Lefty polypeptides, novel members of the transforming growth factor-β (TGF-β) superfamily, are involved in the formation of embryonic lateral patterning. Members of the TGF-β superfamily require processing for their activation, suggesting cleavage to be an essential step for lefty activation. Transfection of different cell lines with lefty resulted in expression of a 42-kDa protein, which was proteolytically processed to release two polypeptides of 34 and 28 kDa. Since members of the proprotein convertase (PC) family cleave different TGF-β factors and are involved in the establishment of embryonic laterality, we studied their role in lefty processing. Cotransfection analysis showed that PC5A processed the lefty precursor to the 34-kDa form in vivo, whereas furin, PACE4, PC5B, and PC7 had a limited activity. None of these PCs showed activity in the processing of the lefty polypeptide to the 28-kDa lefty form. The mutation of the consensus sequences for PC cleavage resulted in expression of a 42-kDa protein, whereas furin, PACE4, PC5B, and PC7 had a limited activity. None of these PCs showed activity in the processing of the lefty polypeptide to the 28-kDa lefty form. The mutation of the consensus sequences for PC cleavage sites to be identified. Mutations of the sequence RGKR to GGKG (amino acids 74–77) and of RHGR to GHGR (amino acids 132–135) prevented the proteolytic processing of the lefty precursor to the 34- and 28-kDa forms, respectively. To identify the biologically active form of lefty, we studied the effect of lefty treatment on pluripotent P19 cells. Lefty did not induce Smad2 or Smad5 phosphorylation, Smad2/Smad4 heterodimerization, or nuclear translocation of Smad2 or Smad4, but activated the MAPK pathway in a time- and dose-dependent fashion. Further analysis showed the 28-kDa (but not the 34-kDa) polypeptide to induce MAPK activity. Surprisingly, the 28-kDa lefty protein was also capable of inducing MAPK activity, indicating that the lefty precursor is biologically active. The data support a molecular model of processing as a mechanism for regulation of lefty signaling.

Vertebrate body organization during embryogenesis is an essential process based on the formation of three axes of asymmetry: anteroposterior, dorsoventral, and lateral (left/right). The coordination of development of these three axes determines the location, structure, and arrangement of different organs. From an embryonic and evolutionary perspective, the lateral (left/right) axis is the last one established during vertebrate development and requires the breakage of the bilateral symmetry. This symmetry-breaking process results in the proper positioning and formation of asymmetric organs like liver, lung, heart, intestine, spleen, and stomach. Moreover, the relative positioning of organs within the body cavity is conserved in all vertebrates, suggesting that the structure and arrangement of these organs are required for their normal function. For example, the asymmetric positioning of the heart allows for more efficient pumping of blood, and the asymmetric development of the digestive system (particularly complex in vertebrates) allows for a more efficient packing of the bowel within the peritoneal cavity. The mechanisms that operate in the lateral symmetry-breaking process have remained surprisingly difficult to elucidate. The current ciliary model of left/right axis determination in vertebrates is based on the so-called “nodal flow.” This flow is produced by a specialized cluster of monocilia present on the ventral surface of the mouse node, which is the mammalian equivalent of the embryonic organizer region identified in Xenopus (1–3). These monocilia, which project into the extraembryonic space, exhibit a type of vortical motion that generates a leftward flow of extraembryonic fluid in the node region. This, so-called nodal flow has been proposed to function as the initiating event in left/right axis formation by causing an initial left/right difference in the relative distribution of extracellular factors (4–6). Therefore, this leftward flow triggers activation of distinct signaling pathways on the left and right sides of the embryo.

Different members of the TGF-β superfamily play an essential role in the establishment of laterality (6–8). The earliest events are the asymmetric expression of nodal and lefty (9–12). Lefty is a novel subfamily of the TGF-β protein superfamily and is composed of lefty-1 and lefty-2 in mouse (11, 12) and their homologs, lefty A and lefty B, in human (13, 14). lefty-1 and lefty-2 both exhibit an asymmetric expression on the left side of...
gastrulating mouse embryos. However, the major expression domains of the two genes are different: lefty-1 expression is predominantly confined to the left side of the ventral neural tube, whereas lefty-2 is strongly expressed in the lateral plate mesoderm on the left side (11, 12). Asymmetric expression of lefty and nodal are also perturbed in mouse mutants with laterality defects (15–18). Furthermore, the knockout mutation of lefty-1 induces a variety of left/right positional defects in visceral organs. The most common feature of lefty-1-deficient mice is bilateral expression of nodal and lefty-2, which results in a thoracic left (rather than right) isomerism (19). These observations support that lefty proteins encode a signal for “leftness.”

