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The Structure of the TGF-β Latency Associated Peptide Region Determines the Ability of the Proprotein Convertase Furin to Cleave TGF-βs

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Abstract The TGF-β family members are generated as latent pre-pro-polypeptides. The active mature peptides are cleaved from the latent forms by cellular proteases. TGF-β1, for instance, is predominantly processed by a substilisin-like proprotein convertase, furin. TGF-β2 has a consensus cleavage site for furin and therefore has been presumed to be cleaved by furin. However, TGF-β2 is often secreted as the latent form, which appears to be inconsistent with its postulated sensitivity to furin. We report here that both the regular (short) form of TGF-β2 and its spliced variant with an additional exon (long form) are insensitive to furin. NIH 3T3 and CHO cells were transfected with expression vectors containing the short or long form of TGF-β2 or a chimeric TGF-β consisting of the TGF-β1 LAP region, the TGF-β2 cleavage site and the TGF-β2 mature peptide. The constructs included a c-*myc* epitope tag in the N-terminal region of the mature peptide. The TGF-βs produced by the transfected cells were analyzed with Western blots and immunocytochemistry. The intracellular proteins harvested from these cells were incubated with furin. Furin only inefficiently cleaved both the long and short forms of TGF-β2, but efficiently processed the chimeric TGF-β. This indicates that the insensitivity of both forms of TGF-β2 to furin is a consequence of the tertiary structure of their LAP regions rather than their cleavage site. This differential processing of TGF-β1 and -β2 may be part of the mechanism that generates isoform-specific functions of the TGF-βs. J. Cell. Biochem. 103: 311–320, 2008.

Key words: TGF-beta2; latency associated peptide; furin; c-*myc* epitope; molecular tagging

The transforming growth factor-betas (TGF-βs) are members of a superfamily of multipotent growth factors. They regulate a wide range of essential cellular activities, including the control of the proliferation, differentiation and functions of many types of cells [Roberts et al., 1990]. Consequently, the dysregulation or dysfunction of the TGF-β system is a feature of an unusually large number of diseases and pathological conditions [Roberts and Sporn, 1993; Blobe et al., 2000]. For instance, TGF-β2 is present at high levels in the aqueous humor of patients with primary open-angle glaucoma (POAG) [Gottanka et al., 2004], and its latent form is secreted in breast cyst fluid at higher levels among women with lower risk of breast cancer [Erbas et al., 1999].

The mammalian TGF-β subfamily contains three isoforms, TGF-β1, -β2, and -β3, of which TGF-β2 is distinctive from the others. TGF-β2 binds to the TGF-β receptors with a lower affinity than the other isoforms, unless betaglycan is present [Rodriguez et al., 1995]. It also lacks the RGD amino-acid sequence, which enables TGF-β1 and -β3 to bind to integrin. Despite these differences, the TGF-β isoforms produce similar effects in vitro [Cordeiro et al., 2000]. However, each of the isoforms appears to regulate a different range of functions in vivo, as evidenced by the distinctive phenotypes of mice with null mutations of the individual isoform [Kulkarni

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et al., 2002]. The distinctive in vivo functions of the TGF-β isoforms are partly a consequence of their different patterns of expression: in many cell types, only one or two TGF-β isoforms are present. However, isoform-specific functions appear to occur even when all three isoforms are present at one location [Kulkarni et al., 2002; McLennan and Koishi, 2002; Makhijani et al., 2005]. Under this circumstance, the specific roles of each isoform must result from some other mechanisms, such as differential activation.

The TGF-βs are initially synthesized as large precursor molecules, which consist of a signal peptide, a latency-associated peptide (LAP) and a mature peptide [Kingsley, 1994]. The LAP regions of the TGF-βs bind to various proteins, thus modulating the subsequent transport and/or processing of the TGF-βs [Miyazono et al., 1991; Munger et al., 1997; Annes et al., 2003]. The amino acid sequences of the TGF-β LAP regions vary between the isoforms, which contrasts markedly to the structural conservation of the mature TGF-β peptides [Annes et al., 2003]. Thus, the LAP regions may contribute to isoform-specific actions. Furthermore, the TGF-β2 has a long and a short form, which are generated by the presence or exclusion of exon 2, encoding for a hydrophobic pocket domain in the LAP region [Koishi et al., 2000]. The inclusion of exon 2 creates a distinct LAP region, and the long form of TGF-β2 may therefore be processed in a manner different from the other TGF-β isoforms.

One of the key post-translational steps is the cleavage of latent-TGF-β at a site between the LAP and the mature peptide. Subtilisin-like proprotein convertases are important processors of protein factors [Nakayama, 1997]. One of them, furin, is the major physiological cleaver of TGF-β1, although other processing systems for the cleavage exist [Dubois et al., 2001]. The role of furin in the cleavage of latent-TGF-β2 is less well proven. The processing of pro-TGF-β2 is sensitive to inhibitors of convertases, which effect furin [Leitlein et al., 2001]. However, there is a poor correlation between the levels of furin and the cleavage of TGF-β2 in various cell lines [Leitlein et al., 2001]. Direct evidence for the involvement of furin in the activation of TGF-β2 is not currently available. We report here that furin only inefficiently cleaves both the long and short forms of TGF-β2, as a consequence of the isoform specific characteristics of their LAP region structures.

