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Fibrillin assembly: dimer formation mediated by amino-terminal sequences

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SUMMARY

We have investigated recombinant fibrillin-1 (profib-1) and fibrillin-2 (glyfib-2) molecules encoding the proline- or glycine-rich regions with flanking domains (exons 9-11), in order to establish whether these sequences might mediate specific molecular recognition events important in fibrillin assembly. Our data demonstrate that both recombinant molecules can form extracellular dimers, but highlight subtle differences in the stability of these dimers. Following expression in COS-1 cells, SDS-PAGE analysis showed that glyfib-2 was present intracellularly as monomers, and extracellularly as monomers and disulphide-bonded dimers. Size fractionation in native non-reducing conditions prior to SDS-PAGE analysis highlighted that glyfib-2 also formed non-covalent associations. In contrast, profib-1 appeared monomeric in cells and medium. Using an in vitro translation system supplemented with semipermeabilised HT1080 cells together with chemical crosslinking, dimers of the fibrillin-1 and fibrillin-2 molecules were detected. Dimerisation was not cell-dependent since molecules translated in the absence of cells dimerised, and was not an intracellular event as judged by proteinase K digestions. A crosslinking and coimmunoprecipitation strategy provided a means of investigating whether molecular chaperones might be involved in preventing dimerisation of translocated molecules. Proteinase K-resistant recombinant molecules associated rapidly with BiP, and thereafter with protein disulphide isomerase and calreticulin. Differences between the two fibrillin isoforms in ability to form stable dimers prompted investigation of the proline- and glycine-rich sequences. Differences in solubility and pI were apparent that may contribute to reduced stability of proline-rich region interactions. These studies suggest that extracellular dimer formation mediated by interactions of the proline- and glycine-rich regions may be a crucial early step in the extracellular assembly of fibrillin into microfibrils.

Key words: Fibrillin, Dimer, Microfibril assembly

INTRODUCTION

Fibrillins are large cysteine-rich glycoproteins (of approx. 350 kDa), which form the molecular scaffold of a class of beaded microfibrils that are key structural elements of dynamic connective tissues (Kielty and Shuttleworth, 1995). These microfibrils are extensible polymers which, during elastic fibre formation, act as a structural lattice for elastin deposition and determine the direction of growth of elastin fibres (Mecham and Heuser, 1991). Linkage of the fibrillin-1 and fibrillin-2 genes to the heritable connective tissue disorders Marfan syndrome and congenital contractural arachnodactyly, respectively, highlights their critical contribution to connective tissue integrity (Ramirez, 1996).

Fibrillins have a cysteine-rich multidomain organisation dominated by calcium-binding consensus sequences (cbEGF-like domains) interspersed with eight-cysteine-containing motifs (TB modules; Pereira et al., 1993; Zhang et al., 1994). The contiguous arrays of cbEGF-like domains form rod-like structures in the presence of calcium (Downing et al., 1996; Reinhardt et al., 1997). Each isoform contains a unique hydrophobic sequence towards the amino terminus that may act as a potential molecular hinge; in fibrillin-1 this sequence is proline-rich and in fibrillin-2 it is glycine-rich. Amino- and carboxy-terminal fibrillin sequences contain furin/PACE proprotein convertase tetrabasic consensus sequences, and processing at these sites may be important regulatory steps in fibrillin assembly (Raghunath et al., 1999; Ritty et al., 1999).

The molecular pathway of fibrillin assembly remains poorly understood, and intermediate assemblies have proved difficult to define due to the large size of these cysteine-rich glycoproteins and their propensity to form disulphide-bonded aggregates (Sakai, 1990). The possibility that dimers may occur has been suggested on the basis of non-reducing SDS-PAGE analysis of metabolically labelled fibrillin immunoprecipitated from cell culture medium (Kielty and Shuttleworth, 1993). The precise arrangement of fibrillin monomers within microfibrils also remains to be established. A parallel head-to-tail alignment model of unstaggered fibrillin monomers with amino- and carboxy-termini at, or close to, the beads has been suggested on the basis of antibody epitope mapping and measured molecular dimensions (Reinhardt et al., 1996). Alternative staggered arrangements based on extrapolation of molecular length from cbEGF-like domain dimensions (Downing et al., 1996), or on alignment of

transglutaminase crosslink sites (Qian and Glanville, 1997), have also been proposed.

In this study, we have investigated human fibrillin assembly using a recombinant approach. We targetted the fibrillin-1 and fibrillin-2 sequences encoding the proline- or glycine-rich regions (exons 10) in order to establish whether these distinctive regions of each molecule might act as molecular recognition sites, and asked whether they mediate formation of intermediates such as dimers intracellularly or extracellularly, and what cellular mechanisms exist to regulate such associations. Our data demonstrate that recombinant fibrillin-1 and fibrillin-2 peptides can form dimers, but highlight subtle differences in dimer stability. These associations may be an early step in the extracellular assembly of fibrillin into microfibrils.

MATERIALS AND METHODS

Fibrillin cDNAs

Two cDNAs were generated by reverse transcriptase-polymerase chain reaction (Fig. 1). They were (1) a three-domain fibrillin-1 cDNA (complete exons 9-11) encoding the first TB module, proline-rich region and EGF domain (profib-1) generated using human dermal fibroblast mRNA, and (2) the corresponding glycine-rich peptide (glyfib-2) generated using human keratinocyte mRNA. These profib-1 and glyfib-2 cDNAs were subcloned into the mammalian expression vector pSecTag (Invitrogen). This vector has a secretion signal peptide from the V-J2-C region of mouse Ig kappa chain that allows efficient translocation and secretion of recombinant proteins from mammalian cells. cDNAs encoding just the proline-rich region of fibrillin-1 and the glycine-rich region of fibrillin-2, respectively, were generated by RT-PCR and then subcloned into inducible prokaryotic pGEX expression vectors. Full sequences of all cDNAs, and their correct vector insertion, were confirmed by dye-terminator automated sequencing.

Generation of recombinant proteins in COS-1 mammalian cells

Profib-1 and glyfib-2 constructs for expression in COS-1 cells had an in-frame c-myc epitope and a six-histidine residue tag at their 3′ end that enables purification using ProBond resin (Invitrogen). COS-1 cells were transfected with the profib-1 and glyfib-2 construct in pSecTag (Invitrogen) by electroporation and incubated for 1 week at 37°C in Dulbecco's modified Eagle medium (DMEM) with 1% fetal calf serum, 1% streptomycin, penicillin and glutamine, which had been pre-cleared with ProBond resin. Cellular debris was removed from the medium by centrifugation at 4000 ***g*** for 15 minutes and the supernatant incubated at 4°C for 18 hours with 2 ml of ProBond resin. Resin was collected in a scintered glass funnel and washed with buffer (20 mM sodium phosphate, pH 7.8) and then with buffer + 500 mM NaCl. Recombinant protein was eluted with buffer containing 200-300 mM imidazole and purified in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl by size fractionation with an AKTA Purifier system using a Superdex 200 PC 3.2/30 column.