Members of the TGF-β superfamily as well as many other proteins are synthesized as large inactive precursor proteins that must be proteolytically processed to release the bioactive polypeptide (20). One of the best studied examples is the proteolytic processing of the TGF-β1 precursor, which is an essential step in the formation of the biologically active TGF-β1 polypeptide. TGF-β1 is expressed as an inactive precursor of 55 kDa, which is cleaved to produce TGF-β1 of 44 kDa and finally a polypeptide of 12.5 kDa, which is biologically active in a homodimer form (21). However, despite its physiological relevance, nothing is known about the endoproteolytic processing of lefty proteins, and the characterization of their biologically active form has not yet been reported. In addition, the identification of the proteases involved in the processing of lefty will shed light on the regulation of the TGF-β network and its modulation during embryonic patterning. Proteins of the TGF-β superfamily are cleaved by members of the proprotein convertase (PC) family of endoproteases (21–23). These endoproteases are Ca²⁺-dependent serine proteases with a consensus cleavage site of RXXR (21–25). Furin was the first convertase to be extensively characterized and is required for the proper processing of several TGF-β proproteins. Furin-deficient LoVo cells fail to cleave TGF-β1, whereas cells transfected with furin regain the ability to properly process TGF-β1 (21). Furthermore, PCs were recently shown to be involved in the establishment of embryonic patterning. Furin is required for ventral closure, axis rotation, formation of the yolksac vasculature, and proper left-sided expression of lefty-2 and pitx2 (26). Mouse embryos lacking PACE4 develop an ambiguous situs with left pulmonary isomerism and/or craniofacial malformations including cyclopia (27). Despite the clear implication of the proprotein convertases in the regulation of TGF-β1, nothing is really known about the processing of the lefty cytokines by these proteases. Here, we analyzed lefty cleavage and studied the in vivo implication of the proprotein convertase family of endoproteases in its processing. We also identified the cleavage sites of the protein and carried out assays to identify the bioactive forms of lefty polypeptides.

EXPERIMENTAL PROCEDURES

Materials—The materials used in these studies included an enhanced chemiluminescence system (Roche Molecular Biochemicals), nitrocellulose membrane (Bio-Rad), Kodak Omnfilm (Sigma), biotin-labeled goat anti-rabbit antisera and ABC reagent (Vector Laboratories, Inc., Burlingame, CA), and protein G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Diphosphorylated myelin basic protein (MBP), anti-phospho-MBP antibody, and immunopuroficiency- purified rabbit anti-MAPK (Erlk1/2) bound to agarose were all obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Unless otherwise indicated, all other chemicals were from either Sigma or Fisher. The mammalian cell lines were from American Type Culture Collection (Manas- sas, VA). The rabbit polyclonal antibodies used in this study were raised against a peptide at the C terminus of the lefty A/B protein (acyetyl-CASDGALVFPRRQLHP) (antibody A353) and a peptide at the N terminus of lefty A (acyetyl-DRAMDEKVLIPAC) (antibody A44). The monocular antibody to the hemagglutinin (HA) tag was obtained from Roche Molecular Biochemicals. The monoclonal and polyclonal antibodies directed against furin, PACE4, PCSA, PC5B, and PC7 were obtained from Alexis Biochemicals (Laufflingen, Switzerland). Anti-Smad2, -4, and -5 antibodies were obtained from Santa Cruz Biotechnology. Recombinant BMP-4 was obtained from R&D Systems (Minneapolis, MN). Recombinant TGF-β1 was obtained from Sigma. Recombinant Escherichia coli lefty A, expressed and refolded from Ser136 to Phe369, was obtained from Regeneron Pharmaceuticals (Tarrytown, NY).

Primer Design and PCR-based Cloning of Human lefty A and lefty B—All constructs obtained by PCR and mutagenesis were checked by sequencing. The coding regions of lefty A (ebal and lefty B cDNAs were cloned by PCR using Marathon-ready cDNA from human pancreas (CLONTECH, Palo Alto, CA) and cloned into pAdCMV5 (Quantum Biotechnologies Inc., Montreal, Canada). The PCR products were separated on 1% agarose gels and purified with a GeneClean kit (Bio 101, Inc., Vista, CA). The PCR products and the plasmids, pcDNA3 (Invitrogen) or HA-pcDNA3, were digested with EcoRI and XhoI (New England Biolabs Inc., Beverly, MA) for lefty A (ebal) or with BamHI and XhoI (New England Biolabs Inc.) for lefty B. The fragments were annealed to the mammalian expression plasmid pcDNA3 with a rapid ligation kit (Stratagene, La Jolla, CA). The sequence of each clone was validated by restriction enzyme digestion and sequencing using Taq DyeDeoxy terminator cycle sequencing reactions in conjunction with an Applied Biosystems Model 373 DNA Sequencer. The plasmid DNAs containing the full-length DNA sequences were prepared using the Promega Wizard miniprep method and used for transfection. Lefty A was tagged at its C terminus with an HA tag (YPYDVPDYAG). The forward primer used was 5′-AGC TGG AGC TGC ACA CCC TGG, and the reverse primer used was 5′-TTT GGA TCA GCC ATC ATC TGG CAC ATC ATA TGG GTA TGG CTG GAG CCT CCT TGG CAC. The sense orientation of the lefty A CDNA was constructed using plasmid pAdCMV5, in which lefty A gene expression is regulated by the cytomegalovirus immediate-early promoter. A 1.2-kilobase pair BamHI/A/II/III lefty A cDNA fragment containing minimal 5′- and 3′-untranslated regions from the plasmid pBluescript SK− lefty A vector was filled in with T4 DNA polymerase and cloned into Pmel1-digested pAdCMV5. Restriction mapping and flanking DNA sequencing confirmed the orientation of the resulting lefty A expression plasmids. All constructs were cloned into the mammalian expression vector pcDNA3. Construction of the cDNAs has been previously described (28–30).

Site-directed Point Mutation of Human lefty A—Site-directed mutagenesis was achieved with a QuikChange™ 1-Day site-directed mutagenesis kit (Stratagene) following the manufacturer’s protocol. The sequences of mutated clones were determined by Taq DyeDeoxy terminator cycle sequencing reactions in conjunction with an Applied Biosys- tems Model 373 DNA Sequencer.

