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Carboxy-terminal conversion of profibrillin to fibrillin at a basic site by PACE/furin-like activity required for incorporation in the matrix

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SUMMARY

Fibrillin-1, the main component of 10-12 nm microfibrils of the extracellular matrix, is synthesized as profibrillin and proteolytically processed to fibrillin. The putative cleavage site has been mapped to the carboxy-terminal domain of profibrillin-1, between amino acids arginine 2731 and serine 2732, by a spontaneous mutation in this recognition site that prevents profibrillin conversion. This site contains a basic amino acid recognition sequence (R-G-R-K-R-R) for proprotein convertases of the furin/PACE family. In this study, we use a mini-profibrillin protein to confirm the cleavage in the carboxy-terminal domain by both fibroblasts and recombinantly expressed furin/PACE, PACE4, PC1/3 and PC2. Site-directed mutagenesis of

amino acids in the consensus recognition motif prevented conversion, thereby identifying the scissile bond and characterizing the basic amino acids required for cleavage. Using a PACE/furin inhibitor, we show that wild-type profibrillin is not incorporated into the extracellular matrix until it is converted to fibrillin. Therefore, profibrillin-1 is the first extracellular matrix protein to be shown to be a substrate for subtilisin-like proteases, and the conversion of profibrillin to fibrillin controls microfibrillogenesis through exclusion of uncleaved profibrillin.

Key words: Fibrillin-1, Microfibril, Furin/PACE

INTRODUCTION

Many growth factors, hormones, neuropeptides and plasma proteins are synthesized as pro-proteins and require proteolytic processing during secretion to yield a biologically active protein. Cleavage often occurs after the paired basic amino acid residues R-R or K-R. The endoproteases responsible for this process are the family of calcium-dependent serine proteases, homologous to the yeast propeptidase Kex2 and bacterial subtilisins (Seidah et al., 1994, 1997). There are currently seven members of this family: furin/PACE (Paired basic Amino acid Cleaving Enzyme), PACE4, and the proprotein or precursor convertases (PCs), PC1/3, PC2, PC4, PC5 and PC7 (Seidah et al., 1994, 1997; Tsuji et al., 1994). The majority of these enzymes use R-X-K/R-R↓ as the cleavage motif, with the arginines in the P1 and P4 position being crucial for efficient processing.

Fibrillin-1 is the major component of 10-12 nm microfibrils of the extracellular matrix, which are widely distributed not only in mammals but also in invertebrates (Sakai et al., 1986; Reber-Muller et al., 1992; Thurmond and Trotter, 1996). Microfibrils are extracellular aggregates that confer critical biomechanical properties on a wide variety of tissues and organ

systems, either alone or in association with elastin. Little is known about how fibrillin-1 aggregates into microfibrils, the control of this process, or the organization (Ramirez, 1996). Mutations in fibrillin-1 cause Marfan syndrome, a connective tissue disorder with skeletal, ocular and cardiovascular complications.

Fibrillin-1 is synthesized as a precursor, profibrillin-1 (350 kDa), which is converted to fibrillin (330 kDa) in a calcium-dependent process (Milewicz et al., 1992; Raghunath et al., 1995). The putative cleavage site was assigned to an R-G-R-K-R-R motif in the unique carboxy-terminal domain by the identification of a point mutation in one fibrillin allele of an individual with isolated skeletal features of Marfan syndrome (Milewicz et al., 1995). This mutation alters the arginine in the P6 position of this motif to a tryptophan (R2726W). Fibroblasts heterozygous for this mutation converted only one-half of secreted profibrillin to fibrillin. In order to further characterize the cleavage site and to confirm that the furin/PACE family is responsible for processing profibrillin, we designed a construct encoding a mini-profibrillin protein, either with (mini-profibrillin) or without (short fibrillin) the unique carboxy-terminal domain. Inhibitors of profibrillin-1 proteolytic processing were also identified to determine the

effect of preventing processing on incorporation of fibrillin-1 into the extracellular matrix. We report that profibrillin is the first extracellular matrix protein identified that is a substrate for proprotein convertases of the subtilisin-like type. Furthermore, our studies indicate that this proteolytic processing is necessary for the incorporation of fibrillin into microfibrils.

MATERIALS AND METHODS

Mini-profibrillin and short-fibrillin constructs and site-directed mutagenesis

Four DNA segments representing the N terminus (exon 1), exons 23-33, exons 30-44 and the C terminus (exons 64-65) of profibrillin-1 were amplified by RT-PCR using control fibroblast RNA and primers tailed with unique restriction sites (Table 1). The DNA fragments were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced. The pGEM-T vector containing the N terminus was digested with *Xba*I and *Bsi*WI and the purified fragment ligated to the pGEM-T vector containing exons 23-33. After identification of correctly ligated clones, the leader sequence and exons 23-33 were removed from pGEM-T using *Apal*I and *Sma*I and inserted into the clone containing exons 30-44. The overlapping region of the two exon constructs contained a common *Sma*I site used to join the fragments in the proper sequence. The carboxyl terminus was inserted using unique *Bsp*EI and *Xba*I sites. The constructs were subcloned with or without the carboxy-terminal sequences into the mammalian expression vector pcDNA3 (Invitrogen, San Diego) and named p(LEEC)DNA3 and p(LEE)DNA3, respectively. The derived proteins were termed mini-profibrillin (with carboxyl terminus) and short-fibrillin (without the carboxyl terminus) (Fig. 1). Short-fibrillin has five additional amino acids (S-G-L-N-H-Stop) added to the carboxyl terminus of the EGF domain encoded by exon 44. Site-directed mutagenesis was done on the wild-type carboxy-terminal sequence in the pGEM-T vector using primers P1-S and P1-AS (Table 1). Primer pairs CTERM-S and P1-AS amplified a 200-bp 5' carboxy-terminal fragment using the wild-type carboxy-terminal clone/pGEM-T as