In some experiments cells were pulsed in medium containing 500 μCi ^{35}S-cysteine for 30 minutes then chased for 30 minutes to 20 hours in cold medium. At each chase-time point, cells were extracted directly into immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100) in the presence of protease inhibitors (2 mM phenylmethanesulphonyl fluoride and 5 mM N-ethylmaleimide), and corresponding medium samples were also collected. Recombinant molecules were then immunoprecipitated from cell extracts and medium using the anti c-myc antibody (1:400) plus 50 μl of protein A Sepharose (10% w/v in PBS) for 18 hours at 4°C. Protein A Sepharose was pelleted by centrifugation at 10,000 ***g*** for 1 minute, then washed twice in immunoprecipitation buffer, once in immunoprecipitation buffer with 500 mM NaCl and once in immunoprecipitation buffer alone. The samples were boiled in SDS-PAGE loading buffer in the presence of 5% (v/v) β-mercaptoethanol for 5 minutes before centrifuging for 1 minute at 10,000 ***g*** and loading the supernatant onto a 15% SDS-PAGE gel.

Western blotting

Recombinant profib-1 and glyfib-2 purified from COS-1 cell medium were separated by 15% SDS-PAGE in the presence or absence of 50 mM dithiothreitol, then stained with Coomassie Brilliant blue or blotted onto nitrocellulose membrane using a semi-dry blotting apparatus. The recombinant molecules were identified by western blotting using a primary antibody to the c-myc epitope (Invitrogen) at a 1:5,000 dilution, a secondary anti-mouse IgG-peroxidase conjugate (Sigma Chemical Company, Dorset, UK) at a 1:3,000 dilution and enhanced chemiluminescence (ECL) detection (Amersham, UK).

Preparation of semi-permeabilised HT1080 cells

Permeabilisation of HT1080 cells was performed as previously described (Lees et al., 1997; Wilson et al., 1995; 1998; Ashworth et al., 1999; Hindson et al., 1999). A 75 cm^3 flask of HT1080 cells was grown to confluency, trypsinised and resuspended in 8 ml KHM buffer (10 mM potassium acetate, 20 mM Hepes, pH 7.2, 2 mM magnesium acetate) containing 100 μg/ml soya-bean trypsin inhibitor and pelleted at 840 ***g*** for 3 minutes. Cells were resuspended in 6 ml KHM buffer containing 40 μg/ml digitonin and incubated in ice for 5 minutes. KHM (8 ml) was added to terminate permeabilisation, the cells pelleted and resuspended in 11 ml of 50 mM Hepes, pH 7.2, 90 mM potassium acetate. After 10 minutes, the cells were pelleted and resuspended in 100 μl KHM. 10 μg/ml staphylococcal nuclease and 1 mM $CaCl_2$ were added and the mixture incubated at room

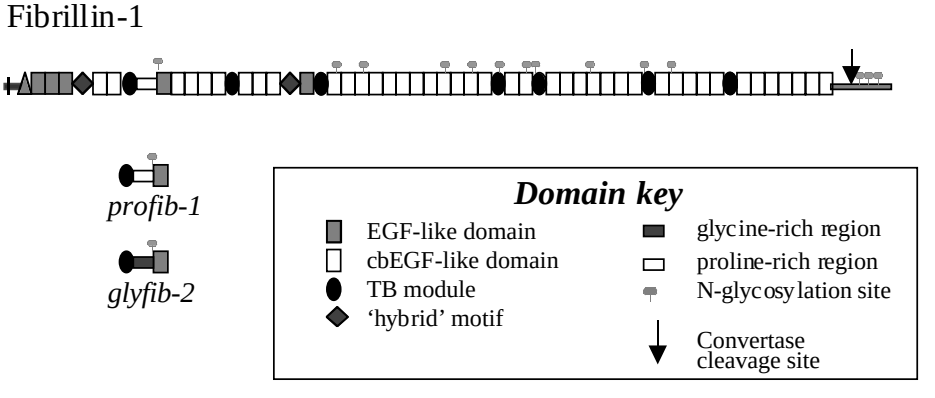

Fig. 1. Human fibrillin-1 and fibrillin-2 cDNAs encoding the recombinant molecules used in this study. Three-domain recombinant molecules were encoded by exons 9-11 of fibrillin-1 (profib-1) and fibrillin-2 (glyfib-2), respectively.

temperature for 12 minutes. The reaction was terminated by adding EGTA to a concentration of 4 mM and the cells pelleted. They were resuspended in 100 μl KHM.

Transcription and in vitro translation

Fibrillin-1 cDNAs subcloned in vector pSecTag were linearised with *Pme*I (New England Biolabs), then purified by phenol-chloroform extraction. Carboxy-terminal tags were not expressed in this system since the DNA is linearised by cleavage in the vector multiple cloning site. Transcription was performed using T7 RNA Polymerase (Promega, Southampton, UK) at 37°C for 3 hours followed by column RNA purification (Qiagen) and elution in 50 μl RNase-free water, with 1 mM dithiothreitol and 40 units RNase inhibitor (Boehringer-Mannheim) added. RNA was heated to 60°C for 10 minutes prior to use, to disrupt secondary structure.

RNA was translated using a rabbit reticulocyte lysate (Flexilysate, Promega, Southampton, UK) for 90 minutes at 30°C. Each reaction contained 35 μl lysate, 1 μl RNase inhibitor, 1 μl amino acids minus cysteine, 1 μl KCl, 2.5 μl ^{35}S-cysteine (25 μCi; ICN) and 2.5 μl prepared RNA. Translations were carried out for up to 90 minutes. The HT 1080 cells were then rigorously washed three times in KHM buffer, then lysed and size-fractionated directly, or boiled in SDS-PAGE loading buffer with or without 5% β-mercaptoethanol, then resolved on 10% or 15% SDS-PAGE gels. In some experiments, samples were removed from translation mixtures at intervals between 5 and 90 minutes and translation was terminated by addition of cyclohexamide to a final concentration of 1 mM.

Proteinase K and endoglycosidase H digestions were carried out on washed cell pellets as previously described (Lees et al., 1997; Wilson et al., 1995, 1998). Size fractionation of native non-reduced labelled recombinant fibrillin was carried out on an AKTA Purifier system using a Superdex 200 PC 3.2/30 column (volume 2.4 ml, fraction size 50 μl, buffer 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl).