Cells, Transfection, and Protein Preparation—Human embryonic kidney 293 cells, a fibroblast cell line (NIH-3T3), a Chinese hamster ovary (CHO) cell line, and RPE40 (a variant of CHO cells deficient in furin) cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 1% antibiotic/antimycotic (Life Technolo- gies, Inc.). For transfection, cells were seeded into 6-well plates (Falcon, Franklin Lakes, NJ) at a concentration of 1.3 × 10⁶ cells/ml and maintained in a CO₂ chamber at 37 °C for ~6 h. When 60% confluent, cells were transfected with lefty A, lefty B, or mutant lefty A cDNA using Superfect transfection reagent (QIAGEN Inc., Valencia, CA) or Fugene (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturers’ protocol.

In transient transfections, serum-free medium was used. The medium was collected 20–24 h after transfection and concentrated 12-fold using Centricon YM-3 centrifugal filter devices (3-kDa protein molecular size cutoff; Amicon, Inc., Beverly, MA). Cells were lysed by addition of 15 μl of Laemmli buffer. Protein concentration was determined with the Bio-Rad protein assay kit a

Affinity Purification of Lefty A Proteins—Lefty A proteins were pu-

rified by affinity purification from culture media of cells transfected

with lefty A. Affinity columns for lefty A were prepared by binding rabbit anti-lefty-1 peptide antibody A353 or A44 to cyanogen-activated Sepha-

rose 4B (referred to as A353 and A44 affinity columns). Cell-free condi-
tions of lefty A-expressing cells were transfected into E. coli and harvested and mixed with 250 μl of anti-lefty A antibody A353 or A44-Sepharose 4B suspension/10 ml of medium. To each 2.5 ml of urine or 250 μl of serum 25 μl of the antibody-Sepharose suspension was added. The

sequences of the primers used for PCR and site-directed muta-
genesis are available upon request.
mixtures were incubated at room temperature for 1 h with gentle shaking. The suspension of bound lefty protein(s) with Sepharose 4B was then poured into a 0.7 × 15-cm column. The column was washed with 20 column volumes of 50 mM Tris-HCl (pH 7.5), 0.2 mM NaCl, and 5 mM EDTA. The bound lefty protein(s) was eluted with 0.5 column volumes of 3 M guanidine HCl in 10 mM Tris-HCl (pH 8.1) and 5 mM EDTA, followed by 0.25 column volumes of 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA. The eluted lefty protein was then dialyzed against three changes of 10 mM sodium phosphate buffer (pH 7.4) at 4 °C and stored at 4 °C. Culture media were first applied to the A353 affinity column. The proteins eluted from the A353 column were then applied to the A44 affinity column and eluted. Eluted and flow-through materials were subjected to Western blotting with antibody A353 to identify the affinity-purified proteins. The affinity column made with antibody A353 bound all lefty proteins (42, 34, and 28 kDa). On the other hand, the affinity column made with antibody A44 bound only the 42-kDa protein, allowing the 34- and 28-kDa proteins to flow through this column (data not shown).

The amount of purified lefty from large-scale purification procedures was determined with the Bio-Rad protein assay kit.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Conditioned media or cell lysates (12–15 μg of protein/lane) were fractionated on a 12% denaturing gel together with a prestained protein ladder (Life Technologies, Inc.) and subsequently blotted onto polyvinylidene difluoride membrane in a Mini-Trans-Blot apparatus (Bio-Rad Laboratories). The blot was stained using either 0.1% amido black or with horseradish peroxidase-conjugated mouse anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). The specific bands were detected with the chemiluminescence system as described by the manufacturer.

Immunoprecipitation—Immunoprecipitations of the transfected convertases were performed as described (30). Briefly, for immunoprecipitations, 2–5 μl of specific antibody was added to each milliliter of cell lysate (~106 cells). The immunoprecipitates were subjected to gel electrophoresis, followed by autoradiography.

Determination of the Effect of Lefty Proteins on the MAPK Pathway—Regulation of the MAPK and JNK pathways was analyzed using the PathDetect™ in vitro signal transduction reporting system (Stratagene). P19 mouse embryonic carcinoma cells were transfected with the respective pathway-specific fusion activator vectors (pcDA-Etk for MAPK and pFA-Jun for JNK pathway; 3 μg/ml of each plasmid) and the luciferase reporter plasmid (pFR-Luc) according to the manufacturer’s instruction. These cells were maintained in a minimal essential medium supplemented with 7.5% bovine calf serum, 2.5% fetal bovine serum, and 1% antibiotic/antimycotic mixture. Twenty-four hours after transfection, cells were incubated for 20 h with the conditioned media of 293 cells stably transfected with wild-type lefty A or lefty B cDNA or the GGGK and GHGR mutant lefty A cDNAs. Cells were then washed with phosphate-buffered saline, and the luciferase activity was quantified using the Promega luciferase assay system following the manufacturer’s recommendation. To ensure equal treatment of cells with the culture media, the media of these cultures were also examined by Western blotting. The blot was stained with the polyclonal antibody to lefty A. The density of the 42-kDa protein measured by scanning laser densitometry was the same in each lane.