template. A 400-bp 3' fragment was amplified likewise using primer pair P1-S and CTERM-AS. Equimolar amounts of the amplified fragments were then used as templates to amplify the full-length carboxy-terminal fragment (584 bp) using the following conditions: denaturation at 94°C for 2 minutes, annealing at 55°C for 3 minutes and extension at 72°C for 1 minute, for 6 cycles. Primers CTERM-S and CTERM-AS were then added to the reaction and 30 cycles were done under standard conditions. Amplified fragments were cloned into pGEM-T and sequenced. The mutant carboxy-terminal fragment was then substituted for the wild-type sequence in the p(LEEC)DNA3 construct using flanking, unique restriction sites (*Bsp*EI and *Xba*I) (Figs 1, 2). The same scheme was used for site-directed mutagenesis to create the mutant P2, P4, P6W and P6A carboxyl terminus clones, using the appropriate primers (Table 1).

Generation of recombinant proteins

To obtain radiolabelled mini-profibrillin and short-fibrillin, a rabbit reticulocyte lysate system (TNT kit; Promega) was employed using 1 µg of plasmid DNA. In vitro transcription/translation was carried out in the presence of [³⁵S]cysteine (Amersham, Arlington Heights, IL). 2 µl of the respective TNT reactions were added to 50 µl of sample buffer containing 10% mercaptoethanol, and proteins were analyzed by SDS-PAGE using a 7% polyacrylamide gel and a buffer system according to Fling and Gregerson (1986). Following synthesis, the TNT reactions were pre-cleared with normal rabbit serum and Protein-G Sepharose (Pharmacia, Piscataway, NJ). The identity of the recombinant proteins was confirmed by immunoprecipitation using a rabbit polyclonal antibody directed against a bacterially expressed fusion protein that contained FBN1 amino acids encoded by nucleotides 4582-4941 (generously provided by Dr Maureen Boxer) (D'Arrigo et al., 1998).

Conversion analyses of recombinant protein

COS-1 cells were transfected with cDNAs for PACE in the expression plasmid pMT3, PACE4 in the expression plasmid pNOT, and PC2 and PC3 both in the expression plasmid pMT3SV2, as previously described (Rehmtulla et al., 1992, 1993; Wise et al., 1990). All constructs for the respective proprotein convertases

Table 1. Primers used

(A) Primers used for the generation of mini-profibrillin and short-fibrillin constructs			
Name	Size (bp)	Sequence	RE sites
Leader-S (L)	199	5'-GAATTCGCCACCATGCGTCGAGGGCGTCTG-3'	<i>Eco</i> RI
Leader-AS		5'-CCCCTCCGGATCCCCTACGGACATTGGGTCTTTAAGC-3'	<i>Bsi</i> WI and <i>Bsp</i> EI
Ex 23-33-S (E)	1466	5'-GACTCGTACGGATATAGATGAATGTGAAGTGTTC-3'	<i>Bsi</i> WI
Ex 23-33-AS		5'-TCCTTCCTTGACACAGACAGCGG-3'	None*
Ex 30-44-S (E)	1764	5'-TTGTGTTATGATGGATTCATGG-3'	None*
Ex 30-44-AS		5'-TCAGTCCGGAATTGCACTGTCTGTGGA-3'	<i>Bsp</i> EI
CTERM-S (C)	584	5'-GTCATCCGGAGCTAGCTCTGGAATGGGCATGGG-3'	<i>Nhe</i> I, <i>Xba</i> I
CTERM-AS		5'-GCTCTAGAGCTAGCTTAATGAAGCAAACCTGGAT-3'	<i>Bsp</i> EI, <i>Nhe</i> I, <i>Bsi</i> WI

**Sma*I site in FBN1 exon 32 cDNA sequence was used.

(B) Primers used for site-directed mutagenesis

Name	Sequence	Amino acid change‡
P1-S	5'-GAAACGGAGcAGCACAAACG-3'	R2731S
P1-AS	5'-CGTTTGTGCTgCTCCGTTTC-3'	
P2-S	5'-GGCAGGAAAgtAGAAGCACAAACG-3'	R2730A
P2-AS	5'-CGTTTGTGCTTCTagcTTTCCTGCC-3'	
P4-S	5'-TACCCCAAACGGGGcgcGAAACGGAGAAGC-3'	R2728A
P4-AS	5'-GCTTCTCCGTTTcgcGCCCCGTTGGGGTA-3'	
P6W-S	5'-GGTACCCCAAAtGGGGCAG-3'	R2726W
P6W-AS	5'-CTGCCCCaTTTGGGGTAGCC-3'	
P6A-S	5'-ATGGCTACCCCAAAGcGGGCAGGAAACGG-3'	R2726A
P6A-AS	5'-CCGTTTCTGCCCCgTGGGGTAGCCAT-3'	

‡Incorporation of the nucleotides in small letters results in the amino acid substitution indicated.

lacked transmembrane domains so that they were secreted in soluble form into the culture medium. Accordingly, conditioned culture media of transfected cells were concentrated up to 36-fold using Microcon-10 spin columns (Amicon, Beverly, MA). Concentrates (10 μ l) were added to 40 μ l of 50 mM sodium acetate buffer, pH 6.0. 2 μ l of TNT products and 3 μ l of 100 mM CaCl₂ (final concentration 6 mM) were added, followed by incubation at 37°C for 16-20 hours. Since both mini-profibrillin and short-fibrillin have three additional dibasic R-R sites in common, 2 μ l of either TNT reaction product were digested in 25 μ l of 0.1 M Tris/HCl/6 mM CaCl₂, pH 7.0, with 0.1 units of a specific R-R/K-R endoprotease (MobiTec, Göttingen, FRG). To monitor conversion by cell cultures, hyperconfluent fibroblasts, LoVo cells or COS-1 cells (10⁶ cells/35 mm dish) were incubated for 16-24 hours with 700 μ l of culture medium (with or without 2% FCS) containing 10 μ l of the respective TNT reactions.