MATERIALS AND METHODS

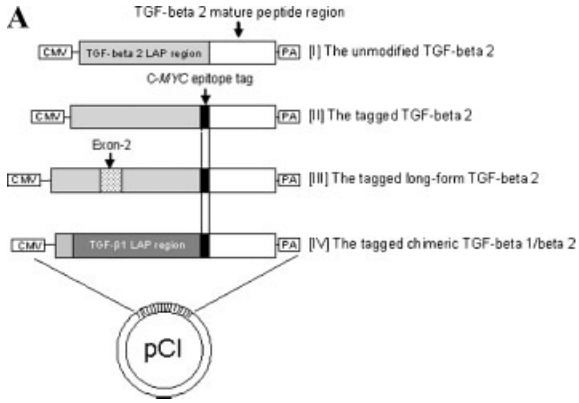
Construction of the TGF-β2 Expression Vectors

Four expression vectors were produced with the structures described in Figure 1A. All of the vectors were generated from a rat TGF-β2 cDNA plasmid clone, which contained the full-length cDNA of the short form of TGF-β2. The vector with the long form of TGF-β2 was produced by PCR amplification of the additional sequence (exon 2), using rat skeletal muscle cDNA as the template (Table I). This cDNA was then inserted into the original short form to generate the native long form of TGF-β2. A hybrid TGF-β molecule was formed by combining the TGF-β1 LAP region and the TGF-β2 mature peptide. The LAP region was generated from a mouse TGF-β1 cDNA clone (pmTGFβ1-1a, a generous gift of Dr Harold L. Moses) by PCR. The edge sequences of the mouse TGF-β1 LAP region and the original TGF-β2 clone were identical. A C-MYC epitope tag, derived from the human C-MYC gene sequence, was inserted into three of the vectors, enabling us to distinguish the TGF-β2 proteins produced from endogenous and transfected genes (Fig. 1A).

The cDNA fragments generated by PCR were fractionated through agarose gel electrophoreses and isolated using a commercial kit (QIAquick Gel Extraction kit, QIAGEN, Hilden, Germany). All of the generated cDNA fragments were inserted into pCI vectors (Promega, WI) using routine molecular biological techniques. The cDNA inserts were verified by sequencing, and the sequence data translated to confirm the amino acid sequences (Fig. 1B).

Transient Expression of the Native or Modified Forms of TGF-β2 Molecules

NIH 3T3 and CHO-K1 cells (5×10^5 cells/well) were plated in 6-well cell culture plates and incubated overnight (16–18 h) in 2.5 ml per well of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA) with 10% calf serum (Invitrogen) or F12-K medium (Invitrogen) with 10% fetal bovine serum (Invitrogen), respectively, at 37°C under an atmosphere of 5% CO_2. These cells were transfected with 2.5 μg DNA/well of the expression vectors described above using LipofectAMINE Plus (Invitrogen) and the protocol provided by the manufacturer. After transfection, the cells were incubated for 24 h. Intracellular protein lysates were then prepared by rinsing the cells with Ca/Mg free



B

[I] Unmodified TGF-β2

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  1 MHYCVLRTFL LLHLVPVALS LSTCSTLDMD QFMRKRIEAI RGQILSKLKL TSPPEDYPEP
 61 DEVPPEVISI YNSTRDLLQE KASRRAAACE RERSDEEYYA KEVYKIDMPS HFPSENAIPP
121 TFYRPYFRIV RFDVSTMEKN ASNLVKAEFR VFRLQNPKAR VAEQRIELYQ ILKSKDLTSP
181 TQRYIDSKVV KTRAEGEWLS FDVTDAVHEW LHHKDRNLGF KISLHCPCCT FIPSNNYIIP
241 NKSQELEARF AGIDGTSTYA SGDQKTIKST RKKSSGKTPH LLLMLLPSYR LESQQSSRRR
301 KR:ALDAAYCF RNVQDNCCLR PLYIDFKRDL GWKWIHEPKG YNANFCAGAC PYLWSSDTQH
361 TKVLSLYNTI NPEASASPCC VSQDLEPLTI LYYIGNTPKI EQLSNMIVKS CKCS
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[II] The tagged TGF-β2