The induction of MAPK activity by lefty was also examined by showing the kinase activity of Erk1/2 as previously described (31, 32). Briefly, P19 cells were treated with affinity-purified 42/28/28-kDa protein mixture, 34/28-kDa protein mixture, 42-kDa lefty A protein, or 26-kDa recombinant E. coli lefty A for the durations shown below. After washing cells with ice-cold phosphate-buffered saline, cells were lysed in cell lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonfyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml peptatin, 1 mM Na3VO4, and 1 mM NaF), and the concentration of protein in the cell lysate was adjusted to 1 μg/μl protein. Five microliters of anti-MAPK antibody-agarose gel slurry was added to 500 μl of cell lysate. The mixture was gently rocked overnight at 4 °C. The beads were collected and washed three times with ice-cold phosphate-buffered saline and incubated with 100 μl of assay buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol). To 10 μl of the immunocomplexes were added 20 μg of dephosphorylated MBP, 10 μl of MBP dilution buffer, and 10 μl of Mg/ATP mixture (500 μM unlabeled ATP and 75 μM MgCl2), and the mixture was incubated for 20 min at 30 °C with constant shaking. Ten microliters of the reaction mixture was added to an equal volume of 2× reducing sample buffer; the mixture was subjected to SDS-polyacrylamide gel electrophoresis; and the proteins were transferred to nitrocellulose membranes. The phosphorylation of MBP was assessed by immunoblotting with 1 μg/ml anti-phospho-MBP.

In Vivo Phosphorylation of Smad Proteins—P19 cells were grown in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 for 24 h. Cells were then washed and incubated for 2 h in Dulbecco’s modified Eagle’s medium without glutamine and phosphate. Cells were incubated for another 2 h in the same medium supplemented with 15 μM/ml [32P]orthophosphate. After cyto kinase treatment, cells were lysed in 1 ml of radioimmunoprecipitation assay buffer, and the samples were subjected to immunoprecipitation with Smad2- or Smad3-specific antibodies. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, followed by blotting and autoradiography.

RESULTS

Identification of the Secreted Lefty Forms—Lefty polypeptides were identified as new members of the TGF-β superfamily (11–14). Cytokinines of the TGF-β superfamily require proper endoproteolytic cleavage for their activity. However, nothing is known about the processing and activation of lefty polypeptides. We cloned the coding regions of human lefty A and lefty B by PCR using Marathon-Ready cDNA from a human pancreas library. To analyze the expression, lefty A was tagged at the carboxyl-terminal domain with HA sequence. Human embryonic kidney 293 cells were transfected with pcDNA3-lefty A-HA, and the expression of lefty was analyzed in the culture media collected from the transfected cells by Western blotting using monoclonal anti-β-HA antibody (Fig. 1A). lefty A transfection induced the expression of three specific forms with respective molecular masses of 42, 34, and 28 kDa. In addition, the location of the HA tag indicates that these fragments correspond to the carboxyl-terminal domain of the lefty A protein. These results strongly support the insight that, as a member of the TGF-β superfamily, lefty A is a secreted protein.

Polyclonal anti-lefty antibody A353 was raised against the peptide corresponding to amino acids 353–366 of lefty A (13, 14). The antibody was purified on a peptide affinity column and characterized by Western blotting and immunoprecipitation of the culture media of control and transfected cells. Antibody A353 showed a specific immunoreactivity against lefty A protein in the culture media of different transfected cell lines, including human embryonic kidney 293 cells and the CHO cell line. Antibody A353 was used to identify lefty forms in conditioned media or cell lysates and to identify lefty forms in conditioned media or cell lysates.
recognized three polypeptides of 42, 34, and 28 kDa in the conditioned media of the transfected cells in the same way that anti-HA antibody reacted with the protein released into the media of both 293 (Fig. 1B) and CHO (Fig. 1C) cells. Blocking experiments with the immunizing peptide also proved the specificity of this antibody. Incubation of the antibody with the immunizing peptide prevents the immunoreactivity of these three polypeptides in a peptide concentration-dependent fashion (33). We did not find immunoreactivity with any endogenous protein in the different cell lines tested. Based on the fact that antibody A353 did not show any reaction with endogenous proteins, we believe that these cells do not express a lefty homolog or that their endogenous expression is very weak. Human 293 cells were transfected with lefty B, and the immunoreactivity of the culture media was probed by Western blotting using antibody A353 (Fig. 1D). In agreement with the 95% identity between lefty A and lefty B, antibody A353 also bound lefty B in the same fashion as it reacted with lefty A. These two proteins exhibit a complete identity in the carboxyl-terminal region used for raising antibody A353. Antibody A353 recognized three polypeptides of 42, 34, and 28 kDa, suggesting that lefty A and lefty B are subjected to the same endoproteolytic processing in cells (Fig. 1D).

Endoproteolytic Processing of Lefty A Polypeptides by Members of the PC Family of Endoproteases—To prove that the polypeptides of 34 and 28 kDa are produced by endoproteolytic processing of the 42-kDa precursor, we studied the proteases involved in the processing of lefty polypeptides. First, we attempted to inhibit lefty processing by using the polypeptide α1-antitrypsin Portland (α1-PDX), a genetically engineered serine protease inhibitor derived from the trypsin inhibitor α1-antitrypsin. α1-PDX was originally reported to selectively inhibit furin and PC5/PC6 activities (34). However, it has recently been shown that α1-PDX can also inhibit other PCs such as PACE4 (35, 36). To prove the implication of serine proteases in the processing of lefty, CHO cells were transfected with lefty A and with α1-PDX, and the conditioned media were analyzed by Western blotting using antibody A353 (Fig. 2A). The serine protease inhibitor prevented the processing of lefty A (Fig. 2A), whereas α1-antitrypsin did not (data not shown), indicating that the precursor of 42 kDa is processed in vivo by a serine protease to release the polypeptides of 34 and 28 kDa.