Fibrillin is thus the only radiolabelled protein generated, although it is not expressed at a high level in this system.

Crosslinking of dimers and immunoprecipitation

Chemical crosslinking and immunoprecipitations were performed as previously described (Lees et al., 1997; Wilson et al., 1998). Profib-1 and glyfib-2 (cDNA linearized with *Pme*I) were translated for 5-90 minutes in separate reactions, and translations were then terminated with 1 mM cyclohexamide. Incubation with bismaleimidohexane (BMH) or dithiobis(succinimidyl proprionate) (DSP; Pierce and Warriner Ltd., Cheshire, UK) was then carried out for 10 minutes at 4°C at final concentrations of 1 mM (from a 50 mM stock solution freshly prepared in DMSO), followed by a further 10 minute incubation with dithiothreitol or glycine to quench the BMH or DSP reactions, respectively. The semi-permeabilised cells were then pelleted and washed three times, and the washed cell pellet was resuspended in 50 μl of KHM buffer. Samples were denatured by boiling for 5 minutes in denaturation buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl with 1% (w/v) SDS and 1% (v/v) Nonidet P-40). Insoluble material was removed by centrifugation at 13,000 ***g*** for 10 minutes and the supernatant was adjusted to a final volume of 1 ml with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100). The following antibodies were used for immunoprecipitations: polyclonal antibodies to PDI (1:400), calreticulin (1:400) and BiP (Grp78) (1:400) (Stressgen Biotechnologies Corp., UK) and a polyclonal antibody to calnexin (1:200) (supplied by Dr S. High, School of Biological Sciences, University of Manchester). Immunoprecipitations were preincubated at 4°C for 40 minutes with 50 μl of protein A Sepharose (10% w/v in PBS) (Sigma Chemical Company, Dorset, UK). Samples were centrifuged for 1 minute at 10,000 ***g***, and the supernatants incubated with primary antibody plus 50 μl of protein A Sepharose (10% w/v in PBS) for 18 hours at 4°C. Protein A Sepharose was pelleted by centrifugation at 10,000 ***g*** for 1 minute, then washed twice in immunoprecipitation buffer, once in immunoprecipitation buffer with 500 mM NaCl and once in immunoprecipitation buffer alone. The samples were boiled in SDS-PAGE loading buffer in the presence of 5% (v/v) β-mercaptoethanol for 5 minutes before centrifuging for 1 minute at 10,000 ***g*** and loading the supernatant onto a 15% SDS-PAGE gel.

Generation of recombinant peptides in prokaryotic cells

The proline- and glycine-rich regions were each expressed as single domains in *E. coli* DH5α cells using the isoprolyl-β-D-thiogalactosidase (IPTG)-inducible prokaryotic pGEX expression vector system. The proline-rich region was expressed using the pGEX-4T-1 vector, and the glycine-rich region using the pGEX-4T-3 vector. Recombinant proteins expressed in this system were produced as glutathione S-transferase (GST) fusion proteins, which were readily purified by glutathione-Sepharose affinity chromatography. Purified recombinant proteins were eluted following thrombin cleavage from the immobilised GST fusion tag.

RESULTS

Expression of recombinant three-domain peptides in COS-1 cells

Three domain profib-1 and glyfib-2 peptides (TB module, proline- or glycine-rich region, EGF-like domain; exons 9-11) were transiently expressed in COS-1 cells using the pSecTag vector, and following secretion were purified from culture medium by nickel resin chromatography. Profib-1 eluted in 20 mM sodium phosphate pH 7.8 containing 300 mM imadazole, whereas glyfib-2 eluted in the column wash (20 mM sodium phosphate, pH 7.8 containing 500 mM NaCl). The peptides were examined by SDS-PAGE in reducing or non-reducing conditions, and detected by Coomassie Blue staining and western blotting using the anti-c-myc antibody. N-terminal peptide sequencing confirmed the identity of both peptides.

In reducing conditions, profib-1 and glyfib-2 each had apparent molecular masses of 32 kDa and were N-glycosylated as judged by increased mobility following N-glycosidase F treatment (not shown). In non-reducing conditions, profib-1 always appeared as a monomer (Fig. 2A), whereas glyfib-2 was present as monomers (28 kDa), dimers (55 kDa) and tetramers (110 kDa) (Fig. 2B). N-terminal sequencing of the glyfib-2 dimer band revealed a single signal.

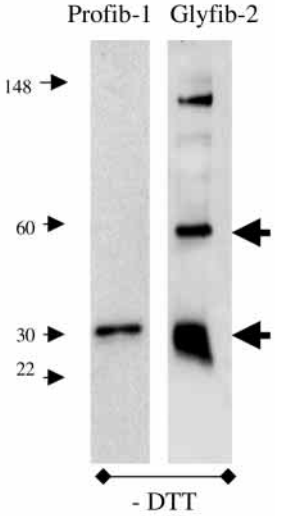

Fig. 2. Western blotting of profib-1 and glyfib-2 secreted from COS-1 cells. Recombinant molecules were purified from the medium of COS-1 cells, electrophoresed on 15% SDS-PAGE gels in non-reducing conditions (–DTT), then western-blotted using an anti-c-myc antibody (see Materials and Methods). (A) profib-1, (B) glyfib-2. The positions of molecular mass marker proteins are shown.