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  1 MHYCVLRTFL LLHLVPVALS LSTCSTLDMD QFMRKRIEAI RGQILSKLKL TSPPEDYPEP
 61 DEVPPEVISI YNSTRDLLQE KASRRAAACE RERSDEEYYA KEVYKIDMPS HFPSENAIPP
121 TFYRPYFRIV RFDVSTMEKN ASNLVKAEFR VFRLQNPKAR VAEQRIELYQ ILKSKDLTSP
181 TQRYIDSKVV KTRAEGEWLS FDVTDAVHEW LHHKDRNLGF KISLHCPCCT FIPSNNYIIP
241 NKSQELEARF AGIDGTSTYA SGDQKTIKST RKKSSGKTPH LLLMLLPSYR LESQQSSRRR
301 KR:ALDAAYCF RN GEQKLISE EDL VQDNCCL RPLYIDFKRD LGWKWIHEPK GYNANFCAGA
361 CPYLWSSDTQ HTKVLSLYNT INPEASASPC CVSQDLEPLT ILYYIGNTPK IEQLSNMIVK
421 SCKCS
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[III] The tagged long-form TGF-β2

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  1 MHYCVLRTFL LLHLVPVALS LSTCSTLDMD QFMRKRIEAI RGQILSKLKL TSPPEDYPEP
 61 DEVPPEVISI YNSTRDLLQE KASRRAAACE RERSDEEYYA KEVYKIDMPS HFPSETVCPV
121 VTTSSGSVGS FCSIQSQVLC GYLDAIPPTF YRPYFRIVRF DVSTMEKNAS NLVKAEFRVF
181 RLQNPKARVA EQRIELYQIL KSKDLTSPTQ RYIDSKVVKT RAEGEWLSFD VTDAVHEWLH
241 HKDRNLGFKI SLHCPCCTFI PSNNYIIPNK SQELEARFAG IDGTSTYASG DQKTIKSTRK
301 KSSGKTPHLL LMLLPSYRLE SQQSSRRRKR :ALDAAYCFRN GEQKLISEED LVQDNCCLRP
361 LYIDFKRDLG WKWIHEPKGY NANFCAGACP YLWSSDTQHT KVLSLYNTIN PEASASPCCV
421 SQDLEPLTIL YYIGNTPKIE QLSNMIVKSC KCS
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[IV] The tagged chimeric TGF-β1/β2

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  1 MHYCVLRTFL LLHLVPVALS LSTCKTIDME LVKRKRIEAI RGQILSKLRL ASPPSQGEVP
 61 PGPLPEAVLA LYNSTRDRVA GESADPEPEP PEADYYAKEV TRVLMVDRNN AIYEKTKDIS
121 HSIYMFFNTS DIREAVPEPP LLSRAELRLQ RLKSSVEQHV ELYQKYSNNS WRYLGNRLLT
181 PTDTPEWLSF KTRAEGEWLS FDVTGVVRQW LNQGDGIQGF RFSAHCSCDS KDNKLHVEIN
241 GISPKRRGDL GTIHDMNRPF LLLMATPLER AQHLHSRRKR :ALDAAYCFRN GEQKLISEED
301 LVQDNCCLRP LYIDFKRDLG WKWIHEPKGY NANFCAGACP YLWSSDTQHT KVLSLYNTIN
361 PEASASPCCV SQDLEPLTIL YYIGNTPKIE QLSNMIVKSC KCS
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Fig. 1. The structures of the expression vectors containing the TGF-β2 derivatives. **A**: Nucleotide sequences. Diagrammatic representation of the structures of the expression vectors: [I] the native short form of TGF-β2; [II] the short-form of TGF-β2 with the c-Myc epitope tag; [III] the long-form of TGF-β2 with the c-Myc epitope tag; [IV] the chimeric molecule containing the TGF-β1 LAP and TGF-β2 mature peptide regions with the c-Myc epitope tag. The white box is the TGF-β2 mature peptide region, the narrow black box is the nucleotide element encoding the c-Myc epitope tag peptide, and the box with dots in [III] indicates the extra exon present in the long form TGF-β2. Boxes with ''CMV'' and ''PA'' are the CMV promoter and poly(A) signal element, respectively. **B**: Amino acid sequences of the TGF-β2 derivatives. The boundary of the propeptide and the mature peptide is notified by '':''. The peptide containing the *c-myc* epitope tag (GEQKLISEEDL) is typed in the bold characters. The extra peptide region of the tagged long-form of TGF-β2 [III] is underlined. The TGF-β1 LAP region in the chimeric TGF-β1/β2 molecule is underlined [IV].

TABLE I. Primer Sequences and PCR Conditions Used to Generate the Modified cDNA Fragments

	Primer sequences: forward primer, reverse primer	Dissociation temperature	Annealing temperature	Cycle number	Sequence codes
Human c-myc tag	5′-CTGCTGTACCTTCATACCGTCTA-3′ 5′-AAGAGGCGAAGGCAGCAAT-TATCCTGCAC**CAGATCTTCTTCA-GAAATAAGTTTTGTTCTCC**ATTCC-TAAAGCAATAGGCGGCATCCA-3′	94°C	58°C	25	Rat TGF-β2: AF153013 Human c-myc: NM_002467
5′ half of the rat TGF-β2 long form	5′-ACATGCACTACTGTGTGCTGA-3′ 5′-CAAGGTACCCACAGAGCACC-3′	94°C	58°C	25	Rat long form TGF-β2: AF153012
3′ half of the rat TGF-β2 long form	5′-CTGTCTGCCCAGTTGTTACA-3′ 5′-AACTCCATAGATATGGGCATGC-3′	94°C	58°C	25	AF153012
The mouse TGF-β1 LAP region	5′-CTCAGTCTGTCTACCTGCAAGAC-CATCGACATGGA-3′ 5′-TTCCGTCGCCGGCTGCTGTGTGCAG-GTGCTGG -3′	94°C	56°C	25	Mouse TGF-β1: NM_011577