Metabolic studies

Hyperconfluent fibroblasts heterozygous for the *FBNI* mutation R2726W and control cultures were depleted for 1 hour in 700 μ l of serum-free MEM lacking cysteine (Milewicz et al., 1995). Then 100 μ Ci of [³⁵S]cysteine were added per culture to pulse the cells for 1 hour. The label was removed and the cells were incubated for 24 hours in complete MEM containing 5% FCS and antibiotics. Parallel cultures were preincubated with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (DecRVKR-CMK) during the depletion step at varying concentrations (Garten et al., 1994). Fresh inhibitor was then added to the chase medium and every 8 hours thereafter. Media were collected and extracellular matrices were harvested as described (Hedman et al., 1979). Briefly, cells were solubilized 3 times with 0.5% desoxycholate, washed once with distilled water and air dried. The extracellular matrix was scraped from the dish into 250 μ l of reducing sample buffer. Media and the first desoxycholate lysate were precipitated with methanol/chloroform and pellets were redissolved in 250 μ l of sample buffer (Wessel and Flugge, 1984). 70 μ l per sample were analyzed by SDS-PAGE and autoradiography performed.

Confocal laser scanning studies of microfibrils deposited in cell culture

Fibroblasts from a normal control were seeded at a density of 10⁶ fibroblasts per 35 mm dish on glass coverslips and allowed to attach for 24 hours. The cells were incubated either in the presence or absence of 100 μ M of the furin inhibitor DecRVKR-CMK, which was replaced every 12 hours for a further 48 hours. The hyperconfluent cultures were fixed for 10 minutes in absolute methanol at -20°C and air dried. After blocking of nonspecific binding sites with 1% bovine serum albumin/10% normal goat serum in PBS, cells were incubated overnight at 23°C with monoclonal antibody mAb69 directed against fibrillin-1 (Maddox et al., 1989). After several washes in PBS, coverslips were incubated for 1 hour at room temperature with FITC-coupled goat anti-mouse IgG (Jackson Immunoresearch). Syto 17 was used as a nuclear counterstain; it has an excitation spectrum comparable to Texas Red (Molecular Probes, Leiden, The Netherlands). Preparations were mounted in Mowiol (Hoechst) in Tris/HCl, pH 8.6. Preparations were examined using a LSM 410 invert device (Carl Zeiss, Oberkochen, Germany) combined with 2 HeNe lasers (543/633 nm) and an argon laser (488 nm) for multicolor fluorescence. A 40-fold oil immersion objective (numerical aperture 1.3) was employed. Extended focus view (10 \times 0.6 μ m) section planes were obtained with identical scanning settings in order to discriminate differences in fluorescence intensities. In addition, single sections (0.6-0.7 μ m) with optimized scanning conditions per field were obtained in order to assess microfibril thickness and density. Photographs were taken on Tmax 3200 ASA (Kodak) using conventional Axiophot (Zeiss, Jena, Germany) epifluorescence equipment.

RESULTS

Characterization of the cleavage of mini-profibrillin-1

To characterize the carboxy-terminal cleavage site of profibrillin-1, we designed a construct (p(LEEC)DNA3) encoding a mini-profibrillin-1 protein with the unique amino and carboxyl termini of profibrillin-1, and a central region of the fibrillin-1 protein encoded by exons 23-44 (Fig. 1). The central region of fibrillin was chosen because we have inferred from the clustering of mutations in neonatal Marfan syndrome in exons 24-32 that this region is critical for microfibril formation (Kainulainen et al., 1994; Putnam et al., 1996). An additional construct (p(LEE)DNA3) lacked the carboxyl terminus and the protein produced by this construct was termed short-fibrillin (Fig. 1). The *in vitro* transcription/translation of p(LEEC)DNA3 and p(LEE)DNA3 produced proteins of 175 and 155 kDa, respectively (Fig. 3). Without reduction, both proteins showed faster migration with blurred bands, indicating that the proteins had spontaneously attained a disulfide-bridge-mediated conformation (data not shown). Both proteins were immunoprecipitated by a polyclonal antibody generated against the protein domains encoded by exons 37-39 (data not shown).

To test the hypothesis that members of the furin/PACE family recognize the cleavage motif in the C terminus, mini-profibrillin was incubated with protein proconvertases furin/PACE, PACE4, PC1/3 and PC2. Exposure to any of the enzymes resulted in the proteolytic processing of mini-profibrillin to a fragment that was 15.8 kDa smaller (termed mini-fibrillin) (Fig. 3). This processing was inhibited in the presence of EGTA. Short-fibrillin was not cleaved, indicating that the cleavage of mini-profibrillin occurred in the carboxyl terminus. Incubation with an R-R/K-R-X endoprotease created additional fragments for both proteins, indicating that the proprotein convertases recognized only the basic amino acid cleavage site in the carboxyl terminus (Fig. 3). Incubation of radiolabelled mini-profibrillin with dermal fibroblasts also resulted in the creation of mini-fibrillin (Fig. 4). Again, short-fibrillin was not detectably trimmed and the cleavage did not occur in the presence of EGTA. Conditioned media from dermal fibroblast cultures also contained converting activity, albeit to a lesser extent (data not shown). Incubation of mini-profibrillin with LoVo cells also resulted in complete conversion of mini-profibrillin to mini-fibrillin (Fig. 4).

