Co-expression of the proprotein-processing endoprotease furin and its substrate transforming growth factor β1 and the differentiation of rat hepatocytes

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Abstract Furin, a member of the yeast Kex2 endoprotease family, converts a number of proproteins to their active forms. The liver produces a number of proproteins having a furin-cleavable site; thus, furin may be involved in growth and differentiation both in the partially hepatectomized liver and in primary cultured hepatocytes. Furin mRNA levels are elevated in tissues regenerated from partially hepatectomized rat liver. In primary culture of rat hepatocytes, furin expression increases gradually with time, and its expression is greatly enhanced by transforming growth factor β1, whose processing from the precursor requires cleavage by furin. Thus, we suggest that the regeneration and differentiation of hepatocytes is dependent upon the co-elevation of furin and transforming growth factor β1 mRNAs.

Key words: Furin; Partial hepatectomy; Hepatocyte; Transforming growth factor β

1. Introduction

Furin, a proprotein-processing enzyme, is a member of the yeast Kex2 family of endoproteases that contain a subtilisin-like serine-protease domain as an active site [1,2]. This enzyme is distributed more or less in virtually all tissues, and high expression is observed in the normal liver [3]. Furin cleaves the carboxy-terminus of the unique consensus sequence -Arg^{-4}-X^{-3}-Lys/Arg^{-2}-Arg^{-1} [RX(K/R)R]. The most essential residues of this sequence are the Args at the −1 and −4 positions. However, Arg^{-6} can replace Arg^{-4}, although this renders the cleavage of RXXX(K/R)R by furin less efficient [4]; thus, for cleavage to occur properly, more basic amino acids are required ahead of the general cleavage site of most propeptide hormones, -$(Lys/Arg)^{-2}$-Arg^{-1} [2]. A number of proproteins are known to contain this furin-cleavable motif [1]: growth factor precursors including platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ), activin A, and insulin-like growth factor-1 (IGF-1); some growth factor proreceptors such as insulin receptor, IGF-1 receptor, and hepatocyte growth factor (HGF) receptor (oncoprotein *MET*); matrix metalloproteinases (MMP) such as stromelysin-3 and membrane-type MMP-1; adhesion molecules such as the cadherin family and integrins α3 and α6; and many plasma proteins including albumin, coagulation factors VII, IX, and X, and von Willebrand factor, and plasminogen activator-1. The liver, including mesenchymal and non-mesenchymal cells, contains these proproteins as listed in Table 1.

Previously, we found that furin is highly expressed during the growth state of many cell types. When furin is highly expressed in well-differentiated pancreatic β cell line MIN6 cells, the β cell-specific differentiated characteristics such as insulin content, expression of prohormone convertases PC2 and PC3, and glucose-regulated insulin secretion, diminish [5]. A β cell line, such as MIN6 and βHC9, with a higher level of insulin content and secretory granules expresses a low level of furin, and exhibits slower cell growth; whereas a β cell line with less-differentiated characteristics, such as βTC3 and RINm5F, expresses a high level of furin and grows much faster than differentiated β cell lines [5,6]. Furin is highly expressed in whole rat islets during the perinatal period, when islet cell replication is most extensive, expression then decreases as development continues toward the adult stage [7]. We also found in the adult rat gastric gland furin-positive cells layered along the proliferative cell zone. By using a gastric surface mucosal cell line, GSM06, we demonstrated that when furin expression is suppressed by its antisense oligonucleotide, cell growth is retarded and expression of the differentiated characteristics, such as the appearance of periodic acid-Schiff-positive materials, is enhanced [8]. In cardiac myocytes, furin was strongly immunostained when the cells underwent hypertrophic growth by stretching [9]. When we infarcted the hearts of rats by ligation of the left descending coronary artery, we found that furin was highly expressed along the cells adjacent to the infarcted area [10]. Thus, we propose that furin is expressed when dividing cells undergo proliferation and non-dividing cells experience hypertrophic growth.