The nucleotide sequence encoding for the *c-myc* epitope tag is described in bold characters. The PCRs were performed with recombinant Taq DNA polymerase (Roche Applied Science, Mannheim, Germany) or ELONGase (Invitrogen), using the buffers and protocols provided by the manufacturers.

Dulbecco's PBS and lysing them by the addition of 1.0 ml per well of a concentrated form of SDS–PAGE sample buffer (4% SDS, 0.25 M Tris-HCl, pH 6.8), which lacked both β-mercaptoethanol and a loading dye.

Western Blot Analysis With Antibodies Specific to TGF-β2 and the c-myc Epitope Tag

Fifty micrograms of each protein lysate were fractionated through 4–12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen). The proteins were transferred from the gels to PVDF membranes (Invitrogen) by electro-blotting system. For the immunochemical detection of target proteins on the Western blots, the blotted membranes were blocked by incubating in 5 w/v% BLOTTO in TBS-T (0.15 M NaCl, 10 mM Tris-HCl pH 8.0 and 0.05 v/v% Tween-20) for 1 h. Then, they were incubated with the rabbit anti-TGF-beta 2 antibody (Cat. No. SC-90, Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 0.05 μg/ml for 3 h at room temperature (r.t.), or a monoclonal anti-C-MYC antibody (9E 10, Developmental Study Hybridoma Bank under the auspices of the NICHD at the University of Iowa, Iowa City, IA 52242, USA) diluted at 1:500 for overnight at 4°C. The anti-TGF-beta 2 antibody was raised against the C-terminal of the TGF-β2 mature peptide region. The monoclonal anti-C-MYC antibody is the mouse monoclonal antibody raised against the human C-MYC epitope tag peptide [Evan et al., 1985]. After the incubation with a primary antibody, the membranes were washed with TBS-T and incubated with a donkey anti-rabbit IgG-HRP conjugate (Cat. No. NA9340, GE-Healthcare, Buckinghamshire, England, UK) diluted at 1:2,000, or a sheep anti-mouse IgG-HRP conjugate (Cat. No. NA9310, GE Healthcare) diluted at 1:2,000 for 1 h at r.t. After the incubation with the secondary antibody, the membranes were washed with TBS (0.15 M NaCl and 10 mM Tris-HCl pH 8.0), and the immunoreactive peptide signals were visualized by using a chemiluminscence detection kit (ECL, GE Healthcare) and being exposed to ECL films (GE Healthcare). After the first detection, the membranes were cleaned for further analysis by incubating them in a buffer containing 100 mM β-mercaptoethanol, 2.0% SDS, 62.5 mM Tris-HCl (pH 6.7) at 55°C for 30 min, and then rinsing them twice with TBS-T at r.t. for 10 min each.

In Vitro Treatment of the Expressed Proteins by Furin Convertase

One hundred micrograms each of the protein lysates were incubated overnight with 2 units of furin (New England Biolabs, Ipswich, MA) at 30°C. The reaction buffer was supplied by the manufacturer and contained 0.1 M HEPES (pH 7.5 at 25°C), 0.5% Triton-X100, 1.0 mM $CaCl_2$ and 1 mM β-mercaptoethanol. The processed proteins were precipitated by adding 4 volumes of cold acetone, followed by incubation overnight at −20°C. One unit of furin cleaves 25 μg of substrate to 95% completion in 6 h (New England Biolabs). Furin is a low abundance enzyme [Schalken et al., 1987; Shapiro et al., 1997], and the use of 2 units for 16 h should efficiently cleave proteins that are natural substrates of furin. The precipitates of the furin-treated proteins were collected by centrifugation at 4°C and dissolved in a SDS-PAGE sample buffer. The protein samples were fractionated through 4–12% NuPAGE Bis-Tris polyacrylamide gels, transferred to PVDF membranes and analyzed by immunochemical detection with anti-TGF-beta 2 or anti-C-MYC antibodies, as described above.

Immunocytochemical Detection of the Proteins Expressed in NIH 3T3 and CHO Cells

For immunocytochemical staining, the transfected cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (PB) for 5 min at 4°C. They were incubated in a solution containing 5 mM glycine and 10 mM PB for 10 min at r.t. and blocked with 5% sheep serum in 10 mM PB for 20 min at r.t. The cells were then incubated overnight with anti-C-MYC antibody diluted at 1:100 with a primary antibody dilution buffer containing 10 mM PB (pH 7.2), 1% bovine serum albumin (BSA), 0.1% Tween-20 and 1% NaCl at 4°C. The cells were washed and then incubated with anti-mouse IgG-HRP conjugate diluted at 1:200 with a secondary-antibody dilution buffer containing 10 mM PB (pH 7.2), 0.1% Tween-20 and 2% NaCl at r.t. for 1 h. After several washes, the immunoreactivity was visualized using AEC (Sigma, St. Louis) as the chromogen.