Characterization of the cleavage site through site-directed mutagenesis of the basic amino acids in the P1, P2, P4 and P6 position

Earlier studies had shown that the furin/PACE family of proteases are all dependent on an arginine in the P1 position. Arginine 2731 in the P1 position of the carboxyl terminus cleavage motif of mini-profibrillin was changed to serine using site-directed mutagenesis to confirm the site of proteolytic processing (Fig. 2). This substitution prevented the cleavage of mini-profibrillin by both fibroblast cultures and proprotein convertases (Fig. 4). Therefore, the site of processing in the carboxy-terminal domain was more precisely mapped to the basic amino acid cleavage consensus sequence.

To further characterize the cleavage site requirements for the PACE/furin-like enzyme processing the mini-profibrillin protein, site-directed mutagenesis was used to alter the basic

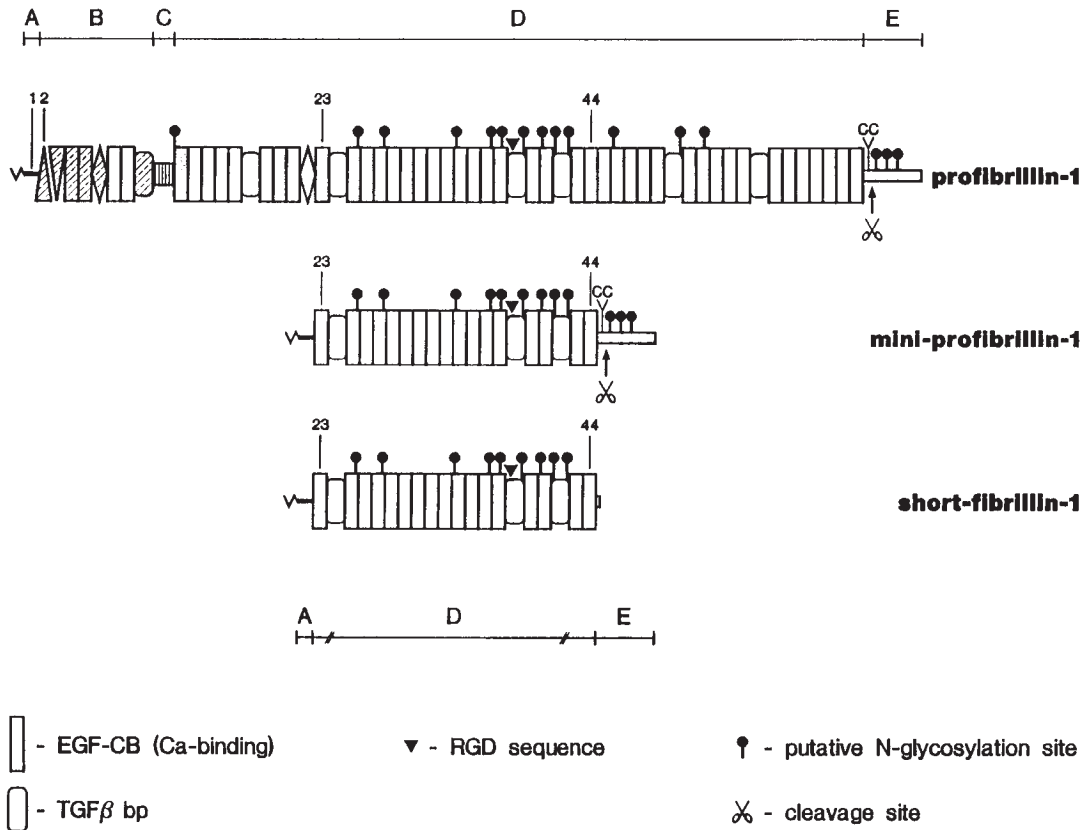


Fig. 1. Structure of wild-type profibrillin-1 (from Pereira et al., 1993) and derived mini-constructs used in this study. The mini-profibrillin construct [p(LEE)DNA3] contains the proper signals for initiation of translation and secretion from the cell. Mini-profibrillin consists of these profibrillin domains: the amino terminus, a central portion encoded by exons 23-44, and the complete carboxyl terminus harboring the putative proteolytic processing site (cleavage site). The short-fibrillin construct [p(LEE)DNA3] predicts a protein lacking the entire carboxyl terminus including the putative cleavage site.

amino acids at the cleavage site. The amino acids in the P1, P2, P4 and P6 positions were altered to neutral amino acids, either serine or alanine (Fig. 2). The P6 arginine was also altered to a tryptophan to recreate the spontaneous *FBNI* mutation that prevented processing of wild-type profibrillin (R2726W) (Milewicz et al., 1995). The recombinant proteins were then exposed to dermal fibroblasts for 24 hours, and the percentage of the protein processed was determined (Fig. 5). Similar to altering the arginine in the P1 position, altering the basic amino acid in the P4 position completely prevented cleavage. Interestingly, altering the P6 arginine to a tryptophan completely prevented cleavage, but altering the P6 arginine to an alanine only slowed the rate of cleavage. Altering the P2 arginine only slightly slowed cleavage. Therefore, the P1 and P4 arginines are required for cleavage, whereas the requirement of the P6 arginine is dependent on the amino acid to which it is altered.