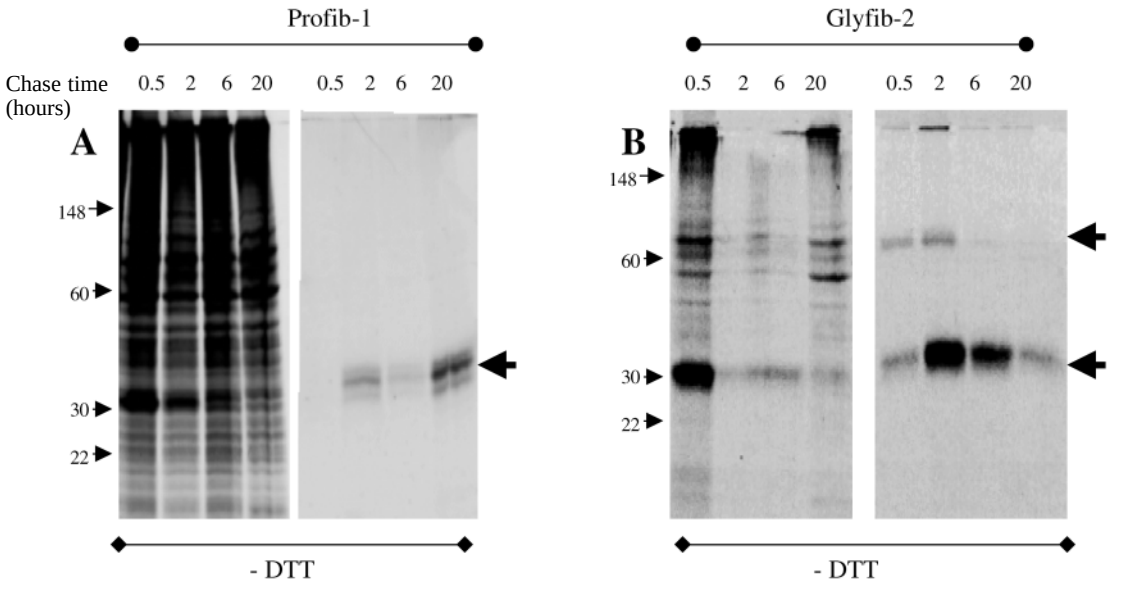

Fig. 3. Pulse-chase analysis of profib-1 (A) and glyfib-2 (B). COS-1 cells expressing profib-1 and glyfib-2, respectively, were pulsed with ^{35}S-cysteine for 30 minutes then chased for up to 20 hours. Cell layer (left lanes) and medium (right lanes) chase samples at the indicated times were immunoprecipitated with anti-c-myc antibody (see Materials and Methods), then electrophoresed on 15% SDS/PAGE gels in non-reducing conditions. Intracellular profib-1 and glyfib-2 appeared monomeric (●). Secreted profib-1 appeared as monomers, whereas secreted glyfib-2 appeared as monomers and dimers.

Pulse-chase analysis of profib-1 and glyfib-2 secretion

Pulse-chase experiments were carried out to establish whether profib-1 and glyfib-2 were secreted from COS-1 cells as monomers and/or dimers or larger assemblies (Fig. 3). SDS-PAGE analysis in non-reducing conditions revealed that newly secreted profib-1 had an apparent molecular mass of 28 kDa, which corresponded to monomers (Fig. 3A). Intracellular glyfib-2 was monomeric, but the secreted form appeared as monomers (28 kDa) and disulphide-bonded dimers (55 kDa) (Fig. 3B).

Size fractionation of profib-1 and glyfib-2

Size fractionation of profib-1 and glyfib-2 purified from COS-1 cell medium was carried out in non-reducing conditions on an AKTA Superdex 200 PC 3.2/30 column in 0.05 M Tris/HCl, pH 7.4, containing 0.15 M NaCl, in order to establish whether any non-covalent molecular associations had occurred (Fig. 4). Column fractions were then analysed by SDS-PAGE in non-reducing conditions, and eluted recombinant molecules were detected by western blotting using anti-c-myc antibody.

Native profib-1 eluted as monomers in fractions 17 and 18, corresponding to 25-35 kDa molecular mass (Fig. 4A). In contrast, native glyfib-2 eluted in a broad molecular mass range, separating on non-reducing SDS-PAGE gels as apparent monomers (fractions 4-24, range 25->200 kDa) and dimers (fractions 4-16, range 50->200 kDa). Most of the peptides that appeared as 55 kDa dimers on non-reducing SDS-PAGE were present in fractions 7-11, which corresponded to >100 kDa by size fractionation (Fig. 4B).

In vitro translation of recombinant fibrillin molecules

Newly synthesised recombinant ^{35}S-labelled three-domain peptides were generated by cell-free translation in the presence or absence of semi-permeabilised HT 1080 cells (Figs 5,6). In the presence of cells, labelled recombinant molecules were translocated into the endoplasmic reticulum, where they became N-glycosylated and were protected from proteinase K digestion except after Triton X-100 treatment (Ashworth et al., 1999; Hindson et al., 1999; Lees et al., 1997; Wilson et al., 1995; 1998). The cDNA constructs used in this expression system did not encode a carboxy-terminal c-myc epitope or histidine tag, which provided a means by which to exclude these sequences from influencing profib-1 or glyfib-2 associations. These recombinant proteins were rapidly produced yet not expressed at high levels, and were easily detectable as the only labelled species present.

When ^{35}S-labelled peptides profib-1 and glyfib-2 were translated for 90 minutes in the presence or absence of HT1080 cells, then resolved on non-reducing SDS-PAGE gels, both profib-1 and glyfib-2 appeared as monomers (25 kDa) plus components with electrophoretic mobilities corresponding to dimers (~55 kDa) and higher molecular mass assemblies (Fig. 5A). The presence of dimers was always more pronounced for glyfib-2 than for pro-fib-1. When the homobifunctional non-reducible crosslinker BMH was added to the profib-1 and glyfib-2 translation mixtures to 'trap' potential non-covalent molecular associations, dimers of profib-1 and glyfib-2 were detected within 10 minutes following the start of translation (Fig. 5B). In the absence of BMH, disulphide-bonded profib-1 and glyfib-2 dimers appeared later (Fig. 5B). That dimers occurred extracellularly was apparent after proteinase K treatment and size fractionation (Fig. 5C). Moreover, dimer formation was not dependent upon the presence of HT1080 cells, since glyfib-2 and profib-1 dimers also occurred in cell-free translation mixes (Fig. 5D).