RESULTS

Successful Generation of the Tagged TGF-β2 Molecules

Four expression vectors containing TGF-β2 derivatives were constructed using a rat cDNA clone as a standard template. The expression vectors contained the native short form of TGF-β2, the long and short forms of TGF-β2 with a c-*myc* epitope tag and lastly, a chimeric hybrid molecule consisting of the TGF-β1 LAP region and the tagged TGF-β2 mature peptide region (Fig. 1A).

The proteins derived from the expression vectors were examined by Western blot analysis using an anti-TGF-beta 2 antibody. The NIH 3T3 transfected with the native short TGF-β2-vector produced 32.5, 44.5, and 54 kDa peptides (Fig. 2A, line 4). The presence of multiple bands is consistent with previous reports, and is thought to arise due to variations in protein processing and/or glycosylation [Madisen et al., 1990; Baillie et al., 1996]. The amount of 12.5 kDa mature peptide detected was minimal, even though the procedure efficiently detects recombinant mature peptide (Fig. 2) and mature peptide in protein extracts [McLennan et al., 1998]. The proteins produced from the short and long TGF-β2 constructs with C-MYC epitope tag mirrored those produced from the native short-TGF-β2, although the proteins were naturally 2–3 and 6–7 kDa larger than the native short-TGF-β2, due to the inserted material. As with the native short form, detectable levels of mature peptide were not present in the cells transfected with the long form of TGF-β2. The vector with the TGF-β1/β2 hybrid molecule was differentially processed, giving rise to 44.5 and 54 kDa proteins, which correspond to the two larger peptides of the native TGF-β2. The smaller 32.5 kDa protein, was not evident with the transfected CHO cells (Fig. 2B), irrespective of which vector they had been transfected with. This may be a reflection of cell-type specific variation of processing systems involved in the TGF-β de novo synthesis.

When the membranes were reprobed with an antibody to c-*myc*, proteins were only detected in the extracts of cells that had been transfected with c-*myc*-containing vectors (Fig. 3). The intensities of the signals generated by the anti-C-MYC antibody tend to be lower than the detection with the anti-TGF-beta 2 antibody. The sizes and abundances of the detected bands corresponded exactly to the bands detected by the anti-TGF-beta 2 antibody (Figs. 2 and 3). This indicates that the c-*myc* insert did not affect the processing of the TGF-β2 molecules.

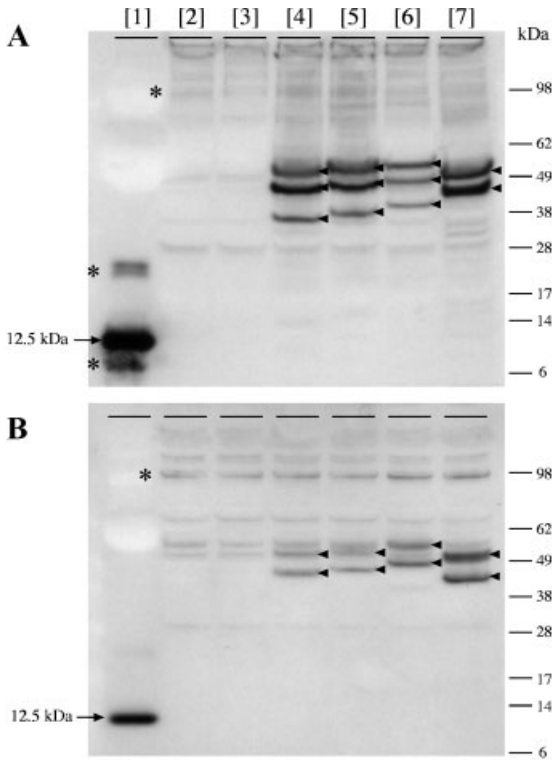

Fig. 2. Western blot analysis of the transfected cells. NIH 3T3 or CHO-K1 cells were transfected with the expression vectors described in Figure 1. The Western blot membranes with intracellular protein lysates were analyzed with anti-TGF-beta 2 antibody as described in Materials and Methods: NIH 3T3 (**A**) and CHO-K1 (**B**) cell lines. The identities of protein samples loaded to the lanes were: [1], recombinant TGF-β2 (10 ng) as a positive control; [2], the lysate from the non-transfected cells; [3], the lysate from the pCI-transfected cells; [4], the lysate from the cells transfected with the unmodified TGF-β2 expression vector ([I]); [5], the lysate from the cells transfected with the c-*myc*-tagged TGF-β2 expression vector ([II]); [6], the lysate from the cells transfected with the c-*myc*-tagged TGF-β2 expression vector ([III]); [7], the lysate from the cells transfected with the c-*myc*-tagged TGF-β1/β2 chimeric form expression vector ([IV]). The arrowheads indicate the peptides detected by the anti-TGF-beta 2 antibody. The asterisks indicate non-specific staining bands.