Profibrillin conversion as a prerequisite for microfibrillogenesis and extracellular matrix deposition

To assess the extent to which unconverted wild-type profibrillin was incorporated into the extracellular matrix of cultured fibroblasts, the irreversible furin peptide-inhibitor DecRVKR-CMK was used to prevent profibrillin proteolytic processing (Garten et al., 1994). This compound inhibited

profibrillin conversion completely at a concentration of 50 μM in control fibroblasts (Fig. 6A). To assure complete inhibition, 75 μM of inhibitor was added every 8 hours during a 24-hour-chase metabolic labeling experiment. The inhibitor did not affect the incorporation of labeled compounds into cellular components (data not shown), general protein synthesis or protein secretion (Fig. 6B), or cell viability as monitored by Trypan Blue. In control cells without inhibitor present, processed fibrillin was the predominant form in the culture medium, and only processed fibrillin could be

Position (P)	6	5	4	3	2	1	C	1'	2'	3'
Wild Type	R	G	R	K	R	R	C	S	T	N
R2731S	R	G	R	K	R	<u>S</u>	C	S	T	N
R2730A	R	G	R	K	<u>A</u>	R	C	S	T	N
R2728A	R	G	<u>A</u>	K	R	R	C	S	T	N
R2726A	<u>A</u>	G	R	K	R	R	C	S	T	N
R2726W*	<u>W</u>	G	R	K	R	R	C	S	T	N

Fig. 2. Cleavage site (C) in the carboxyl terminus of profibrillin-1. The P position nomenclature is assigned according to Schechter and Berger (1967). The amino acids altered are underlined and in bold. The asterisk indicates the mutation that recreates the spontaneous *FBNI* mutation (R2726W) that prevents profibrillin cleavage (Milewicz et al., 1995).

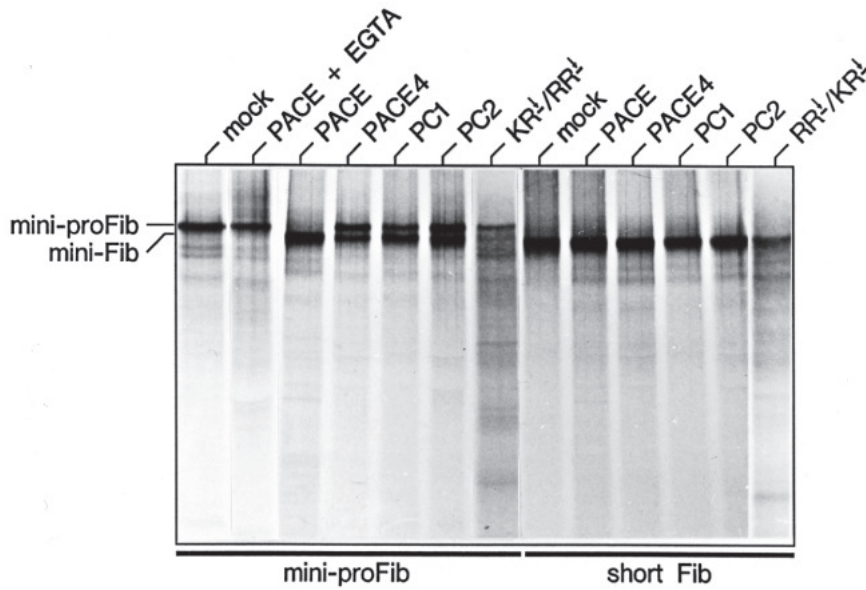


Fig. 3. In vitro cleavage of mini-profibrillin (mini-proFib) and short-fibrillin (short-Fib) by four proprotein convertases. Incubation of mini-profibrillin with any of the proteases results in the cleavage of mini-profibrillin to a single fragment (mini-fibrillin), approximately 20 kDa smaller (lanes 3-6 from the left). No cleavage occurs in the presence of either culture media from mock-transfected COS-1 cells or EGTA (lanes 1 and 2). The short-fibrillin protein is not cleaved (lanes 9-12). In contrast, an Arg-Arg/Lys-Arg-X endoprotease produces several fragments with both proteins (lanes 7 and 13). In vitro transcription/translation was performed with a rabbit reticulocyte lysate system using 1 µg of each plasmid DNA in the presence of [³⁵S]cysteine. Proteins were analyzed by SDS-PAGE using a 7% polyacrylamide gel in the presence of 10% mercaptoethanol. The removed propeptide of miniprofibrillin is not visible because it does not contain cysteine.

recovered from the extracellular matrix by a stringent extraction procedure (Fig. 6B) (Hedman et al., 1979). In cultures treated with DecRVKR-CMK, however, only profibrillin was present in the culture medium, demonstrating the efficient inhibition of profibrillin conversion. Analysis of the extracellular matrix of treated cultures revealed the absence of fibrillin and only trace amounts of unconverted profibrillin (Fig. 6B). We also studied fibrillin deposition in the fibroblast strain harboring the R2726W mutation in *FBN1* that eliminates the P6 arginine in the cleavage site of wild-type profibrillin. Culture media obtained from these cells contained profibrillin and fibrillin, whereas the extracellular matrix of these cells predominantly contained fibrillin and only trace amounts of profibrillin. Addition of the furin inhibitor greatly diminished fibrillin deposition in the extracellular matrix.

Morphological effects of impaired profibrillin conversion in cell culture

The presence of the furin inhibitor caused a diminished microfibrillar meshwork, which was only faintly immunofluorescent under identical excitation conditions (Fig. 7a,b). Optimized excitation by laser scanning, however, revealed that the density and wovenness of the fibrillin-containing microfibrillar network was comparable to that of the control cells (Fig. 7c,d). Using standard immunofluorescence techniques, individual microfibrils were thinner (Fig. 7g,h).