The liver has an enormous capacity to regenerate following either partial hepatectomy or massive liver damage by severe hepatitis [11,12]. During its regenerative growth, a number of growth factors and growth-related proteins are expressed in a cascade fashion. After regeneration, the liver produces a massive amount of plasma proteins to maintain homeostasis. As listed in Table 1, many proproteins produced in the liver contain a furin-specific cleavage site. However, some growth factors and proreceptors, such as PDGF, IGF-1, and c-Met, induce hepatocytic growth, whereas others, such as TGFβ and activin A, induce cell differentiation. As for plasma proteins, some proproteins including complements are produced in the acute-phase response to injury, while others such as albumin are produced primarily from the normal-state liver. However, we do not yet know whether furin expressed for the conversion of proproteins derives from the growing or the differentiated state of the hepatocytes. The present study demonstrates changes in the expression of furin and its substrate

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TGFβ after a partial hepatectomy and during the primary culturing of the hepatocytes.

2. Materials and methods

2.1. Materials

Recombinant TGFβ1 was obtained from King Jyozo (Himeji, Japan), EGF from Collaborative Biomedical Products (Bedford, MA, USA). Male Wistar rats (150–200 g) were purchased from Imai Experimental Animal Farm (Saitama, Japan).

2.2. Surgical procedures

Rats were maintained under controlled light (7:00 to 19:00 h) with food and water provided ad libitum. Partial (70%) hepatectomy was performed under anesthesia with diethyl ether, as described previously [13]. After opening the peritoneal cavity, the left lateral and left median lobes were excised from the liver. Control operations were performed using an identical procedure without excision of any liver tissue. At an indicated time after the operation, the rats were killed and their livers were removed for RNA isolation.

2.3. Hepatocyte isolation and culture

Hepatocytes were prepared by perfusion of the liver with collagenase [14]. Cells were plated close to confluence ($6–7\times10^4/cm^2$) on a 90-mm collagen-coated dish in Williams' E medium, containing 5% fetal bovine serum, 10 nM insulin, 1 nM dexamethasone, streptomycin and penicillin. After 3 h, the cells were washed with phosphate-buffered saline, then incubated in fresh, serum-free Williams' E medium, containing 0.1% bovine serum albumin (BSA) with EGF (10 ng/ml) or TGFβ1 (usually at 100 pM; in other cases at an indicated concentration). Culture media were replaced every 24 h.

2.4. RNA extraction and hybridization

Total RNA was extracted from primary cultured hepatocytes or partially hepatectomized liver with TRIzol reagent (Life Technologies, Grand Island, NY, USA). For Northern blot analysis, total RNA (10 μg) was heat-denatured and electrophoresed on a 1% agarose gel, then transferred to a nylon membrane (Hybond-N, Amersham Japan, Tokyo, Japan). Hybridization was performed in a solution consisting of 5×SSPE (20×SSPE = 3.0 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 10×Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 50% formamide, 1.4% sodium dodecyl sulfate (SDS), and 0.1 mg/ml salmon sperm DNA with either a mouse furin cDNA probe (a 924-bp fragment cut with *Bam*HI [5]) or a rat TGFβ1 cDNA probe (a 490-bp fragment cut with *Eco*RI and *Bgl*I [15]), labeled with [α-^{32}P]dCTP, using a *redi* prime random primer labeling kit (Amersham Japan, Tokyo). After washing, the membrane was exposed to X-ray film with an intensifying screen at −80°C.

3. Results

3.1. Furin expression after partial hepatectomy

Total RNA derived from the remaining liver after the partial hepatectomy or after the control operation was subjected to Northern blot analysis for furin expression. After a control operation, furin mRNA levels remained relatively constant up to 168 h (7 days) (Fig. 1A); however, after partial hepatectomy, furin expression decreased at 3–6 h, returned to control levels at 12–18 h, then gradually increased from 24–168 h (Fig. 1B). This decrease of furin mRNA was confirmed by a total of eight experiments. Furthermore, 18S and 28S ribosomal RNAs were equally stained with ethidium bromide at each point (Fig. 1C). Because the proliferation of hepatocytes is maximal 24 h after partial hepatectomy, 3–6-h points are in the middle of the G1 phase of the cell cycle [11]. At this phase,