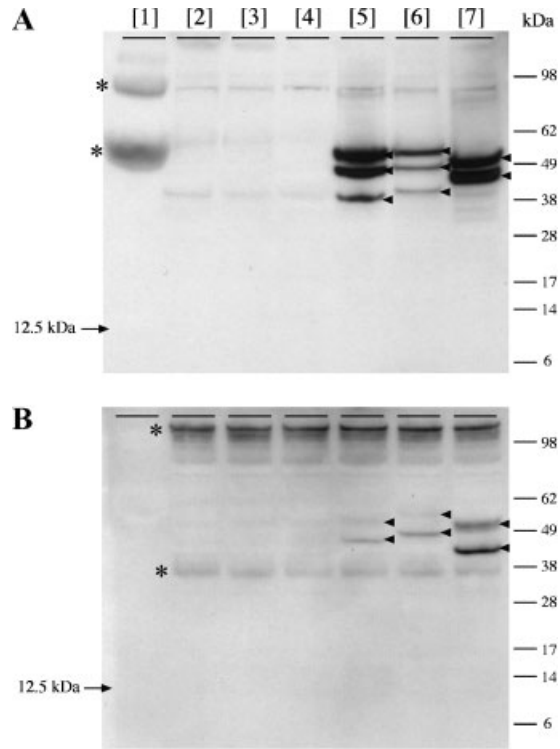

Fig. 3. Western blot analysis with anti-C-MYC antibody. The membranes analyzed with anti-TGF-beta 2 antibody (Fig. 2) were stripped and re-probed with the anti-C-MYC antibody as described in Materials and Methods: NIH 3T3 (**A**) and CHO-K1 (**B**) cell lines. The identities of protein samples loaded to the lanes were identical to Fig. 2: [1], recombinant TGF-β2 (10 ng) as a positive control; [2], the lysate from the non-transfected cells; [3], the lysate from the pCI-transfected cells; [4], the lysate from the cells transfected with the unmodified TGF-β2 expression vector ([I]); [5], the lysate from the cells transfected with the c-*myc*-tagged TGF-β2 expression vector ([II]); [6], the lysate from the cells transfected with the c-*myc*-tagged TGF-β2 expression vector ([III]); [7], the lysate from the cells transfected with the c-*myc*-tagged TGF-β1/β2 chimeric form expression vector ([IV]). The arrowheads indicate the peptides detected by the anti-C-MYC antibody. The asterisks indicate non-specific staining bands.

The Intracellular Localization of the Tagged TGF-β2 Molecule

The intracellular locations of the native and tagged TGF-β2 proteins were examined by immunocytochemistry, using anti-C-MYC and anti-TGF-beta 2 antibodies. The proteins detected by both antibodies were widely distributed in the cytoplasm in NIH 3T3 and CHO-K1 cells, with both antibodies having the same localization. The results with the anti-C-MYC antibody were illustrated in Figure 4.

The Susceptibility of the Proteins to Furin, a Proprotein Convertase

As noted above, none of the TGF-β2 derivatives generated in the transfected cells were cleaved to any significant extent. The susceptibility of the various proteins to furin convertase was therefore examined by incubating the intracellular protein lysates with recombinant furin, and examining the proteins by Western blot analysis. Furin convertase cleaved all of the TGF-β2 derivatives, but only very inefficiently:

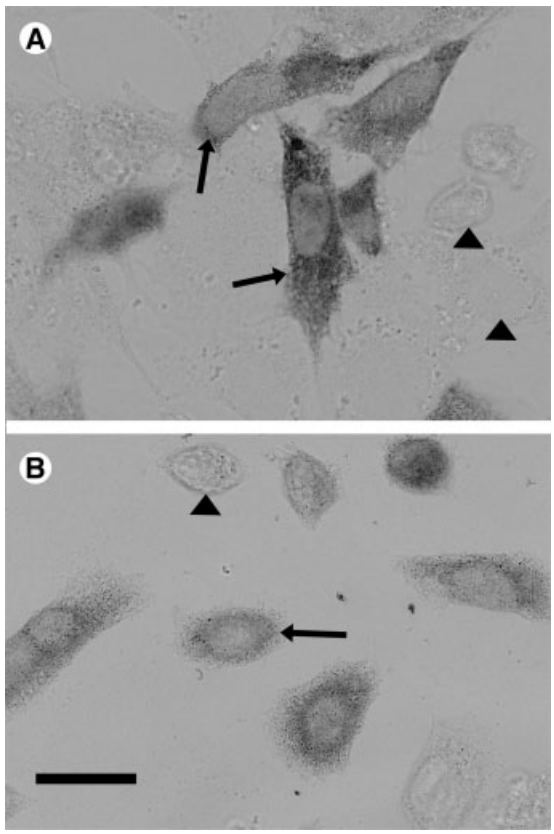

Fig. 4. Photomicrographs of NIH 3T3 and CHO cells transfected with the tagged-TGF-β2 expression vector. NIH 3T3 and CHO-K1 cells were transfected with the expression vector containing the short-form of TGF-β2 cDNA with the c-*myc* epitope tag ([II] in Fig. 1a). The location of the tagged-TGF-β2 was detected using the anti-C-MYC antibody at 24 h after transfection. **A**: NIH 3T3 and **B**: CHO-K1. The arrows indicate the cells that expressed the vector-derived proteins. The triangle markers indicate the cells that were not transfected. The bar in (B) represents 15 μm.