DISCUSSION

In this study we have demonstrated that profibrillin undergoes carboxy-terminal cleavage by a furin/PACE-like protease. The

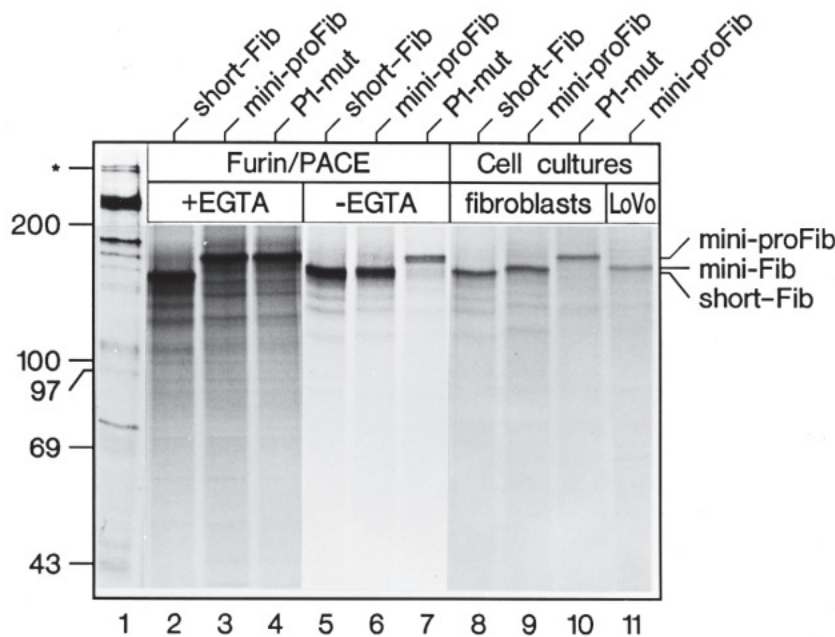
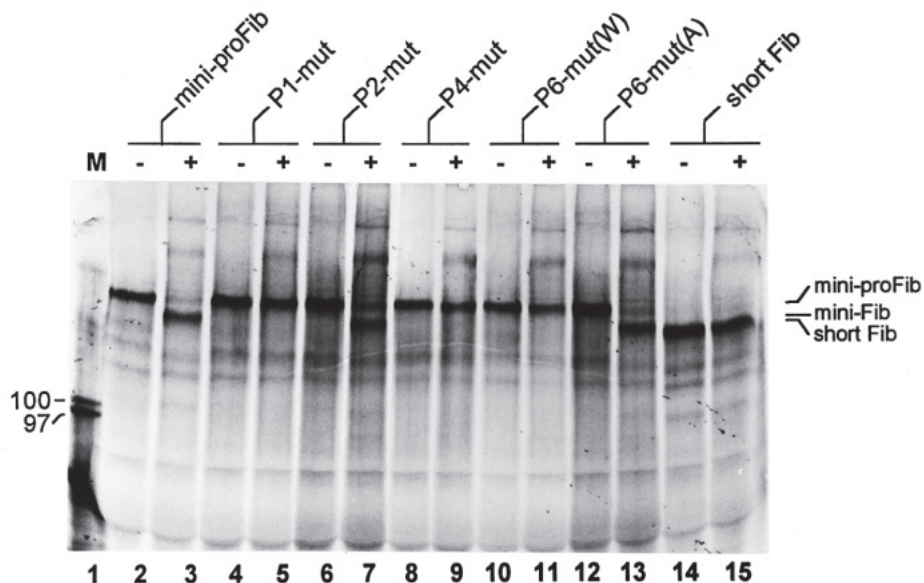


Fig. 4. Substitution of a serine for arginine in the P1 position abrogates conversion of mini-profibrillin. PACE digests of radiolabelled recombinant proteins were performed as in Fig. 3 (lanes 5-7). Lanes 2-4 show PACE digests in the presence of EGTA. The mutagenized mini-profibrillin with a substitution of the P1 arginine by serine (P1-mut) cannot be cleaved by PACE. When these proteins were added to hyperconfluent fibroblast cultures or the cell line LoVo, mini-profibrillin (mini-proFib, lanes 9 and 11) was cleaved to mini-fibrillin. The short-fibrillin (short Fib, lane 8) and the P1-mutated mini-profibrillin were not cleaved (lane 10). Note that short-fibrillin migrates slightly faster than mini-fibrillin. This is due to a predicted difference of 39 amino acids between the two proteins. Lane 1 shows culture medium from metabolically labelled fibroblasts; the asterisk indicates wild-type profibrillin and fibrillin. The positions of molecular mass markers (kDa) are shown.

Fig. 5. Analysis of mini-profibrillin processing by hyperconfluent dermal fibroblasts. Following *in vitro* transcription/translation, peptide products were exposed to hyperconfluent dermal fibroblasts, or incubated at 37°C without exposure to fibroblasts, for 24 hours. The samples were separated by SDS-PAGE and visualized by autoradiography. Lanes are labeled M (molecular mass markers), -, without and +, with exposure to fibroblasts. Molecular masses of ¹⁴C-labeled markers are indicated on the left side, the migration of mini-proFib and mini-Fib on the right side. Specific mutants are indicated above the figure.



lipophilic and intracellularly active furin-inhibitor decanoyl-R-V-K-R-chloromethylketone inhibited profibrillin conversion in cell culture (Garten et al., 1994). The inhibition could occur in three ways: inhibition of secreted or cell membrane-associated furin/PACE, cross-inhibition of the genuine profibrillin convertase, or inhibition of the activation of the genuine profibrillin convertase by furin/PACE (proprotein convertases contain the R-X-K-R motif near the amino terminus and cleavage at this site is required for activation). Further support that a furin-like enzyme is responsible for the cleavage is the

fact that recombinantly expressed furin/PACE, PACE4, PC1/3 and PC2 were all able to cleave the mini-profibrillin protein, although activation of another protease by these expressed enzymes cannot be excluded. The scissile bond was identified between the P1 arginine 2731 and the P1' serine 2732 by site-directed mutagenesis of the arginine, which inhibited processing by both fibroblasts and furin/PACE. The basic amino acid requirements at the cleavage site in profibrillin were further characterized by altering the P2, P4 and P6 basic amino acids, then testing the ability of human fibroblasts to cleave the