Because of the implication of the PCs in the processing of other cytokines of the TGF-β superfamily, we have analyzed the in vivo processing of lefty proteins by members of this family of endoproteases. Furin was the first convertase to be extensively characterized and is required for the proper processing of pro-TGF-β1 (21) and BMP (37, 38). We studied the lefty processing in furin-deficient CHO cells (RPE40 cells). RPE40 cells were transfected with lefty A, and the culture media were collected and subjected to Western blotting (Fig. 2B). The culture media from furin-deficient RPE40 cells showed the typical pattern of three polypeptides of 42, 34, and 28 kDa, suggesting that furin is not essential to the processing of lefty. Other members of the convertase family of endoproteases were cotransfected with lefty A in CHO cells, and their effect on lefty processing was analyzed by Western blotting of the conditioned media using antibody A353 (Fig. 2C). Among the proteases, PC5A induced the in vivo processing of the 42-kDa lefty precursor primarily to the 34-kDa form. Furin, PACE4, and PC5B exhibited a limited effect, whereas PC7 did not affect lefty processing of the precursor. Although all convertases were expressed properly (Fig. 2D), none of these proteases enhanced the processing of the 34-kDa polypeptide to the 28-kDa form (Fig. 2C).

Identification of the Endoproteolytic Cleavage Sites of Lefty

A—To validate that the PC family of endoproteases is involved in the in vivo endoproteolytic processing of lefty polypeptides, we identified the cleavage sites according to the consensus sequences required for convertase cleavage. We suggested the sequences GRKR (aa 74–77) and RHGR (aa 132–135) as potential cleavage sites for the processing of lefty proteins by the convertases (Fig. 3A) (13). These sequences are conserved in both lefty A and lefty B, suggesting the same processing for these two proteins. To prove these sequences to be cleavage sites in vivo, we analyzed the effect of the mutations of GRKR to GGKG (aa 74–77) and of RHGR to GHGR (aa 132–135) on the processing of lefty. 293 cells were transfected with the lefty A mutants, and culture media were analyzed by Western blotting (Fig. 3B). The mutation of RGKR to GGKG (aa 74–77) prevented the processing of the 42-kDa lefty precursor to the 34-kDa form, whereas the mutation of RHGR to GHGR (aa 132–135) abolished the processing of the 34-kDa polypeptide to the 28-kDa form. Moreover, the mutation of RGKR to GGKG (aa 74–77) did not inhibit the formation of the 28-kDa polypeptide, suggesting that the two proteolytic cleavages are independent. Therefore, the cleavage at Arg135 does not require cleavage at Lys77 or the formation of the 34-kDa polypeptide.
These results also rule out the possibility that these point mutations prevented the proteolytic cleavage in another site.

**Biological Activity of Lefty Polypeptides—** Members of the TGF-β family signal through the Smad and MAPK pathways (40–49). To study the biological role of the endoproteolytic processing in lefty signaling, we tested the ability of lefty to signal through the Smad and MAPK pathways. Upon stimulation by TGF-β, Smad2/3 is transphosphorylated by the activated TGF-β receptors. The phosphorylated Smad2/3 protein heterodimerizes with Smad4, and the complexes formed accumulate in the nucleus (40–49). BMP, on the other hand, leads to the phosphorylation of Smad5. To test the effect of lefty on the Smad pathway, the effect of lefty on phosphorylation of Smad2 and Smad5 was compared with the activity of TGF-β1 and BMP-4. TGF-β1 induced the phosphorylation of Smad2, and BMP-4 induced the phosphorylation of Smad5. On the other hand, lefty A did not have any effect on Smad2 or Smad5 phosphorylation (Fig. 4, A and B). We next examined the effect of lefty on heterodimerization of Smad2 with Smad4. Smad2 was immunoprecipitated from the cytosolic fraction of P19 cells treated with lefty and with TGF-β1. The immunoprecipitates were subjected to Western blotting for Smad2 and Smad4. Smad2 was immunoprecipitated from the cytosolic fraction of P19 cells treated with lefty and with TGF-β1. Although TGF-β induced heterodimerization of Smad4 with Smad2, lefty had no effect (Fig. 4C). P19 cells were then treated with lefty and with TGF-β1. After 1 h of treatment, the nuclear lysates of these cells were subjected to Western blotting for Smad2 and Smad4. Although TGF-β1 led to the nuclear accumulation of Smad proteins, lefty did not change the amount of either of these Smad proteins in the cytosol (data not shown) or in the nuclei of the treated cells (Fig. 4D).

Since lefty did not seem to directly signal through the Smad pathway, we analyzed the phosphorylation and subsequent activation of MAPK after lefty treatment. We first used the conditioned culture media from control and lefty A-transfected 293 cells and analyzed their effects on both the MAPK and JNK kinase pathways in P19 cells. The activity of the MAPK and JNK kinase pathways in P19 cells was visualized in vivo using the PathDetect™ luciferase reporting system. The culture media from control and lefty A-transfected 293 cells were collected...
were used to treat the serum-starved pluripotent mouse P19 embryonic carcinoma cells, and the activation of the MAPK pathway was analyzed in vivo using the PathDetectTM luciferase reporting system as indicated under “Experimental Procedures.” The error bars show the S.D. of three different experiments.

and used for the treatment of P19 cells. The culture media from *lefty A*-transfected 293 cells induced ∼4-fold activation of the MAPK pathway in P19 cells compared with treatment with the culture media from control cells (Fig. 5). We analyzed whether the activation of the MAPK pathway is specifically due to the lefty polypeptides or other factors present in the culture media of transfected cells. The lefty proteins present in the culture media were “blocked” by increasing the concentration of anti-lefty antibody A353, and their activity was analyzed by the treatment of P19 cells. The anti-lefty antibody inhibited the MAPK activity of the culture media in a concentration-dependent fashion (Fig. 5). These results show that lefty factors rather than other factors present in the culture media of transfected 293 cells induced the activation of the MAPK pathway in the pluripotent P19 cells. In addition, the results also provide evidence that human lefty factors have biological activity in mouse cells, unlike other cytokines with a species-specific activity. In contrast to these results, we did not detect any effect of the culture media of lefty-transfected cells on the JNK pathway (data not shown).