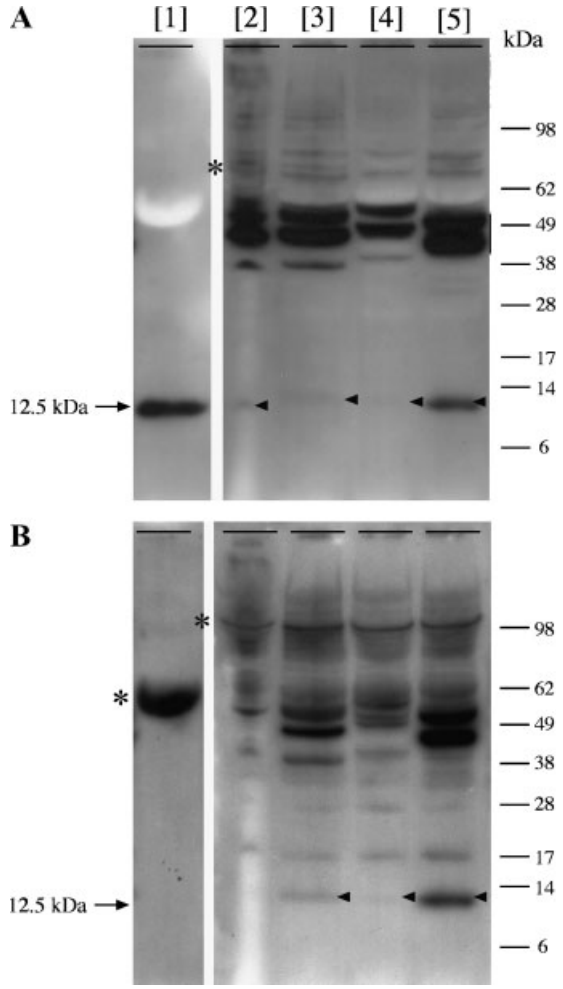

Fig. 5. Cleavage by furin convertase. The intracellular protein lysates prepared from the transfected NIH3T3 cells were processed with furin and analyzed by Western blot immunochemical detection as described in Materials and Methods. The proteins were transferred to PVDF membranes and examined with anti-TGF-beta 2 (**A**) or anti-C-MYC (**B**) antibodies. The identities of the samples are: [1], recombinant TGF-β2 (10 ng) as a control; [2], the lysate from the cells transfected with the unmodified TGF-β2 expression vector ([I]); [3], the lysate from the cells transfected with the c-*myc*-tagged TGF-β2 expression vector ([II]); [4], the lysate from the cells transfected with the c-*myc*-tagged TGF-β2 expression vector ([III]); [5], the lysate from the cells transfected with the c-*myc*-tagged TGF-β1/β2 chimeric form expression vector ([IV]). The arrows indicate peptides with sizes consistent with TGF-β2 mature peptide region. The asterisks indicate non-specific staining bands.

the bands corresponding to the large uncleaved forms of TGF-β2 remained strong, whereas the bands corresponding to the mature peptides (12.5–13 kDa) were weak, particularly for the long form of TGF-β2 (Fig. 5). Similar results were obtained with both the anti-C-MYC and anti-TGF-beta 2 antibodies.

In marked contrast to the long and short-TGF-β2 proteins, furin convertase treatment of the chimeric TGF-β1/β2 hybrid molecule resulted in the presence of an intense 13 kDa protein, which was detected by both antibodies. This indicates that furin convertase efficiently cleaved the chimeric molecule.

DISCUSSION

This study demonstrates that the cleavage of the TGF-β2 mature peptide from its pro-form is via a process distinct from that of TGF-β1. This creates a potential mechanism by which TGF-β2 and -β1 can mediate different physiological

functions at the same site, through differential activation processes.

