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Pericellular proteins of the developing mouse tendon: a proteomic analysis

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Abstract

Tendon fibroblasts synthesize and assemble collagen fibrils, the basic structural unit of tendons. Regulation of fibrillogenesis is essential for tendon development and function. Fibril assembly begins within extracellular micro-domains associated with the fibroblast surface. We hypothesize that molecules crucial to the regulation of fibril assembly are membrane-associated and/or within the pericellular micro-environment. This report defines proteins in the surfaceome, *i.e.*, plasma membrane and pericellular matrix, from mouse flexor digitorum longus tendons. Proteomic analysis identified a set of surfaceome molecules including collagens, fibronectin, integrins, proteoglycans and receptors in extracts from mouse tendons at postnatal day 1, a developmental stage when collagen protofibril nucleation and initial steps in fibril assembly predominate. The proteomic results were validated for molecules identified with a small number of unique peptides and/or low sequence coverage. For these analyses, proteins were selected based on their potential roles in fibril nucleation, *i.e.*, collagen V; organization of fibrillogenesis, *i.e.*, integrins and fibronectin; and known localization to the plasma membrane with potential to impact matrix assembly, *i.e.*, CD44, syndecan-1, epidermal growth factor receptor, and matrix metalloproteinase 25. These molecules all were detected in extracts of the developing tendon, demonstrating that the surfaceome included molecules hypothesized to regulate fibrillogenesis as well as many with no known function in this capacity. This report, therefore, generates an unbiased set of cell surface-associated molecules, providing a resource to identify novel or unexpected regulatory molecules involved in collagen fibril and matrix assembly.

Keywords

Fibroblast surface; collagen fibrillogenesis; development; extracellular matrix; surfaceome

INTRODUCTION

Collagen fibrillogenesis involves a sequence of events including initiation of fibril assembly, linear and lateral fibril growth, and maturation [1-4]. Macromolecular interactions have been implicated in the coordinate regulation of this sequence of events. During tendon development, collagen fibril assembly begins in extracellular micro-domains associated with

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DECLARATION OF INTEREST

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the fibroblast surface [2, 5, 6]. Nucleators, *e.g.*, collagens V and XI, are essential for initiating collagen fibrillogenesis [7-9]. Molecules such as fibronectin and integrins have been implicated in determining the sites of fibril assembly and organizing fibrils in the developing extracellular matrix [10-12]. Other regulatory molecules such as tenascin-X [13, 14], membrane-bound proteoglycans, *e.g.*, syndecans [15], and small leucine rich proteoglycans (SLRPs) [16-18], have been shown to influence assembly. The localization of CD44 (a cell-surface glycoprotein important for cell adhesion and migration and known as a receptor for hyaluronan), Epidermal growth factor receptor (or EGFR, which is known to bind the matrix proteoglycan decorin) and Matrix metalloproteinase 25 (or MMP25, an integral membrane enzyme that degrades molecules in the normal function and the structural remodeling of tissues) at the cell membrane also potentially involves them in the cell-surface associated fibrillogenesis steps, although such involvement hasn't been shown. All of these molecular interactions coordinately determine tissue-specific fibril and matrix structure and function [19].

Compartmentalizing the initial steps in collagen fibrillogenesis to micro-domains associated with the cell surface provides a mechanism to regulate fibrillogenesis and position the assembling fibrils in the developing matrix. We hypothesize that surfaceome molecules are involved in a series of regulatory interactions required for the critical initial steps in fibril assembly. This includes regulation of fibril nucleation as well as efficient processing and positioning necessary for tissue-specific matrix assembly. In addition, receptor-mediated interactions would be expected to be involved in feedback influencing fibroblast behavior.

A definition of the proteins at the surface of the tendon fibroblast at different developmental stages and in different tissues is unavailable. This report utilizes an unbiased proteomic approach to define a set of potential proteins associated with the tendon fibroblast surface. Tendons from P1 mice, an early developmental stage characterized by initial assembly of protofibrils and their deposition into the developing extracellular matrix were used. The tendon extracts were enriched for proteins associated with the tendon fibroblast surfaceome. These proteins included those with known roles, candidate proteins, and proteins with no appreciated function in fibril and extracellular matrix assembly. This will contribute to the elucidation of protein interactions involved in the regulation of the early events in tendon collagen fibrillogenesis.