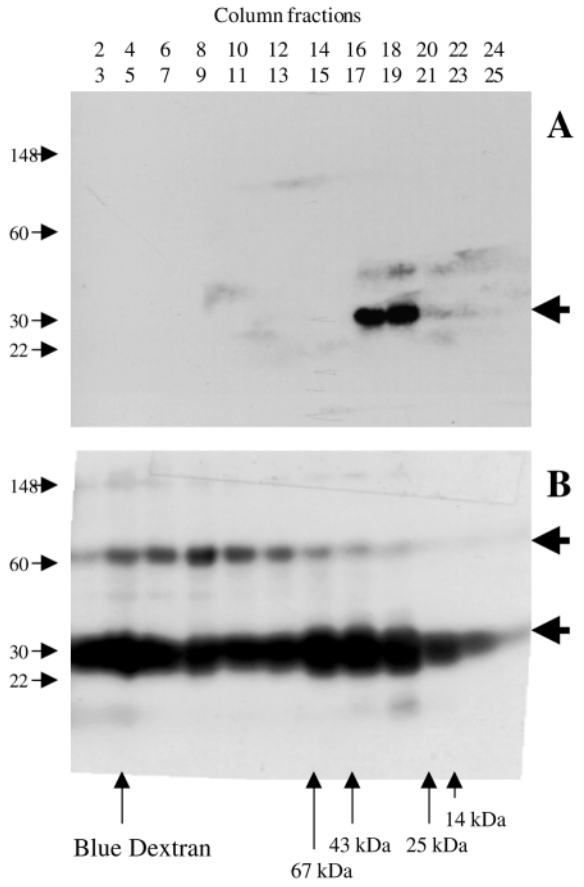

Fig. 4. Size fractionation of profib-1 (A) and glyfib-2 (B) prior to SDS-PAGE. Secreted profib-1 and glyfib-2 from COS-1 cell medium were size-fractionated in native non-reducing conditions on an AKTA Purifier system using a Superdex 200 PC 3.2/30 column, prior to SDS-PAGE analysis on 15% gels in non-reducing conditions. (A) Profib-1 eluted as monomers (fractions 16-19) of 30-35 kDa. (B) Glyfib-2 eluted in a broad size range from monomers to >200 kDa, but appeared on SDS-PAGE gels as monomers and dimers. The elution positions of marker proteins from the column are shown at the bottom.

Association of three domain peptides with molecular chaperones

The possibility that chaperones might limit intracellular dimer formation was examined using the semi-permeabilised cell expression system together with a crosslinking/coimmunoprecipitation strategy (Fig. 6). Coimmunoprecipitation experiments were carried out with antibodies to the candidate molecular chaperones protein disulphide isomerase (PDI), calreticulin, calnexin and BiP, in order to examine whether high molecular mass bands apparent after BMH crosslinking (see Fig. 5B) were fibrillin-chaperone complexes.

BMH-treated ^{35}S-labelled translocated profib-1 and glyfib-2 coimmunoprecipitated with antibodies specific for PDI (58 kDa) and calreticulin (48 kDa), but not calnexin (Fig. 6A). Crosslinked PDI complexes appeared as two distinct labelled bands of 90 kDa and 110 kDa, and calreticulin complexes had molecular masses of 100 kDa and 130 kDa. These fibrillin-chaperone complexes were detected within 10-15 minutes of the initiation of translation, but became more prominent by 90 minutes translation. Using the reducible crosslinker DSP, ^{35}S-labelled profib-1 and glyfib-2 coimmunoprecipitated with PDI (Fig. 6B), BiP (Fig. 6B) and calreticulin (not shown), respectively. Time-course experiments confirmed that BiP associations were prominent within 5 minutes, whereas PDI-fibrillin and calreticulin-fibrillin complexes were present from 15 minutes.

Analysis of proline- and glycine-rich regions

Primary structural analysis of the proline- and glycine-rich regions was carried out to assess potential molecular differences that might influence molecular association. Hydropathy analysis confirmed the predominantly hydrophobic character of the proline- and glycine-rich regions, but highlighted gross differences in the arrangement of hydrophobic residues (Fig. 7A). In addition, the pI of the proline-rich region was shown to be 8.5 whereas that of the glycine-rich region was 5.8.

The proline- and glycine-rich regions alone were expressed in *E. coli* DH5α cells as GST fusion proteins using pGEX expression vectors, in order to compare solubilities and aggregation potential (Fig. 7B). Since neither sequence contained cysteine residues with the potential to form spurious disulphide bonds or putative N-glycosylation sites, the recombinant peptides were likely to adopt a native folding arrangement. The molecular masses of the proline-rich region fusion protein and the glycine-rich region fusion protein, as judged by SDS-PAGE, were 35.0 kDa, and the GST fusion protein alone was 29.0 kDa.

When the recombinant peptides were cleaved from immobilised GST, marked differences in solubility were immediately apparent. Profib-1 was purified as a soluble protein of approx. 6 kDa following thrombin cleavage and elution from the affinity column, and resolved on SDS-PAGE with no evidence of aggregation (Fig. 7B). In contrast, thrombin-cleaved glycine-rich region rapidly precipitated out of solution.

A concentrated sample of the proline-rich region (8 mg/ml D_2O) was subjected to 1-D NMR. This analysis revealed that the proline-rich region in solution was unstructured and unrestricted by secondary structures such as α-helices and β-sheets, and that this peptide probably has a random coil structure (data not shown).

DISCUSSION

Linear structural extracellular matrix polymers such as collagen fibres form in the extracellular space by tightly regulated self-assembly processes, driven largely by primary structure (Kielty et al., 1993). In this way, the formation of fibrillin-rich microfibrils is probably initiated by self-assembly of the fibrillin scaffold. However, primary structural information has not readily translated into an understanding of how fibrillin molecules associate to form microfibrils. As a consequence, the basic structural biology of microfibrils and genotype-to-phenotype correlations in Marfan syndrome and related diseases remains poorly resolved. Biosynthetic and extraction studies have indicated that newly secreted fibrillin is rapidly incorporated into an extensive, disulphide bonded fibrillar network (Gibson et al., 1989; Sakai, 1990). However,

difficulties in electrophoretic resolution of full-length fibrillin molecules and a tendency to form disulphide-bonded aggregates have, to date, both precluded characterisation of assembly intermediates and predicted the existence of specific molecular recognition events to direct correct alignment of newly synthesised fibrillin molecules. Using a recombinant approach, we have focussed on the potential involvement of the distinctive proline- and glycine-rich regions of fibrillin-1 and