Furin appears to be the main and physiological pro-protein convertase of pro-TGF-β1, as furin-deficient cells, or cells transfected with a furin inhibitor, have significantly impaired production of mature TGF-β1 [Dubois et al., 1995; Dubois et al., 2001]. Furin is a subtilisin-like endoprotease that cleaves pro-growth factors that contain a basic amino acid motif, RXXR [Hosaka et al., 1991; Molloy et al., 1992]. All of the mammalian TGF-β isoforms contain this motif at their cleavage sites, although the amino acid sequences are slightly different. The motif in TGF-β1 is RHRR/A and RKKR/A in TGFβ2 and TGF-β3 [Roberts and Sporn, 1990]. The RHRR/A and RKKR/A sites in other proteins are both efficiently cleaved by furin [Lazure et al., 1998; Dufour et al., 2001], As a consequence, furin was expected to cleave the proforms of all of the TGF-βs. However, both the pro-long-TGF-β2 and pro-short-TGF-β2 were inefficiently cleaved by furin in this study. In contrast, furin efficiently cleaved a chimeric molecule that consisted of the TGF-β1 LAP region, the TGF-β2 cleavage site and the TGF-β2 mature peptide (Fig. 5). This confirms that furin is able to cleave the RKKR/A site in pro-TGF-β2, but that the structure of the TGF-β2 LAP region inhibits furin.

The inability of furin to cleave TGF-β2 could arise through at least two mechanisms. First, the tertiary structure of pro-TGF-β2 is likely to be distinct from pro-TGF-β1, as the amino acid sequences of their LAP regions are distinct [Annes et al., 2003]. This could prevent furin cleaving the consensus site, by preventing furin interacting with the consensus site. Equally, the LAP region is known to interact with binding proteins [Miyazono et al., 1991; Hyytiainen et al., 2004]. Consequently, the failure of furin to cleave TGF-β2 could be due to the association of a binding protein with the TGF-β2 LAP, which could limit access of furin to its consensus site.

TGF-β2 contrasts with TGF-β1 in that it is frequently secreted in its proform, both in vitro and in vivo [Lioubin et al., 1991; Erbas et al., 1999; McCormick, 2001]. This has been paradoxical as furin is concentrated in the trans-Golgi [Takahashi et al., 1995; Nakayama, 1997], and would therefore have been expected to cleave pro-TGF-β2. The demonstration here that TGF-β2 is insensitive to furin may explain this paradox, as it opens the possibility that pro-TGF-β2 can be processed through the Golgi, without cleavage. However, the fact that TGF-β2 can be secreted in its pro-form does not imply that it is never cleaved intracellularly. In this and other studies, mature TGF-β2 was not detected in intracellular proteins. However, the presence of the smaller less abundant 32.5 kDa in NIH 3T3 cells (Fig. 2A) is consistent with some type of intracellular processing. This includes the secretion of the mature TGF-β2 without co-secretion of the LAP region. In this context, it is worth noting that our data does not exclude a role for furin in the processing of TGF-β2. As noted above, the motif site in the TGF-β2 connected with TGF-β1 LAP is very efficiently cleaved by furin, providing that inhibition from the LAP region is not present. This creates the opportunity for cell-type-specific or context-dependent cleavage of pro-TGF-β2, depending on which proteins are bound to it.

The physiological significance of TGF-β2 being secreted in its pro-form is unclear. Cleavage may simply occur extracellularly as part of the activation process. Alternatively, the pro- and cleaved forms of TGF-β2 could have different functions, in a manner similar to the neurotrophins. The pro- and mature neurotrophins have differing affinities for the low (p75) and high-affinity (trk) neurotrophin receptors, which in some circumstances can switch them from being anti-apoptotic (mature form) to pro-apoptotic (pro-form) [Lee et al., 2001; Chao and Bothwell, 2002; Lu, 2003].

The long form of pro-TGF-β2 has a hydrophobic pocket size domain in its LAP region, which is absent in the short form [Koishi et al., 2000]. Both forms of TGF-β2 were similarly processed in this study. Their intracellular distributions were grossly similar and neither was efficiently cleaved by furin. This may indicate that the long and short forms of TGF-β2 are only differentially processed in certain cell types, or that the function of the insert lies outside of the parameters examined here. One such function could be differential intracellular trafficking pathway of TGF-β2.

The expression vectors produced for this study are suitable for multiple types of investigations. The insertion of the *c-myc* tag will enable the fate of TGF-β2 produced from the transfected vectors to be distinguished from endogenous TGF-β2. The *c-myc* epitope tag was inserted near the N-terminal of the mature

peptide, just after the putative cleavage site (Fig. 1B). The site was chosen as even slight modification of the C-terminal of the TGF-β mature peptide affects its function [Wakefield et al., 1991; Qian et al., 1996]. This strategy worked well. The number and size of the polypeptides produced from the *c-myc*-containing vectors were consistent with those produced from the vector with the native form of TGF-β2. Similarly, the anti-TGF-beta 2 and anti-C-MYC antibodies produced similar patterns of immunoreactivity. This suggests that the epitope tag did not alter the de novo synthesis or protein processing of the TGF-β2 polypeptides.

The TGF-βs mediate an extra-ordinarily broad range of functions, many of which are cell-type specific and/or context-specific. This appears to necessitate that the regulation of the TGF-βs is multi-faceted. The demonstration that the LAP region influences the cleavage of the pro-TGF-βs adds a potential further mechanism to account for the diversity of TGF-β functions.

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