To further validate the results obtained from the reporter assays and to determine the time kinetics of the response, we examined the kinase activity of Erk1/2 after treatment with lefty. To reduce the basal MAPK activity, P19 cells were starved in culture for 2 days in the absence of serum. These cells were incubated with lefty proteins (42, 34, and 28 kDa) purified from the culture media of *lefty A*-transfected cells. Erk1/2 in the treated P19 cells was immunoprecipitated with Erk1/2-specific antibody, and the kinase activity of the immunocomplexes was tested on dephosphorylated MBP. The phosphorylation of MBP was then assessed by Western blotting.

The results show that lefty proteins induced MAPK activation in a time-dependent manner. Lefty proteins induced MAPK activation as early as 5 min, and the activity continued to progressively increase over a period of 60 min (Fig. 6A). This response to lefty could be blocked by antibody A353 (data not shown). To determine whether the induction of MAPK was due to the 42-kDa or cleaved forms of lefty, the 42-kDa protein was affinity-purified and separated from the 34- and 28-kDa proteins using the A44 affinity column, and the experiments were repeated using the 42-kDa purified protein and the 34/28-kDa protein mixture. The 34/28-kDa protein mixture induced MAPK activity; but compared with the effect of the 42/34/28-kDa protein mixture, the effect was delayed. MAPK activation was visualized after 30 min of incubation of the cells with lefty proteins, and the response became pronounced after 60 min of incubation (Fig. 6B). In contrast, purified 42-kDa lefty vigorously activated MAPK after 5 and 15 min, and this response quickly diminished to undetectable levels 30 and 60 min after incubation (Fig. 6C). These findings show that the 42-kDa protein led to early MAPK activation, whereas the 34/28-kDa protein mixture induced a delayed response. These results suggest that the activation of the MAPK pathway in P19 cells is related to the endoproteolytic processing of lefty.

To further identify the bioactive cleaved form(s) of lefty, we analyzed the biological activity of the different lefty polypeptides. 293 cells were transfected with control wild-type *lefty A*, its mutant forms (GGKG (aa 74–77) and GHGR (aa 132–135)), and *lefty B*. Forty-eight hours after transfection, cultured media were collected and used for the treatment of P19 cells after checking the lefty protein expression. The effect of the conditioned media from 293 cells on the MAPK pathway in P19 cells was quantified by measuring luciferase activity (Fig. 7A). The conditioned media of 293 cells transfected with wild-type *lefty A* or *lefty B* used as a control induced an ∼2.5-fold increase in the activation of the MAPK pathway in P19 cells. Moreover, the conditioned media from 293 cells transfected with the GGKG (aa 74–77) *lefty A* mutant induced MAPK pathway activation in P19 cells in a fashion similar to that of the conditioned media from 293 cells transfected with control wild-type *lefty A*. However, the conditioned media from 293 cells transfected with the GHGR (aa 132–135) *lefty A* mutant failed to induce the activation of the MAPK pathway in P19 cells. The results show that the MAPK activation was primarily due to the 28-kDa form of lefty since the GHGR (aa 132–135) mutation, which prevented the formation of the 34-kDa form, had no effect on MAPK activation, whereas the GGKG (aa 74–77) mutation, which
were transfected with pcDNA3 empty vector (Control), lefty A, lefty B, and the mutant GGKG (aa 74–77) and GHGR (aa 132–135) forms of lefty A. The conditioned media were used for the treatment of serum-starved P19 cells, and the activation of the MAPK pathway was quantitated using the PathDetect™ reporting system. The error bars show the S.D. of three different experiments. B, P19 cells were incubated without and with the indicated amounts of recombinant E. coli lefty A. The activation of the MAPK pathway was quantitated using the PathDetect™ reporting system. The error bars show the S.D. of three different experiments. C, P19 cells were incubated without (−) and with (+) recombinant E. coli lefty A (10 ng/ml) for the durations shown. Cells incubated without lefty (−) served to show the basal activity of MAPK after 30 min. After cytokine treatment, Erk1/2 was immunoprecipitated, and the kinase activity of the immunocomplexes on dephosphorylated MBP was assessed by immunoblotting using an antibody reactive with phosphorylated MBP as described under “Results.”

Some data suggested that lefty-1, the mouse homolog of human lefty A, may act as an inhibitor of BMP signaling (12). Since BMP is known to induce MAPK activation, we compared the effect of BMP and lefty alone and together on MAPK activation. Consistent with previous results, recombinant lefty (10 ng/ml) induced a delayed time-dependent response (Fig. 8A). Used at the same dose, BMP induced a response within 15 min, which diminished in 30 min (Fig. 8B). Addition of BMP to lefty enhanced the MAPK activation of lefty at an early time point (5 min), but diminished the response at later time points (Fig. 8C). These findings show that the combination of the two cytokines produced a more balanced response in MAPK activity compared with the effect of each individual cytokine.