Table 1
A list of liver-produced proproteins with a furin-cleavable site

Proprotein	Propeptide										Bioactive peptide
					−6		−4			−1	+1
Growth factors											
PDGF-A	-K	R	P	L	P	I	**R**	**R**	K	**R**	SIE-
PDGF-B	-E	L	E	S	L	A	**R**	G	**R**	**R**	SLG-
IGF-1	-A	P	L	K	P	A	**K**	S	A	**R**	SVR-
TGF-β	-Q	H	L	Q	S	S	**R**	H	**R**	**R**	ALD-
Activin A	-E	D	H	P	H	**R**	**R**	**R**	**R**	**R**	GLE-
PTHrP	-V	E	G	S	L	**R**	**R**	L	K	**R**	AVS-
Plasma proteins											
Albumin					**R**	G	V	F	**R**	**R**	DAH-
Factor VII	-A	H	G	V	L	H	**R**	**R**	**R**	**R**	ANA-
Factor IX	-A	N	K	I	L	N	**R**	P	K	**R**	YNS-
Factor X	-G	K	Q	T	L	E	**R**	**R**	K	**R**	SVA-
von Willebrand factor	-S	S	P	L	S	H	**R**	S	K	**R**	SLS-
Tissue-type plasminogen activator	-I	H	A	R	F	**R**	**R**	G	A	**R**	SVQ-
Prothrombin	-A	R	S	L	L	Q	**R**	V	**R**	**R**	ANT-
Protein C	-M	E	K	K	**R**	S	H	L	K	**R**	DTE-
Matrix metalloproteinases (MMP)											
Stromelysin-3	-G	L	S	A	**R**	N	**R**	Q	K	**R**	FVL-
Membrane-type MMP-1	-E	I	K	A	N	V	**R**	**R**	K	**R**	YAI-
Adhesion molecules											
E-cadherin	-S	S	P	G	L	**R**	**R**	Q	K	**R**	DWV-
Integrin α3 H-L chain	-R	P	S	S	P	Q	**R**	**R**	**R**	**R**	QLD-
Integrin α6 H-L chain	-T	E	S	H	N	S	**R**	**K**	K	**R**	EIT-
Complements											
C_3 β-α chains	-C	P	Q	P	A	A	**R**	**R**	**R**	**R**	SVQ-
C_4 β-α chains	-P	K	E	K	T	T	**R**	**K**	K	**R**	NVN-
C_4 α-γ chains	-F	E	G	R	**R**	N	**R**	**R**	**R**	**R**	EAP-
C_5 β-α chains	-P	C	K	E	I	L	**R**	P	**R**	**R**	TLQ-
Pro-receptors											
Insulin receptor α-β chains	-F	V	P	R	P	S	**R**	**K**	**R**	**R**	SLG-
HGF receptor (c-Met) α-β chains	-C	I	L	T	E	K	**R**	**K**	K	**R**	STK-

A furin-cleavable consensus sequence is Arg^{-4}-X-$(Lys/Arg)^{-2}$-Arg^{-1}. Albumin does not possess Arg^{-4}, but instead possess Arg^{-6}. Basic residues from −1 to −6 positions are written in bold.

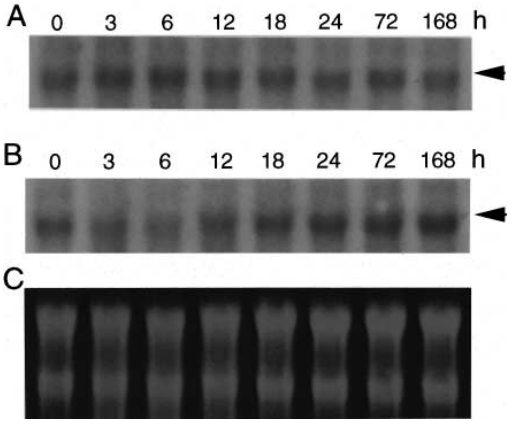

Fig. 1. Northern blot analyses of furin mRNAs after partial hepatectomy. A: Furin mRNAs from control-operated livers. B: Furin mRNAs from the partially hepatectomized liver. Time (h) after the operation is indicated at the top of the panel. The arrowhead indicates 28S rRNA. The size of furin mRNA is approximately 4.5 kb. C: 18S and 28S rRNA bands stained with ethidium bromide.

furin was under-expressed in the partially resected liver. Because furin mRNA levels became elevated toward the 168-h point, furin may be involved in the production of proproteins related to cell differentiation.