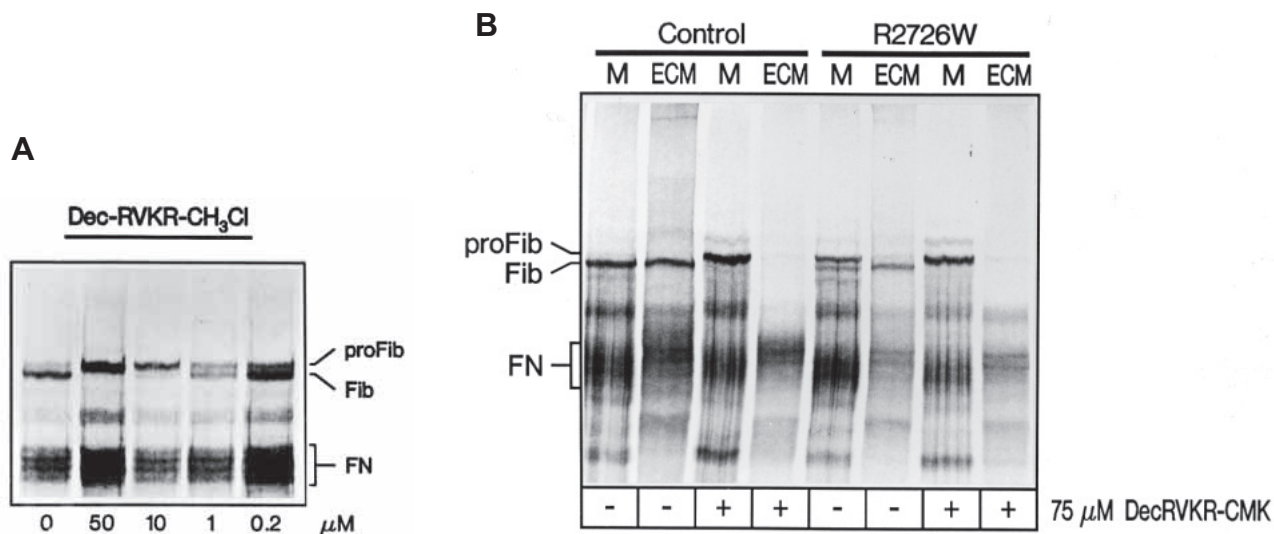


Fig. 6. (A) Variable amounts of the furin-inhibitor DecRVKR-CMK were applied to confluent fibroblast cultures as a single dose during a 4-hour pulse period with [³⁵S]cysteine, after preincubation for 1 hour. There is a clear dose-dependent inhibition of the conversion of profibrillin (proFib) to fibrillin (Fib), which is completely inhibited at 50 μM. FN, fibronectin. (B) Only converted profibrillin is deposited into the extracellular matrix. Cells from normal dermal fibroblasts and the fibroblast line heterozygous for the R2726W mutation (P6 mutation of the profibrillin cleavage site) were pulsed for 1 hour with [³⁵S]cysteine and chased for 24 hours. The furin inhibitor DecRVKR-CMK was added after preincubation of the cells for 1 hour and fresh inhibitor was added every 8 hours. The presence of profibrillin (proFib) and fibrillin (Fib) was monitored in culture medium (M) and extracellular matrix extracts (ECM). When conversion of profibrillin to fibrillin was blocked, no fibrillin or profibrillin was deposited in the extracellular matrix. Note that the deposition of the extracellular matrix fibronectin (FN), which does not contain a tetrabasic cleavage site for proprotein convertases of the furin/PACE family, is not affected.