MATERIALS AND METHODS

Animals

Flexor digitorum longus (FDL) tendons from hind limbs of 12 to 16 C57BL/6 mice at P1 were used per experiment according to an IACUC approved protocol at the University of South Florida.

Tendon Extracts

Two different extractions were utilized to enrich for molecules of the FDL tendon fibroblast surfaceome. The experimental approach is presented in **Fig. 1S**. First, a mild detergent extraction was used to enrich for proteins from the pericellular environment, generating a 'cell surface-associated extract'. The procedure was optimized to enrich for readily soluble plasma membrane, surface-associated and extracellular matrix proteins, rather than for cytoplasmic proteins. Based on the results from the surfaceome of *Leptospira* solubilized by mild Triton X [20], we chose a detergent class (Brij) shown to be less stringent in a report on cells in monolayer culture [21], (where extraction was performed on ice for 30 min or at 37°C for 10 min and detection of selected marker proteins assessed "the graded selectivity of the detergents [including 1% Brij 96, 2% Brij 58, 2% Brij 98, 2% Triton, 2% Tween 20,

or 8% CHAPS]” that were used). Tendons were dissected, rinsed in PBS and extracted in a 15-fold excess (volume/wet weight) buffer containing 50 mM Tris-HCl, pH 7, 0.15 M NaCl, 1X Halt Protease Inhibitor cocktail (Fisher Scientific), 0.5% Brij-97 for 24 hrs at 4°C. The resulting extract was clarified by centrifugation and total protein in the sample was measured using the detergent-compatible Lowry protein assay kit (BioRad) with BSA as a standard. A second extraction targeted membrane proteins, using the Pierce Mem-PER kit for extraction and selective enrichment of integral and attached hydrophobic proteins from mammalian membranes, generated the ‘membrane protein extract’. Tendons from P1 mice (20 mg wet weight) were rinsed with 200 µl of ice cold PBS followed by disruption in 200 µl PBS with a Sonic Dismembrator Model 100 tissue grinder (Fisher). The homogenate was transferred to a new tube and centrifuged at 5000xg for 5 min at 4°C. Membrane proteins were isolated from the pellet using a Mem-PER kit and a PAGE-Prep kit (Pierce Biotech) was used to prepare the samples for SDS-PAGE, according to the manufacturer's instructions.

Proteomic Analysis

An entire gel lane containing electrophoresed extract was excised into 24-30 uniform transverse slices. Following in-gel tryptic digestion, peptides were extracted and concentrated using vacuum centrifugation. A nanoflow liquid chromatograph (1100, Agilent, Santa Clara, CA) coupled to an electrospray ion trap mass spectrometer fitted with a chip-based ion source (HCT Ultra, Agilent, Santa Clara, CA) was used for tandem mass spectrometry peptide sequencing. Following capture on a C18 reverse phase trap column, peptides were separated using a 30 minute gradient from 5% B to 50% B (A: 2% acetonitrile/0.1% formic acid; B: 90% acetonitrile/0.1% formic acid). The trap and analytical columns were contained on the Agilent Protein ID Chip (G4240-62002), which has a 40 nl enrichment column and a 75 µm × 150 mm analytical column; both are packed with 5 µm SB-Zorbax C-18 reverse phase media. Loading was performed at 4 µl/min followed by valve switching and LC-MS/MS at 300 nl/min. For each MS scan, 5 tandem mass spectra were acquired; prior precursors are excluded for 60 sec.

Database Search—Mascot (www.matrixscience.com) was set up to search the current Swiss-Prot *Mus musculus* database (16338 entries), assuming the digestion enzyme to be trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 2.5 Da. Carbamido-methylation of cysteine, oxidation of methionine, and deamidation of asparagine and glutamine were selected as variable modifications, and as many as 2 missed tryptic cleavages were allowed. The reverse database also was searched to determine false discovery rates (FDR) for identified proteins.