**DISCUSSION**

There is a great interest in understanding the vertebrate body organization that determines the location, structure, and arrangement of visceral organs. Different members of the TGF-β superfamily play essential roles in the establishment of embryonic patterning (6–8). Among these, lefty is a novel subfamily of the TGF-β factors. Both lefty-1 and lefty-2 are expressed on the left side during gastrulation and encode a signal for leftness (11, 12, 19). However, despite their physiological role, nothing is really known about the regulation and processing of these new factors. In addition, members of the PC family of endopeptidases were shown to be involved in the establishment of embryonic patterning (26, 27). Since these proteases are also required for the activation of the cytokines of the TGF-β superfamily (20–25), we studied their role in the processing of lefty proteins and identified their cleavage sites.
Furthermore, we assessed the biological activity of lefty polypeptides to identify the bioactive forms of lefty.

Transfection of human embryonic kidney 293 cells and the CHO cell line with lefty A and lefty B led to the secretion of three polypeptides of 42, 34, and 28 kDa. The 28- and 34-kDa polypeptides correspond to the carboxyl-terminal domain of the lefty proteins, which indicates that lefty A and lefty B are subjected to the same endoproteolytic processing, in agreement with the 95% identity between these proteins. We did not find any immunoreactivity with endogenous proteins in the different cell lines tested, suggesting that these cell lines do not express endogenous lefty or its possible homolog(s) or that their expression is very weak. The specificity of the three lefty polypeptides was analyzed by Western blotting using monoclonal anti-HA antibody and purified polyclonal anti-lefty antibody A353. Addition of extra amino acids to the C terminus of TGF-β interfered with the normal dimerization of the protein product and totally inhibited the normal proteolytic processing and glycosylation of the precursor protein (50). However, addition of the HA tag to the C terminus of lefty did not interfere with the processing of lefty. The difference in the processing of TGF-β and lefty might be related to the fact that the C terminus of lefty extends 12 amino acids beyond the C terminus of most other TGF-β family members (13).

Since the proproteins of the TGF-β superfamily are cleaved by members of the PC family, we studied their role in lefty processing. We first showed that α₁-PDX prevented lefty processing. α₁-PDX was originally reported to selectively inhibit furin and PC5/PC6 activities (34). However, it has recently been shown that α₁-PDX can also inhibit other PCs like PACE4 (35, 36). The serine protease inhibitor α₁-PDX interfered with processing of lefty and prevented the formation of the 34- and 28-kDa lefty polypeptides, indicating that lefty A and lefty B are expressed as a 42-kDa precursor that is proteolytically processed by a PC. We analyzed lefty processing in furin-deficient RPE40 cells. The results showed that furin is not required for processing of lefty; hence, we studied the potential role of other PC endoproteases. Among these proteases, furin, PACE4, PC5B, and PC7 showed a very limited or no effect on the processing of the lefty precursor. Recently, it was reported that PCs are involved in the establishment of embryonic patterning (26). The knockout mutation of furin and PACE4 induces a variety of left/right positional defects and perturbs the left-sided expression of lefty proteins. Furin is required for ventral closure, axis rotation, formation of the yolk sac vasculature, and proper left-sided expression of lefty-2 and pits2 (26).

Mouse embryos lacking PACE4 develop an ambiguous situs combined with left pulmonary isomerism and/or craniofacial malformations including cyclopia (27). Our results support the insight that furin and PACE4 act by induction of the processing of cytokines other than lefty proteins. Indeed, furin and PACE4 promote maturation of other members of the TGF-β family such as nodal and BMP in tissue culture cells (51–55). Furin also accounts for TGF-β1 and endoglin maturation (20–27, 34–39, 49). Despite the presence of several perfect Arg-X-(Lys/ Arg)-Arg consensus sequences for PC cleavage (51–53), furin and PC4 could not cleave lefty proteins in vivo (13). Other than this consensus sequence, additional structural features are presumably needed for the specificity of the PC endoproteases. Likewise, PC endoproteases are modulated in vivo by the specificity of substrate and the pattern of expression.

Our data strongly suggest that PC5A is involved in the processing of lefty proteins in vivo. PC5A produced the in vivo processing of the 42-kDa lefty precursor to the 34-kDa form. Furthermore, early detection of PC5/PC6A mRNA in the neural tube spatially matches the predominant lefty expression on the left side of the neural tube (12, 54). Interestingly, lefty and PC5/PC6A are coexpressed in different regions of the developing nervous system during embryo implantation and in the female reproductive tract, suggesting PC5A as the candidate enzyme for processing of lefty in vivo (14, 5, 56). None of the PC endoproteases tested including PC5A were able to induce in vivo the formation of the biologically active 28-kDa form. To validate that the proprotein convertase family of endoproteases is involved in the in vivo endoproteolytic processing of lefty polypeptides, we identified and mutated the potential consensus sequences required for convertase cleavage. The mutations of the PC consensus sequences RGKR (aa 74–77) and RHGR (aa 132–135) prevented the formation of the 34- and 28-kDa lefty polypeptides, respectively. Taken together, we suggest that PC5A induces cleavage at Arg77 to release the 34-kDa polypeptide. The 28-kDa lefty is produced by cleavage of the 42-kDa lefty polypeptide, likely by a serine protease that may be a new member of the PC family of endoproteases.