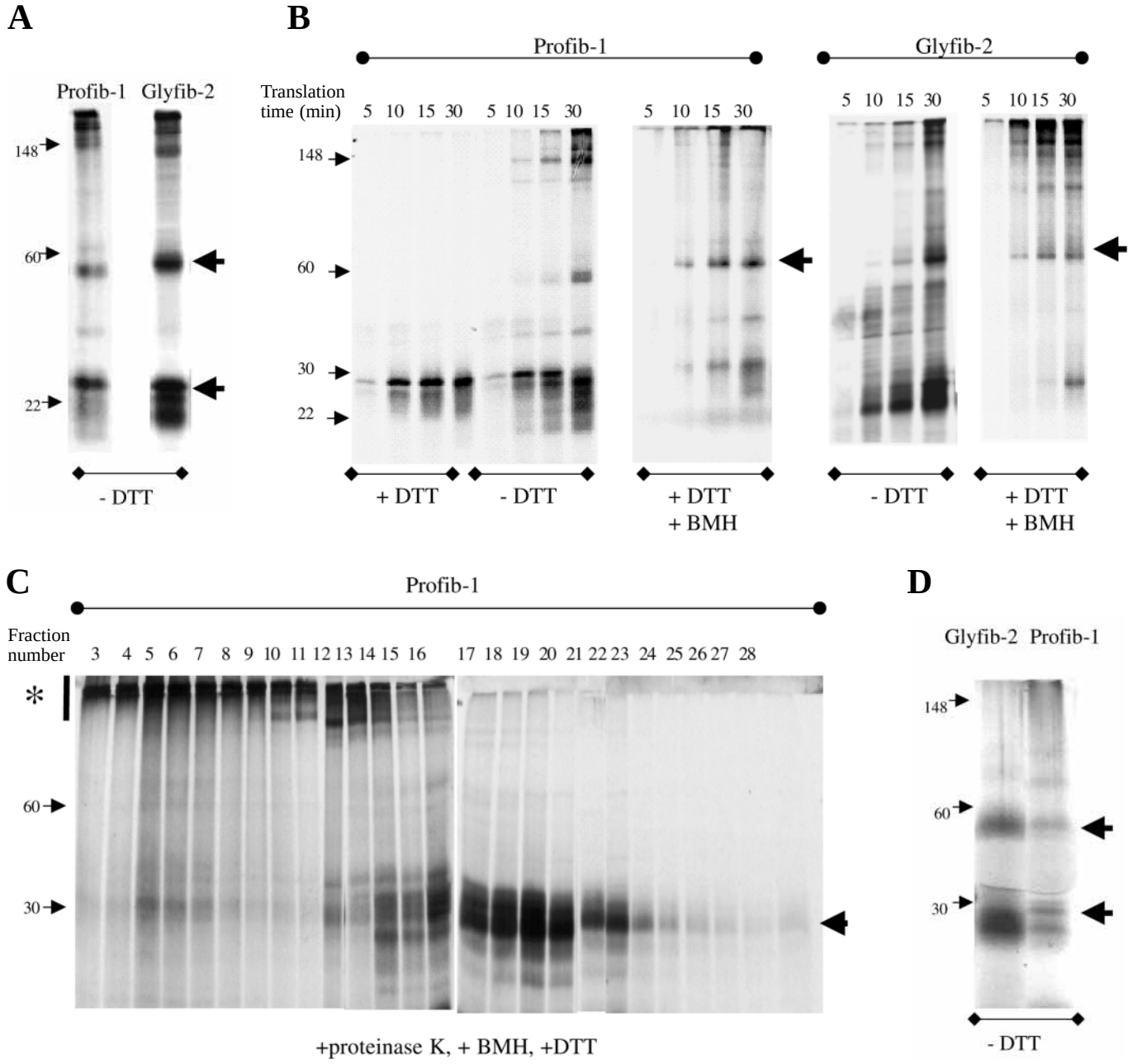

Fig. 5. Electrophoretic analysis of profib-1 and glyfib-2 expressed in an in vitro translation system supplemented with semi-permeabilised HT1080 cells. ^{35}S-labelled profib-1 and glyfib-2 were translated for up to 90 minutes in the presence of semi-permeabilised HT1080 cells, then the cells were rigorously washed prior to SDS-PAGE analysis. In some experiments, washed cells were treated with proteinase K prior to electrophoresis (see Materials and Methods). In each experiment, gels for profib-1 and glyfib-2 were exposed for similar times, usually between 24 and 48 hours. (A) In non-reducing conditions (–DTT) after translation for 90 minutes, profib-1 and glyfib-2 both appeared as monomers (25 kDa), dimers (55 kDa) and high molecular mass components. (B) Time-course experiments in reducing (+DTT) and non-reducing conditions (–DTT) in the presence of the non-reducible crosslinker, BMH, dimers were present from 10 minutes of translation. In the absence of crosslinker, disulphide-bonded dimers subsequently became apparent. (C) Size fractionation of profib-1 on an AKTA Purifier system using a Superdex 200 PC 3.2/30 column (see Materials and Methods) after 90 minutes translation, followed by treatment with BMH crosslinker and proteinase K treatment. Monomers were present, as well as high molecular mass bands (*). No dimers were detected after proteinase K treatment. (D) When profib-1 and glyfib-2 were translated for 90 minutes in non-reducing conditions in the absence of cells, dimers of each molecule were detected.

fibrillin-2 in molecular recognition and association. The mammalian expression systems used generated secreted N-glycosylated peptides, in vitro translated peptides and translocated intracellular N-glycosylated peptides.

SDS-PAGE was initially used to monitor the three-domain peptides profib-1 and glyfib-2 purified from COS-1 cell medium. This analysis highlighted a marked difference between the two molecules. Profib-1 was monomeric in reducing and non-reducing conditions, whereas glyfib-2 was present as monomers and disulphide-bonded dimers. Subsequent pulse-chase experments confirmed that the profib-1 was secreted as monomers, whereas intracellular glyfib-2 was monomeric but extracellular glyfib-2 was detected as monomers and dimers. Thus, formation of glyfib-2 dimers is apparently an extracellular event. Size fractionation in native non-reducing conditions was then conducted prior to non-reducing SDS-PAGE analysis to determine whether any non disulphide-bonded assemblies were present. The elution position of profib-1 corresponded to monomers only, but glyfib-2 eluted at positions consistent with monomers, dimers and larger assemblies, indicating that some non-covalent assemblies were present, including the non-covalent association of some disulphide bonded dimers into tetramers. Thus in this system, secreted glyfib-2, but not profib-1, had the potential to associate to form defined dimers, at least some of which subsequently become disulphide-bonded. Since these associations are extracellular, the cell probably has stringent controls in place to prevent potentially catastrophic intracellular aggregation.