Protein Identification—Scaffold (version 3_00_03, Proteome Software Inc., Portland, OR, www.proteomesoftware.com) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability as specified by the Peptide Prophet algorithm [22]. Peptide identifications also were required to exceed specific database search engine thresholds. Mascot identifications required ion scores greater than the associated identity scores and 30, 35 and 40 for double, triple and quadruple charged peptides, respectively. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 1 unique peptide. Confidence filters were relaxed to 1 peptide to include less confident identifications that might prove promising for further investigation. Protein probabilities were assigned by the Protein Prophet algorithm [23]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Protein Classification—All classification of proteins by compartment or molecular function was according to the Gene Ontology annotations from the National Center for Biotechnology Information (NCBI) as displayed in the Scaffold data for each protein. These annotations are publicly available (www.ncbi.nlm.nih.gov/gene) and can be downloaded and updated in Scaffold whenever new information becomes available from the database.

Classification based on roles in fibril and matrix assembly was devised after our own literature searches in Pubmed (www.ncbi.nlm.nih.gov/pubmed/) for studies in which data indicated such roles for these proteins of interest. No statistical analyses were performed; the searches were done *in silico* with the aim of researching published information on the proteins' locations, *e.g.*, matrix; structures, *e.g.*, glycoproteins; and functions, *e.g.*, receptor or regulatory.

Electrophoresis and Immuno-blots

The extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and processed using standard immuno-blot procedures. SDS-PAGE was performed using the NuPAGE Protein gel system (Invitrogen) with 4-12% Bis-Tris gels. Immuno-blots were done with I-Block (AP Biosystems, 0.5% in PBS) for blocking and ECL kits (Pierce) for development. Developed bands were visualized and imaged using a Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad) with Quantity One 1-D Analysis™ Software.

Antibodies

All primary antibodies were used at 1:500 in Western blots. HRP-conjugated secondary antibodies (Invitrogen) were used at 1:5000. Anti-fibronectin and integrin antibodies were from Santa Cruz Biotechnology; anti-type V collagen antibodies were produced as described previously [7]; anti-CD44 antibodies were from BD Biosciences; anti-Epidermal Growth Factor (EGFR) and syndecan-1 antibodies were from Invitrogen; and anti-Matrix Metalloproteinase (MMP)25 antibodies were from LifeSpan BioSciences.

RESULTS

We hypothesize that the fibroblast surfaceome contains proteins involved in regulation of tendon collagen fibril assembly. An unbiased proteomic approach was used to identify surfaceome proteins in the developing tendon. An overview of the mass spectrometry data is shown in **Table 1**, with total spectra, peptides and identified proteins presented.

Cell surface-associated extract of P1 tendon

The cell surface-associated extract contained proteins with an expected large range of molecular weight, from below 20 kDa to above 260 kDa (**Fig. 1A**). This extract was analyzed by mass spectrometry to identify the proteins present. This analysis demonstrated that the extract contained at least 453 identifiable proteins; (False discovery rate (FDR): Protein-level 1.7%, Peptide-level 0.2%) (**Fig. 1A, Table 1S**).

Cell surface-associated proteins identified by LC-MS/MS were further analyzed and grouped based on whether they are intracellular, present in the plasma membrane or in the extracellular matrix. Of the 453 molecules, 98 were from the plasma membrane and extracellular matrix. These 98 proteins were further organized into functional groups (**Fig. 1A, Table 2**). The identified proteins have molecular functions from binding, transport and structure, to signal transduction, enzyme regulation and catalytic activity. These proteins from the cell surface extract provide a data set from which to extract candidate regulatory molecules in the cell-surface mediated steps of tendon collagen fibrillogenesis. All classification of proteins by compartment, *i.e.*, intracellular, plasma membrane or

extracellular matrix or molecular function was derived from the Gene Ontology annotations (NCBI) in Scaffold. Identified molecules included many that we hypothesized to be a part of the surfaceome. Collagens I, V, VI, XI and XII as well as proteoglycans (SLRPs, syndecan) were identified. In addition, membrane proteins such as the hyaluronan receptor (CD44), tenomodulin, and EGFR; as well as a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-1, MMP25, and tolloid-like protein1 were present in the extracts. Other proteins that influence cell behavior also were identified, *i.e.*, growth factors (development and differentiation enhancing factor [DDEF]-2, insulin growth factor [IGF]-1) and adhesion proteins (integrins, fibronectin).

