 cells. However, Smad7 did not affect the transactivation of ngn3 by activin A and HGF. This demonstrated that Smad-dependent signals, although commonly considered the main signaling pathway for activin A, are not required for the transactivation of ngn3 by activin A and HGF.

Instead of Smad-dependent signaling, the TAK1-MKK3-p38 MAPK pathway was involved in this transactivation process. Several studies have recently revealed a role for p38 MAPK in TGF-β signaling (21, 28). Our findings show that the p38 MAPK pathway can play a role in activin A signaling as well. The existence of other, non-Smad pathways for TGF-β signaling is reminiscent of Wingless/Wnt molecules, which also can signal through more than one pathway (29). Recent studies indicate that known components of the canonical Wingless/Wnt signaling pathway can function independently of β-catenin to establish planer cell polarity through a JNK or RhoA signaling cascade (30–32). Like Wnt signals, diverse sets of signals might be needed to mediate the various cellular responses to activin A.

Our data clearly show the importance of p38 MAPK in the signal transduction pathway for activin A- and HGF-induced expression of ngn3. However, because transfection of the dominant-negative forms of TAK1, MKK3, and p38 MAPK specific inhibitor did not completely inhibit activation of the NEUROG3 promoter, other pathways that do not involve TAK1-
MKK3-p38 MAPK may also contribute to NEUROG3 promoter activation. Previous studies have shown that RAS GTPase, Rho-like GTPase (including Rho, Rac, and Cdc42), and protein phosphatase 2A are activated by TGF-β (33–36). In this study, we detected the activation of Erk soon after the addition of activin A and HGF to AR42J-B13 cells, although its functional role was not investigated. Thus, assessment of other signal transduction pathways affected by activin A and HGF will be needed to complete our understanding of this process.

HGF is a multifunctional cytokine that has been shown to have a variety of effects on different cells. The receptor for HGF is a heterodimeric tyrosine kinase encoded by the c-met proto-oncogene (37, 38). c-met signaling is mediated by autophosphorylation of its own receptor, which leads to marked up-regulation of its kinase activity. Previous studies have shown that MAPK, the signal transducer and activator of transcription 3 (STAT3), and phosphatidylinositol 3-kinase are involved in HGF signaling (39–41), but no previous data have suggested that HGF activates the TAK1-MKK3 signaling pathway. Muller et al. (42) found that HGF alone could activate p38 MAPK in MLP29 cells. We found that HGF by itself activates p38 MAPK in AR42J-B13 cells.2 These results suggest that activation of p38 MAPK by HGF might enhance the activation of the p38 MAPK pathway might play an important role in determining the fate of early pancreatic precursor cells. Considering the fact that HGF controls the initiation of islet cell formation, a detailed understanding of these signaling pathways may eventually contribute to a method for generating new islet cells from pancreatic precursor cells.

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