The difference between profib-1 and glyfib-2 in ability to

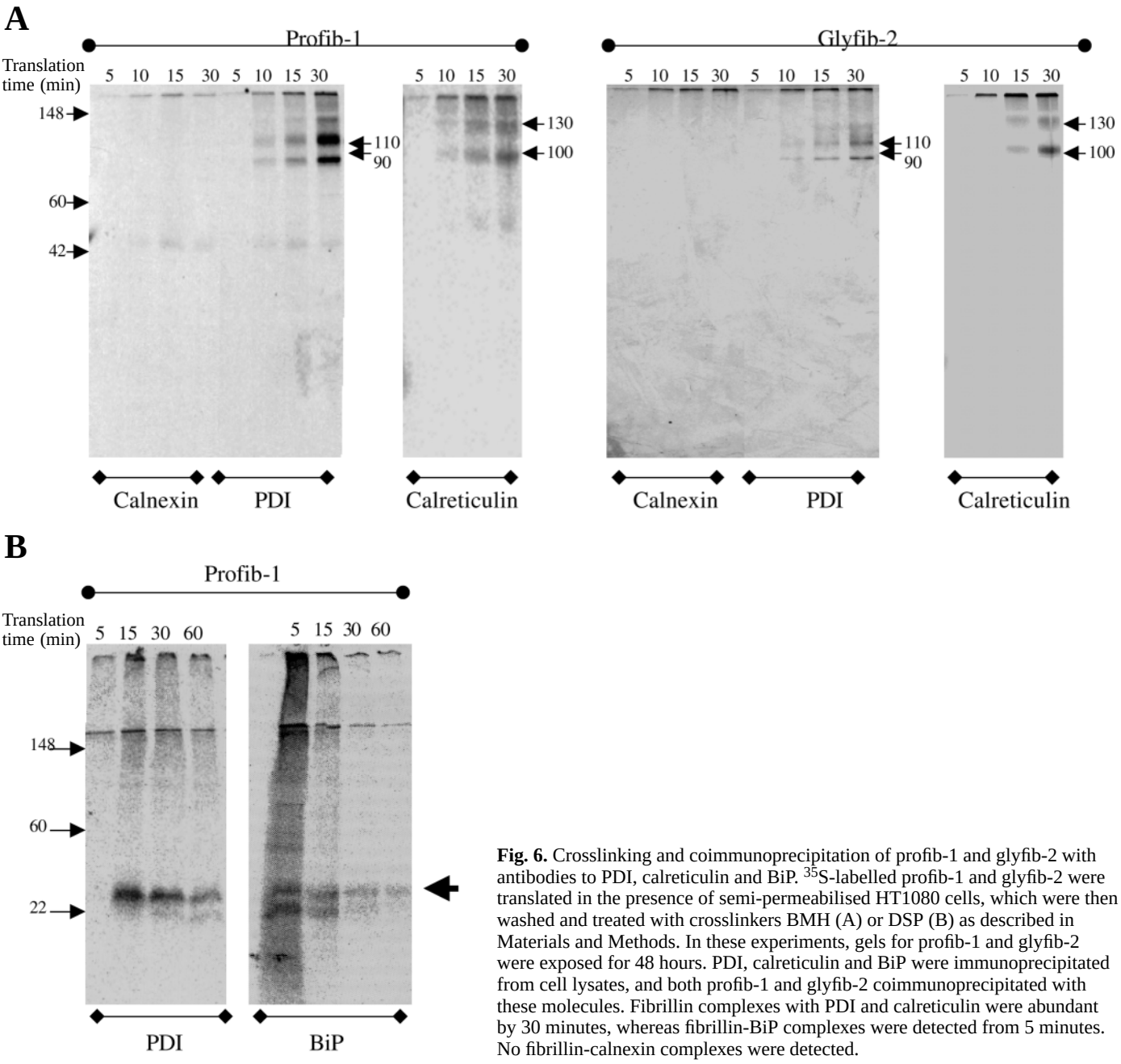

Fig. 6. Crosslinking and coimmunoprecipitation of profib-1 and glyfib-2 with antibodies to PDI, calreticulin and BiP. ^{35}S-labelled profib-1 and glyfib-2 were translated in the presence of semi-permeabilised HT1080 cells, which were then washed and treated with crosslinkers BMH (A) or DSP (B) as described in Materials and Methods. In these experiments, gels for profib-1 and glyfib-2 were exposed for 48 hours. PDI, calreticulin and BiP were immunoprecipitated from cell lysates, and both profib-1 and glyfib-2 coimmunoprecipitated with these molecules. Fibrillin complexes with PDI and calreticulin were abundant by 30 minutes, whereas fibrillin-BiP complexes were detected from 5 minutes. No fibrillin-calnexin complexes were detected.