Membrane-associated extract of P1 tendon

To ensure optimal representation of the low-solubility membrane proteins, a separate membrane extract was generated to complement the proteomic profile from the surface extract. A broad range (20–260 kDa) in molecular weight of proteins in a membrane fraction of P1 mouse FDL tendons was observed (**Fig. 1B**). As with the surface-associated proteins, the plasma membrane proteins were further annotated to provide an overview of their molecular functions. The complete list of 291 proteins identified, including 204 on intracellular membranes, is presented in **Table 2S**. The identified membrane proteins included molecules with important functions in the organization and regulation of collagen fibril formation, including plasma membrane-bound annexins, CD44, CD109, epidermal growth factor receptor, integrins $\alpha 1/2/5/6/7/V$ and $\beta 1/2$ (of which $\alpha 1$ and $\beta 2$ are collagen receptors and $\alpha 5 \beta 1$ is a fibronectin receptor), syndecan-1, MMP25, and tenomodulin.

The P1 tendon fibroblast surfaceome

Proteomic profiles of the cell-associated and membrane-associated extracts were analyzed separately. These data sets were then combined and all molecules designated by Gene Ontology annotation as intracellular were excluded from further analysis. The resulting compiled set of cell surface-associated and membrane proteins identified represent the surfaceome of P1 tendon fibroblasts (**Table 2**). The 66 plasma membrane and extracellular matrix molecules that were identified only in the surface-associated extract (**Fig. 1A**, **Tables 2 and 1S**), the 55 plasma membrane molecules identified only in the membrane extract (**Fig. 1B**, **Tables 2 and 2S**), and the 32 plasma membrane and extracellular matrix molecules identified in both extracts (**Tables 2, 1S, 2S**), are all part of the surfaceome.

Proteins in the P1 tendon surfaceome that have been implicated in different aspects of regulation of extracellular matrix assembly and development across various tissues are presented in **Table 3**. In contrast to the objective classifications of the previous tables, these molecules were classified based on our analysis. This was done to evaluate the value of the proteomics screen to both characterize the general surfaceome of a model tissue and to identify candidate proteins that may be involved in regulation of tendon fibrillogenesis. There were some noticeable absences in our extracts of molecules already known to be involved in fibrillogenesis such as $\alpha 2(V)$, $\alpha 3(VI)$, N- and C-propeptidases. The mass spectrometry approach we used in this report detects peptides, many of which can be found in multiple protein isoforms. The absence of identified unique peptides for expected species does not rule out the possibility that they are present at detectable levels. Additionally, issues with protein copy number may impact the ability to observe these species. Such qualitative protein experiments, therefore, yield valuable data to serve as starting points for identifying relevant protein species, but cannot declare that an undetected protein is indeed absent from this tissue. Targeted mass spectrometry such as in Multiple Reaction Monitoring can be utilized to address absence / presence / quantification of particular proteins of interest. Even such experiments, however, require detectable unique

(proteotypic) peptides; it is sometimes quite difficult to distinguish between two highly identical protein species.

From the molecules we detected, proteins (**asterisks in Table 3**) were selected for immunoblot analysis to further validate the proteomic screen. First, molecules that were identified by a single peptide (*i.e.*, CD44, integrin $\alpha 1$, syndecan-1, EGFR and MMP25) or with low sequence coverage (*i.e.*, integrins $\alpha 2/5$; collagen V), as indicated in **Table 2**, were analyzed. Second, proteins of interest as potential regulators of tendon extracellular matrix assembly, specifically at the cell surface-mediated early stages of fibrillogenesis, including integrin $\alpha 1$ and fibronectin, were examined. Immuno-blots for these diverse molecules in the extract confirmed their presence and the validity of the proteomics screen (**Fig. 2**). Our data, therefore, define an unbiased data set comprising the cell surface-associated proteome or surfaceome of developing tendons. This provides a resource from which to identify novel or unexpected regulatory molecules involved in tendon collagen fibril and matrix assembly.

DISCUSSION

The fibroblast surface is a key site in the regulation of collagen fibrillogenesis. The importance of regulatory interactions involving collagens I and V in initiation of fibril assembly have been reported [24]. We hypothesized that the tendon fibroblast surfaceome would include nucleators, such as collagen V, organizers of early collagen assembly, such as integrins, and membrane-bound molecules involved in fibril and matrix assembly. The presence of these diverse molecules at the fibroblast surface allows the cell to orchestrate the focal regulation and positioning of newly assembled protofibrils into the developing matrix. We anticipated that the surfaceome would include a variety of proteins involved in essential developmental functions such as signal transduction, cell adhesion, and matrix turnover. Molecules involved in all of these processes were identified in the surfaceome of early developing mouse tendons.