3.2. *Furin expression in primary cultured hepatocytes*

The gene expression pattern of a primary culture of hepatocytes in the presence of a cocktail of insulin, glucocorticoid, and EGF roughly simulates that of partially resected liver [16]. In the presence of 10 ng/ml EGF, hepatocytes initially grow, then display differentiated features. Furin mRNA was elevated approaching the 72-h point post culture (Fig. 2A). Furin is known to convert pro-TGFβ to its active form [17], which then induces a differentiated state of hepatocytes. When TGFβ (final 100 pM) was further added to the culture medium, furin mRNA levels were markedly elevated, even at the 24-h point, increasing up to 72 h post culture (Fig. 2B). This furin expression was TGFβ1 dose-dependent up to 300 pM (Fig. 2C), and the effect was significant at concentrations

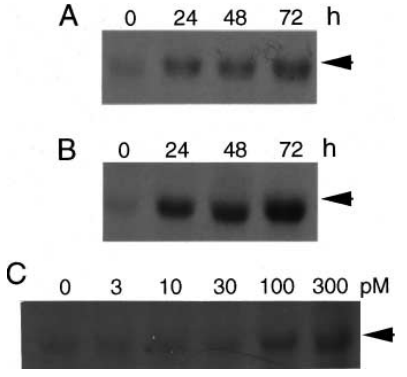

Fig. 2. Northern blot analyses of furin mRNAs in primary cultured hepatocytes. A: Furin mRNAs after the addition of 10 ng/ml EGF to the culture. B: Furin mRNAs after the addition of 10 ng/ml EGF and 0.1 nM TGFβ1 to the culture. Time (h) after the addition of EGF or TGFβ1 is indicated at the top of the panel. C: Expression of furin mRNAs after the addition of increasing amounts of TGFβ1 for 72 h. TGFβ1 dosage is indicated at the top of the panel. The arrowhead indicates 28S rRNA.

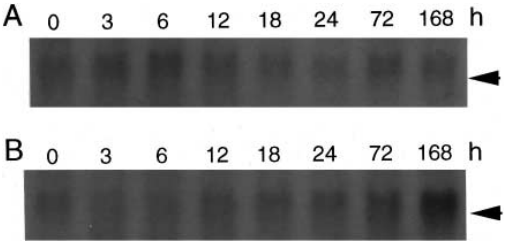

Fig. 3. Northern blot analyses of TGFβ1 mRNAs after partial hepatectomy. A: TGFβ1 mRNAs from control-operated livers. B: TGFβ1 mRNAs from the partially hepatectomized liver. Time (h) after the operation is indicated at the top of the panel. The arrowhead indicates 18S rRNA. The size of TGFβ1 mRNA is approximately 2.5 kb.

greater than 100 pM TGFβ1. Thus, TGFβ is a strong inducer of furin in hepatocytes.

3.3. *TGFβ1 expression after partial hepatectomy*

When furin is expressed, its substrate proproteins may be co-expressed. After partial hepatectomy, furin expression increased up to the 168-h point, as already shown in Fig. 1B; likewise, TGFβ1 mRNA expression increased toward the 168-h point in partially hepatectomized rat livers (Fig. 3B), but stayed at the same low level in the control rat livers (Fig. 3A). Thus, although the increase of furin mRNA occurred earlier than did that of TGFβ1 mRNA, both furin and TGFβ mRNAs co-elevated after partial hepatectomy.

3.4. *TGFβ1 expression in primary cultured hepatocytes*

In the primary culturing of hepatocytes using EGF, TGFβ1 expression was only faintly visible at the 72-h point (Fig. 4A). Adding TGFβ1 to the culture resulted in increased levels of TGFβ1 mRNA expression at the 72-h point (Fig. 4B), similar to the furin mRNA expression levels (Fig. 2B). Also, TGFβ1 expression was TGFβ1 dose-dependent up to 300 pM (Fig. 4C), and the TGFβ1 effect was noted above 100 pM TGFβ1. Thus, TGFβ1 and furin mRNAs were co-elevated in partially hepatectomized liver as well as in primary cultured hepatocytes.