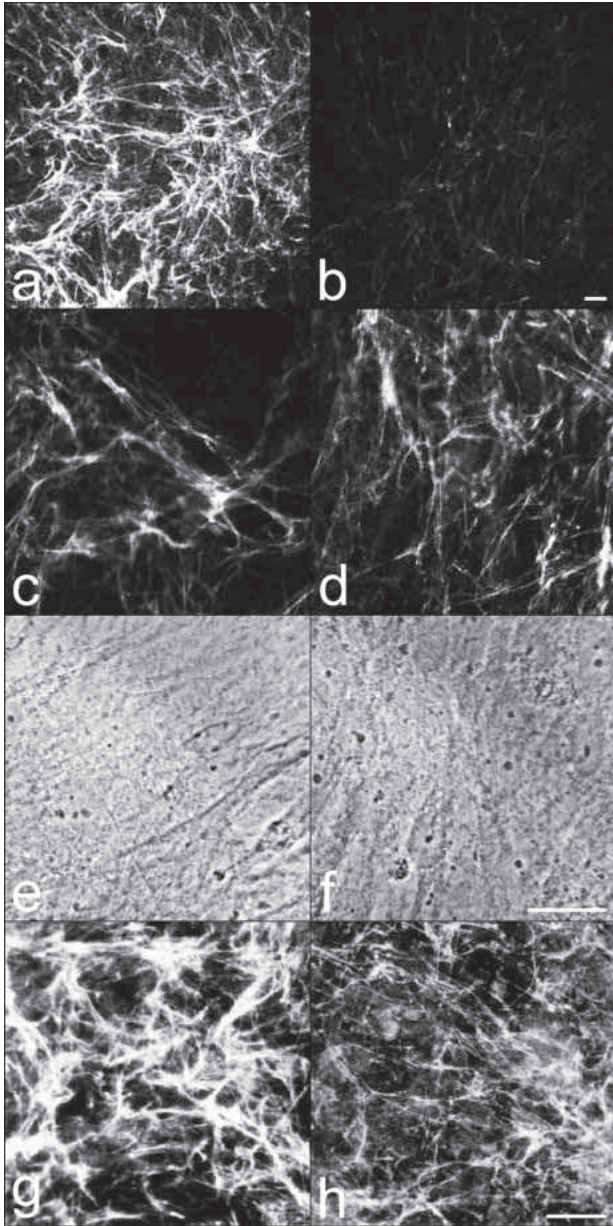


Fig. 7. Morphological appearance of the microfibrillar matrix in cultured fibroblasts after inhibition of the profibrillin to fibrillin conversion. Hyperconfluent normal fibroblasts were treated with 100 μM of the furin inhibitor DecRVKR-CMK (b,d,f,h) every 12 hours for a further 48 hours, and the control culture with vehicle only (a,c,e,g). The microfibrillar meshwork was visualized by indirect immunofluorescence labeling of fibrillin (a-d). (a,b) An extended focus view as obtained by confocal laser scanning under identical excitation conditions. This analysis reveals markedly diminished microfibril deposition in the treated culture (b) in comparison to the control (a). Analysis of a 0.6 μm optical section at high magnification of the control culture (c) and with increased laser output for optimized signal yield in the treated culture (d) reveals that the density and woveness of the microfibrillar matrix is mainly comparable with a prevalence of thinner microfibrils giving a sharp linear immunostaining signal (d). (e,f) Phase-contrast images scanned from the identical areas as in a-d, demonstrating comparable cell density and cell shape in the treated cultures. (g,h) Conventional immunofluorescence with automatic exposure time reveals the overall appearance of thinned microfibrils in the treated cultures. Bars, 20 μm .

mutant mini-profibrillin protein. The P4 basic amino acid was required for cleavage; altering this amino acid also completely prevented cleavage. Altering the P6 arginine slowed cleavage when it was mutated to an alanine, but completely prevented cleavage when mutated to a tryptophan. These results help to validate the experimental system, since the spontaneous profibrillin mutation (R2726W), altering the P6 arginine to a tryptophan, prevents processing of profibrillin (Milewicz et al., 1995). The characterization of the cleavage site allowed the exclusion of PC1/3 and PC2 as profibrillin convertases since these enzymes required only dibasic amino acids at the cleavage site (either R-R or K-R). In general, it is found that furin/PACE prefers to cleave precursors at the general R/K-X-R-X-R/K-R \downarrow motif (Rehemtulla et al., 1992; Seidah et al., 1997), similar to the characterized motif for profibrillin. The basic amino acids required for PACE4 cleavage are similar to those for furin/PACE (Rehemtulla et al., 1993). PC5 has been shown to best cleave precursors with R-X-X-R \downarrow . The cleavage motifs for the other convertases have not been well characterized.

It should be noted that the amino terminus of profibrillin-1 also contained an R-X-K-R-R site, predicting the removal of about 20 amino acids (Pereira et al., 1993). Recent observations suggested that this cleavage site was used with the eukaryotically expressed amino-terminal half of fibrillin-1 that contained the addition of the BM40 leader sequence before the regular fibrillin leader sequence (Reinhardt et al., 1996). We have also studied the cleavage of the amino-terminal domain of the mini-profibrillin construct by both CHO-K1 cells and baculovirus cells (unpublished). Amino-terminal sequencing of secreted protein revealed leader sequence processing at two sites: between glycine 24 and alanine 25, and between alanine 27 and asparagine 28. Both eukaryotic systems also cleaved the protein in the carboxy-terminal domain of the protein. Further studies are needed to determine if the amino-terminal site is cleaved *in vitro*, and the role this cleavage plays in the incorporation of fibrillin into the matrix.

Based on the basic amino acids required for cleavage, only PC1/3 and PC2 can be excluded as the enzymes responsible for processing profibrillin. PC4 is expressed in germ cells, so it is not a likely candidate for the convertase responsible for profibrillin processing. Furin/PACE, the best-characterized member of the subtilisin-like family, is ubiquitously expressed and is a membrane-bound enzyme that is active in the trans-Golgi network, but is known to cycle between the trans-Golgi and the cell surface (Klimpel et al., 1992). Recombinant overexpressed PACE can be secreted into the medium, suggesting that a similar pathway may exist for cells that naturally express PACE (Vey, 1994; Creemers et al., 1996). PC5 and PC7 are widely expressed and are located in dense core secretory granules and the trans-Golgi network. PC5 does not have a transmembrane domain, whereas PC7 does. PACE4 is also widely expressed but little is known about its cellular localization. Of interest is the fact that LoVo cells synthesize primarily PACE4 and an inactive furin/PACE, but are shown here to be able to cleave the mini-profibrillin protein (Takahashi et al., 1995; Seidah et al., 1994). Therefore, many of the furin-like enzymes, including PC5, PC7 and PACE4, are candidates for the enzyme responsible for profibrillin processing. The ability of many of the expressed furin/PACE-like enzymes to properly process the mini-profibrillin indicates

that the enzyme responsible for the proteolytic processing may be dependent on which of the enzymes are expressed by the cells that are synthesizing profibrillin.

The exclusion of profibrillin from the matrix in the presence of the furin inhibitor provides evidence that profibrillin processing is necessary for matrix incorporation. The observation that fewer microfibrils are formed when profibrillin processing is prevented by the furin inhibitor also helps to establish that profibrillin processing is necessary for the incorporation of fibrillin into microfibrils, although the furin inhibitor could potentially be acting on other proteins necessary for microfibril formation. The clinical relevance of the proprotein convertases is significant, since these enzymes are involved in the processing of neuropeptides, neurotrophins, growth factors, renin, endothelin, HIV, insulin and related peptides, and parathormone and parathormone-like peptides, and matrix metalloproteases (Seidah et al., 1994; Pei and Weiss, 1995). The evidence presented here adds profibrillin to the list of proteins that are processed by the PACE/furin family of enzymes, the first extracellular matrix protein that has been shown to be processed by these enzymes. Impaired profibrillin processing, such as that due to the R2726W mutation identified in an individual with isolated skeletal features of Marfan syndrome, suggests that the availability of fibrillin for microfibrillogenesis during skeletal development is crucial (Milewicz et al., 1995). This fact raises the possibility that the profibrillin convertase is a modulator of skeletal growth. Accordingly, mutations can be envisaged that impair profibrillin convertase and result in skeletal features of Marfan syndrome.

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