There was considerable repetition of intracellular molecules identified by mass spectrometry in the surfaceome extracts, *e.g.*, multiple types of proteasomal, ribosomal, keratin, translation initiation/elongation factors, histone proteins and heterogeneous nuclear ribonucleoproteins. In fact, the majority of the proteins in both extracts are from the intracellular compartment (Fig. 1). It is important to note that the number of proteins does not correlate with the amount of protein present. There are far more intracellular proteins than extracellular proteins. Also, the extracts are not clean- we are not isolating specific proteins, we are enriching for hydrophobic proteins that may be present on the inside or outside of the cell membrane. Additionally, these proteins interact with other proteins and some “sticky” uninvolved proteins would be brought through the extraction process. Only 22% (98 of 453, **Table 1**) of the proteins identified in this study could be classified as surfaceome proteins. However, in a study on compartmentalization of proteins in the genomes of pathogens [25], the average percentage of extracellular proteins in the 15 genomes used was 15.7%. Our data showing 22% of the extracted proteins being extracellular or plasma membrane-bound, therefore, indicate enrichment for surfaceome proteins in our extracts. It would be more appropriate to make this comparison using data from higher organisms, but we are unaware of any published reports of the proportion of surface-associated to intracellular molecules in higher genomes.

Plasma membrane proteins are expressed at low levels [26] and have low solubility, so the limited dynamic range of the LC-MS/MS in this study might have caused underrepresentation of the diversity of membrane proteins identified in the cell-associated extract. To overcome this limitation, a proteomic approach aimed at identification of membrane proteins usually involves a step for enrichment of the extract for such membrane proteins,

which was done in this study (**Table 2S**). For the cellular extract, high abundance proteins may mask the presence of low abundance species. For the membrane extracts, we would reduce the presence of abundant hydrophilic species, enabling us to see a larger population of proteins (including those coming from inside the cell). This might explain why 30% (87 of 291) of the proteins in the membrane extract are plasma membrane-bound and not intracellular, compared to 22% in the cellular extract. In characterizing the set of proteins on the tendon fibroblast membrane, matrix receptors (such as CD44, syndecan-1 and EGFR), tendon regulatory proteins (such as collagen V and MMP25) as well as surface adhesion molecules (such as fibronectin and integrins) were strong candidates for the hypothesized surface-related proteins important in very early collagen fibril formation; these were all identified in our membrane extracts (**Tables 2, 2S**).

It has been proposed [27], based on studies with fibronectin-deficient mouse fibroblasts [11] and vascular smooth muscle cells [10], that most, if not all, of the collagen assembly in cultured cells is regulated by cell-surface molecules. Our study probes tissues using proteomics and immuno-blotting of selected proteins implicated in collagen fibrillogenesis in various tissues. We found expression of matrix receptors, *i.e.*, integrins 1, 2, 5, and 11; hyaluronan receptor CD44; syndecan-1; fibronectin; and collagen V; and developmentally regulatory proteins EGFR and MMP25. We were interested in these particular proteins because they either have vital roles in the nucleation of collagen assembly, *i.e.*, collagen V, or are known to be proteins with roles in matrix assembly in normal development or in remodeling of different tissues, *i.e.*, integrins, fibronectin, receptors, and syndecan-1 [12, 28-32].

Further studies will elucidate interactions among these proteins, *e.g.*, between collagen V and the membrane-bound molecules (**Fig. 3**). This will be important in defining mechanisms for tethering of collagen V to the fibroblast surface, since this collagen would need to be near the fibroblast surface, linking nucleation of collagen assembly directly to cell defined micro-domains. We have hypothesized that without this physical link, the normal functions of collagen V could be disrupted; we have also shown that any disruption in this integral collagen is known to cause defective tendon structure and function [33]. Integrins could be involved in indirectly linking collagen assembly to the cell surface through an adaptor molecule such as fibronectin, which in turn could associate with the fibrils (**Fig. 3**). Such interaction of an initiator collagen during assembly (*e.g.*, collagen V) with a membrane-bound molecule or receptor has been mentioned [34]. Collagen fibrillogenesis may require that collagens physically interact at the cell surface with fibronectin and its integrin partners, as treatment with a fibronectin receptor blocking antibody inhibits collagen assembly *in vitro* [10]. Future characterization of these potential interactions using liquid assays such as immunoprecipitation and solid phase assays such as surface plasmon resonance will define any interplay among molecules of interest during matrix assembly.