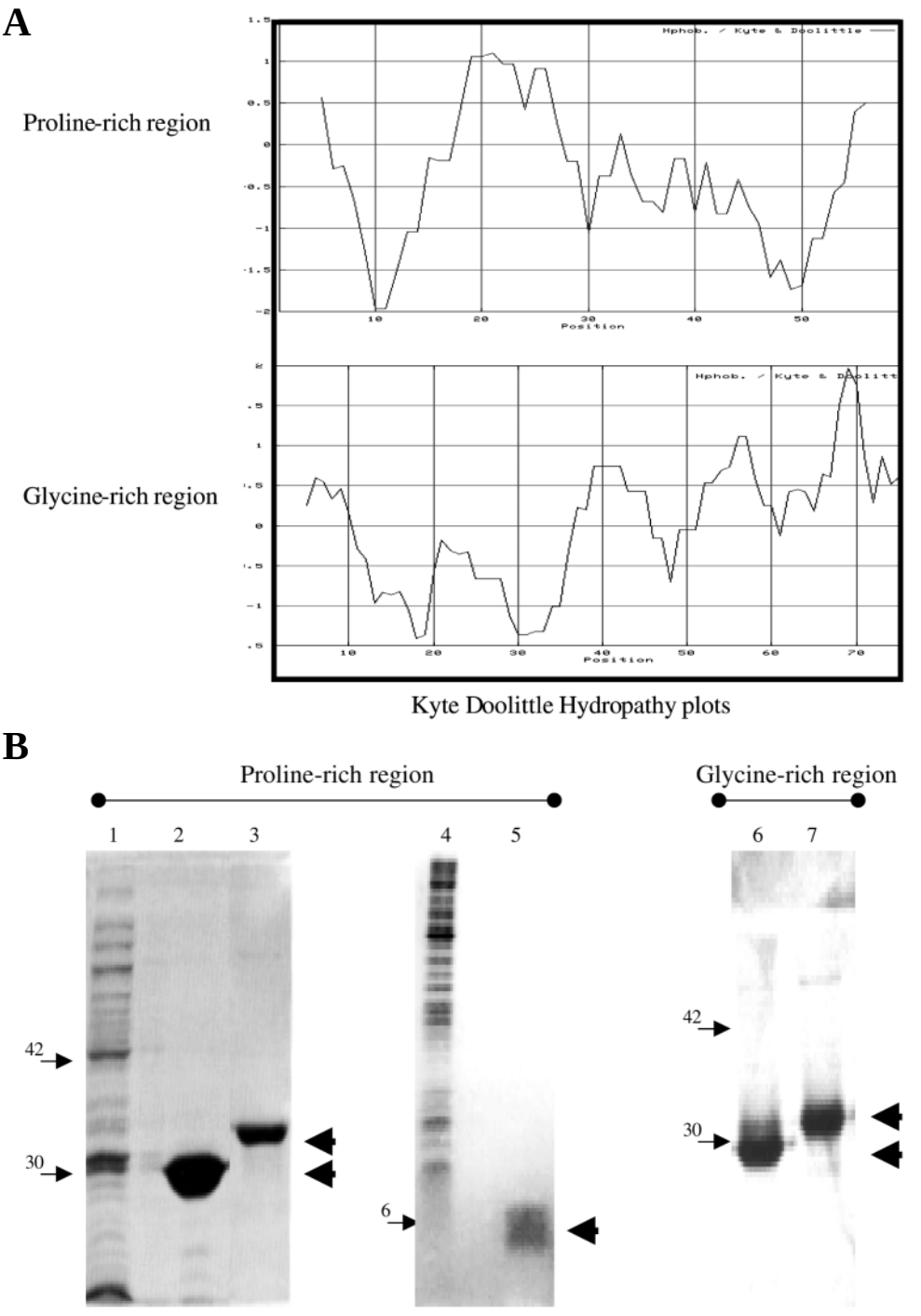

Fig. 7. Analysis of proline- and glycine-rich regions. The proline- and glycine-rich regions of fibrillin-1 and fibrillin-2, respectively, were analysed in terms of primary structural features (A) and following recombinant expression in a prokaryotic (pGEX) expression system. (A) Hydropathy analysis revealed distinct distributions of hydrophobic residues. (B) The proline-rich region (lanes 1-3) and glycine-rich region (lanes 6,7) were each expressed and purified as GST fusion proteins in *E. coli* cells. The proline-rich region was further purified as a 6 kDa soluble molecule following thrombin cleavage from the fusion protein (lanes 4,5). The glycine-rich region could not be recovered in soluble form.

dimerise suggests that fundamental differences may exist in their route or rate of assembly. In order to investigate further these differences, a second recombinant expression system was utilised. Both three-domain peptides were generated using an in vitro translation system in the presence or absence of semi-permeabilised HT1080 cells that contained intact secretory pathway organelles (Lees et al., 1997; Wilson et al., 1995; 1998). The preparation of recombinant labelled fibrillin molecules in this system had several advantages. Fibrillin was the only translated protein and it could be rapidly generated in radiolabelled form. Since it is well known that the semi-permeabilised cells effectively recapitulate post-translational modifications within the endoplasmic reticulum, the system allowed examination of potential associations of newly translocated fibrillin peptides, after proteinase K treatment, with molecular chaperones which might constitute key cellular controls on assembly. It also had the advantage of not being a high-level expression system, which could potentially encourage aberrant molecular associations. Moreover, by leaving out cells, it was possible to assess whether cells were required for dimerisation.

Using the in vitro translation system with semi-

permeabilised HT 1080 cells in combination with a BMH crosslinking strategy, evidence was obtained that both profib-1 and glyfib-2 could rapidly dimerise and that disulphide bonding of these dimers was a subsequent event. Proteinase K treatments revealed that dimers comprised untranslocated material. Interestingly, there were always more dimers in the glyfib-2 preparations. The in vitro translation experiments in the absence of cells confirmed that the appearance of dimers was not cell-dependent. It is not clear why profib-1 dimers were detected in this expression system but not in the COS-1 cell system; enhanced sensitivity to detection may be a factor, but another possibility is that the folding of secreted profib-1 may somehow alter dimerisation efficacy and matrix incorporation.

The demonstration of dimers in both mammalian recombinant systems confirmed that ability to dimerise is not an artefact of the in vitro translation/semi-permeabilised cell system or of high-level protein expression, but a fundamental property of the peptides themselves. Peptides expressed in the in vitro translation system differed from those secreted from COS-1 cells in not having carboxy-terminal c-myc and histidine tags. Since dimers formed using both expression systems, the presence of tags does not influence their ability to dimerise. Both peptides have an N-glycosylation site at the junction of the proline- and glycine-rich regions and the following EGF-like domain, but since peptides expressed in the absence of cells were not N-glycosylated but still able to dimerise, this modification appears not to influence dimerisation.

Our evidence that dimers form extracellularly suggests the existence of cellular mechanisms that may prevent intracellular dimerisation. The association of BiP with newly synthesised fibrillin molecules shortly after translocation suggests a role for this ATP-dependent molecular chaperone in controlling such intracellular associations. BiP is known to interact with hydrophobic sequences (Hartl, 1996), and its rapid association with the newly translocated peptides suggests it recognises and binds the predominantly hydrophobic proline- and glycine-rich regions shortly after translocation. Recent data indicate that PDI, in addition to its well-characterised role as a subunit of prolyl hydroxylase, regulates disulphide bond formation in the endoplasmic reticulum and acts as a molecule chaperone in suppressing aberrant aggregation (Wilson et al., 1998). The association of PDI with the three-domain peptides may be important in both contexts. A key role for calreticulin within the endoplasmic reticulum is to moderate glycosidase-driven glucose trimming of N-linked carbohydrates (Helenius et al., 1997). Calreticulin interactions with the three-domain peptides may regulate trimming of their single N-linked carbohydrate, and its presence may inhibit dimeric associations. These molecular chaperone associations may be crucial in preventing intracellular aggregation. Failure to demonstrate interactions between fibrillin and membrane-bound calnexin reinforces the specificity of the other chaperone associations identified.

The molecular basis of the differences between secreted profib-1 and glyfib-2 in dimer formation is not known, but the proline- and glycine-rich regions themselves are implicated as the major sequence variation between the two molecules. When these sequences alone were expressed, marked solubility differences were identified. Hydropathy analysis indicated variations between the two peptides in distribution of hydrophobic residues and pI values. These analyses indicate that the proline- and glycine-rich regions have fundamentally different chemical properties.

In summary, our data show that recombinant fibrillin-1 molecules encompassing the proline- and glycine-rich regions and flanking domains can dimerise, and have highlighted subtle differences between these regions of the fibrillin-1 and fibrillin-2 isoforms. We propose that fibrillin molecules are secreted as monomers, and that extracellular conditions or loss of chaperones lead to dimer formation mediated by specific recognition of the proline- or glycine-rich regions. Thereafter, dimers can become covalently stabilised through disulphide exchange reactions, which may involve strategically aligned TB modules. Dimers may form the basic assembly intermediate of linear assembly, a process regulated, at least in part, by terminal processing events. The physiological significance of tetramers is not yet clear, although their appearance does indicate the potential for higher order associations. Some support for the concept of dimers as intermediates of assembly comes from ultrastructural studies of microfibrils, where the interbead regions often appear as two defined strands (Keene et al., 1991; Reinhardt et al., 1996; Hanssen et al., 1998; Sherratt et al., 1999). These studies have provided important new insights into fibrillin assembly. Future studies will be directed to investigating whether or not heterodimers can form.

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