4. Discussion

This study demonstrated that furin is highly expressed with the differentiation of hepatocytes, and its expression is greatly enhanced by the same TGFβ1 that is known to inhibit the growth of hepatocytes and to induce their differentiation [11,12]. This expression mode of furin is contrary to our previous findings which detected expression in the growing state of pancreatic β cells, gastric surface mucous cells, and cardiac myocytes, as described in Section 1 [5–10].

The liver produces a number of proproteins with furin-cleavable sites, as listed in Table 1 [1]. Some of these, such as PDGF, IGF1 and c-Met, are involved in the liver regeneration. Because furin expression was high in normal and regenerated liver and low shortly after partial hepatectomy (Fig. 1), these proproteins may be cleaved by other Kex2 family endoproteases, such as PACE4 [18]. Several bioactive proteins are known to induce hepatocyte differentiation in an autocrine/paracrine fashion. The most well-known proprotein is TGFβ, which inhibits the growth and induces the differentiation of hepatocytes [11,12,19]. TGFβ is produced as a latent

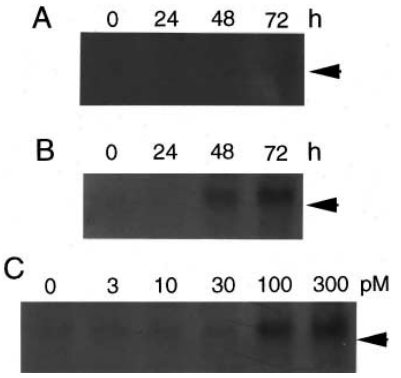

Fig. 4. Northern blot analyses of TGFβ1 mRNAs in primary cultured hepatocytes. A: TGFβ1 mRNAs after the addition of 10 ng/ml EGF to the culture. B: TGFβ1 mRNAs after the addition of 10 ng/ml EGF and 0.1 nM TGFβ1 to the culture. Time (h) after the addition of EGF or TGFβ1 is indicated at the top of the panel. C: Expression of TGFβ1 mRNAs after the addition of increasing amounts of TGFβ1 over 72 h. TGFβ1 dosage is indicated at the top of the panel. The arrowhead indicates 18S rRNAs.

form composed of three components: a 25-kDa TGFβ dimer, a cleaved propeptide dimer non-covalently bound to TGFβ, and a latent TGFβ-binding protein (LTBP). The latent form of TGFβ requires two processing reactions for maturation: first, cleavage of TGFβ propeptide precursor by furin [17], and second, removal of LTBP possibly by plasmin [20]. Thus, furin is required for the activation of pro-TGFβ. Furthermore, TGFβ1 is known to upregulate its own expression [21]. Previously, we suggested that whenever furin is expressed, its substrate proproteins will be co-expressed in the same cell; brain natriuretic peptide is one such proprotein that is co-expressed with furin in the hypertrophic cardiac myocytes by stretching [9,10]. TGFβ1 is reported to induce the expression of furin in cultured rat synovial cells as early as 3–48 h after the addition of TGFβ1 [22]. We also confirmed that TGFβ1 induces the expression of both furin and TGFβ1 in partially hepatectomized liver and also in primary cultured hepatocytes.

In the liver, most of the TGFβ1 is produced by endothelial cells and Kupffer cells, a smaller amount by fat-storing cells (also called lipocytes and Ito cells), and a much smaller amount by hepatocytes [23]. In our experiments, TGFβ1 as well as furin expression was faintly visible in cultured hepatocytes unless TGFβ1 had been added to the culture. Since the normal liver and regenerated liver express a high level of furin, we need to examine whether furin distribution is similar to TGFβ1 distribution in the liver cell types. Although TGFβ1 production was not high, hepatocytes are known to continue producing TGFβ1 over 72 h compared with an hourly rise of TGFβ1 in the other three liver cell types in primary culture [23]. In hepatocytes, TGFβ induced expression of furin and additional levels of TGFβ. Thus, induced furin may activate not only pro-TGFβ but also other proproteins, such as the plasma proteins, which are instrumental for maintaining homeostasis.

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