To identify the biologically active form of lefty, we tested the activity of various lefty polypeptides. The members of the TGF-β superfamily are pleiotropic factors that modulate diverse cellular responses in a variety of cell types. TGF-β signals through two distinct mechanisms, MAPK activation and the Smad-mediated signaling pathway. The latter includes Smad2/3 phosphorylation and is followed by heterodimerization of Smad2/3 with Smad4, nuclear accumulation of these complexes, and subsequent gene transcriptional activity (41–49). The former cascade consists of Ras, MAPKs, MKK4, MEK1, the MAPKs SAPK and Erk, and the specific AP1 proteins Fra-2 and JunD (57). Lefty fails to induce phosphorylation of Smad2; heterodimerization of Smad2 with Smad4; and nuclear accumulation of these heteromeric complexes, which is essential to the transcriptional activity of TGF-β (41–49). Likewise, lefty does not lead to the phosphorylation of Smad5, which is involved in BMP-mediated signaling (58). Thus, the biological role of lefty does not appear to directly depend on Smad-mediated signaling. However, we have observed that lefty perturbs the TGF-β signaling by inhibiting the phosphorylation of R-Smad following activation of the TGF-β receptor as well as events that lie downstream from R-Smad phosphorylation, including heterodimerization of R-Smad proteins with Smad4 and nuclear translocation of the R-Smad:Smad4 complex. Lefty represses TGF-β-induced expression of reporter genes for the p21, cdc25, and connective tissue growth factor promoters and of a reporter gene driven by the SBE. Thus, lefty provides a repressed state of TGF-β-responsive genes and participates in negative regulation of TGF-β signaling by inhibition of phosphorylation of R-Smad proteins (59). Moreover, lefty also inhibits BMP-induced phosphorylation of Smad5 as well as BMP-mediated gene transcription, suggesting that lefty is an inhibitor of BMP actions (59). Interestingly, injection of lefty into mouse blastocysts leads to neurogenesis, a function attributable to BMP inhibitors such as chordin, noggin, and follistatin (12, 60–63). Antivin, a lefty-related gene product, acts as a specific competitive inhibitor for activin during embryogenesis in zebrafish, and the lefty-related factor Xatv acts as a feedback inhibitor of nodal signaling in induction of mesoderm and left/right axis development in Xenopus (60, 64, 65). These findings imply that lefty is an inhibitor member of the TGF-β family.

In contrast to these inhibitory activities, lefty proteins directly induce MAPK activation. This activity is inducible by both the 42-kDa and cleaved forms of the protein. However, the 42-kDa protein induces a quick response, whereas the effect induced by the cleaved forms is delayed, taking as long as 30 min to become detectable. This latter response appears to be
the effect of the 28-kDa lefty protein since the mutation that prevents secretion of the 28-kDa (but not the 34-kDa) form leads to the loss of this MAPK-inducing activity. Moreover, this effect is reproducible by the recombinant E. coli lefty polypeptide (aa 136–369) corresponding to the 28-kDa native lefty protein. These results show that, in contrast to the TGF-β precursor, the precursor of the lefty protein is biologically active. Moreover, the data show that the precursor induces a response that is distinctly different, both temporally and in terms of its magnitude, from that induced by its cleaved forms. These findings establish that processing of lefty leads to formation of two different forms: a 28-kDa form that induces MAPK activation and a 34-kDa protein that is inactive. Since PCSA processes lefty to its 34-kDa form, which is incapable of inducing MAPK activity, the processing by this enzyme could provide a mechanism for regulating lefty function by producing a bias toward production of its inactive form. The results reported here also suggest that lefty proteins, like other members of the TGF-β superfamily, are expressed as a precursor that is processed for activation. The best studied example is TGF-β1, which is expressed as an inactive precursor of 55 kDa. The TGF-β1 precursor is cleaved to produce pro-TGF-β1 of 44 kDa and finally a polypeptide of 12.5 kDa, which becomes biologically active in a homodimer form. However, in contradistinction to the TGF-β precursor, the 42-kDa lefty protein is biologically active and competent to drive the activation of MAPK within minutes in treated cells.

The kinetics of MAPK activation by the 42-kDa protein or the 42/34/28-kDa protein mixture of lefty was similar to that observed with TGF-β and BMP. Both TGF-β1 and TGF-β2 lead to rapid, dose-dependent activation of Erk1. This activation takes place within 5–10 min after addition of the growth factor to exponentially proliferating cultures of intestinal epithelial cells (66). Similarly, the BMP-2-induced Erk activation shows a latent but sustained activity during osteoblastic differentiation (58). The effect of recombinant E. coli lefty and BMP-4 on P19 cells was delayed and became apparent after only 15–30 min of treatment of P19 cells. The immediate effect of lefty on MAPK activation was enhanced by BMP, although this effect later became less pronounced or appeared to be inhibited by BMP. A similar inhibitory effect of BMP on the activity of both platelet-derived growth factor- and epidermal growth factor-stimulated MAPKs has been demonstrated in mesangial cells (66, 67). MAPK is a key regulator of multiple cellular processes, including cell growth, apoptosis, and differentiation, in a wide range of cell types (68–71). For example, macrophage apoptosis, chondrocyte cell growth, and inhibition of DNA synthesis in intestinal epithelial cells induced by TGF-β were all found to be mediated by MAPK activation (70, 72). Because of the ability to induce MAPK activation, lefty is a suitable candidate for modulation of growth, apoptosis, and/or differentiation of target cells.

In summary, our results indicate that the lefty protein is endoproteolytically processed to release two cleaved forms. The lefty protein is expressed as a precursor of 42 kDa that is cleaved at Arg77 and Arg155 to release polypeptides of 34 and 28 kDa, respectively. Lefty fails to induce activation of the Smad pathway, but leads to MAPK activation. The 42- and 28-kDa lefty proteins induce MAPK activation, but the 34-kDa protein does not exhibit this activity. Among the PCGproteases known, PCSA is the only enzyme that is able to induce the processing of the lefty protein to the 34-kDa form, suggesting processing as a means for regulating the function of lefty in vivo. The data support a molecular model of processing as a mechanism for regulation of lefty signaling.

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