As a resource for unrelated research (*i.e.*, research unrelated to tendon collagen fibrillogenesis), we believe the value of this work is in the unexpected molecules that were seen in the tendon surfaceome (Table 2). Some of these are: neprilysin and nicastrin, which are involved in the degradation of amyloid beta protein and contribute to Alzheimer pathology; inversin, the altered protein in cystic renal disorders; ezrin, which has structural and regulatory roles in the cytoskeletal/plasma membrane interplay and which also binds directly to CD44. Researchers interested in these molecules could discover novel roles in collagen fibrillogenesis, general matrix assembly, or other connective tissue regulatory roles.

CONCLUSIONS

We have defined a set of proteins included in the fibroblast surfaceome. The surfaceome contains diverse proteins including collagens, fibronectin, integrins and other membrane-bound receptors that we hypothesized would be present based on possible roles in collagen fibrillogenesis. This unbiased data set can serve as a starting point for more targeted experiments, *e.g.* first identifying proteins of interest in the surfaceome of the developing tendon, then characterizing any change in quantitative or spatial expression pattern (by Western blot and densitometry or by immunohistochemistry / immunoelectron microscopy) of those proteins in a particular animal model, during aging or in a disease process. It is also an excellent source for finding those unanticipated molecules that might not have previously been regarded as matrix regulatory molecules but could now be pursued. For our interests, future areas of pursuit would involve elucidation of the roles of candidate proteins in regulation of tendon-specific fibrillogenesis. Collagen V, fibronectin and the $\alpha 1$, $\alpha 2$, $\alpha 5$ and $\beta 1$ integrins are appealing starting points. Other studies could address differential proteomic profiles with advancing age or after injury, and the association with changes in tendon regulatory proteins during collagen assembly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1. Annotation of proteins in cell surface-associated and membrane-associated extracts of the developing tendon

(**A**) LC-MS/MS of the surface-associated extract (gel on left) identified 453 proteins, 22% (98) were in the plasma membrane or extracellular matrix. The cellular compartments of the proteins are shown (pie chart). The plasma membrane and extracellular matrix proteins, collectively the surfaceome are classified based on molecular function (bar graph). (**B**) LC-MS/MS of the membrane extract (gel on left) identified 291 proteins, 30% (87) were in the plasma membrane, with diverse molecular functions (bar graph). The protein annotations are from Gene Ontology annotations in [NCBI](#). Identified proteins function in binding, transport, structure, signal transduction, enzyme control and catalytic activity.

FIG. 2. Expression of selected proteins important in assembly of collagen and extracellular matrix

An extract of the developing FDL was probed for the integrins $\alpha 1$, $\alpha 2$, $\alpha 5$ and $\beta 1$; collagen V; fibronectin (abbreviated here as Fn); the matrix receptors CD44 and syndecan-1; the receptor EGFR; and the protease MMP25 (with the processed enzyme and the larger proenzyme visible). All except integrin $\alpha 1$ and fibronectin were identified in the proteomics by no more than 2 peptides and/or with limited sequence coverage. These molecules were present in immuno-blots of the extracts and validate the proteomics data.

FIG. 3. Model of interactions involving matrix receptors, glycoproteins and collagen in surfaceome mediated regulation of tendon-specific fibril assembly

Based on the data from this work, we show the tendon fibroblast expressing diverse macromolecules that are expressed at the cell surface in early tendon development. Of these, the regulatory molecules, *e.g.*, collagen V and integrins regulate critical steps in fibril assembly, deposition, and organization within the developing extracellular matrix. Glycoproteins could act as adaptor molecules for these interactions, *e.g.*, fibronectin.

Table 1

Summary Data from LC-MS/MS Analysis of Developing Tendons

	-----Total-----			
	Spectra	Unique Peptides	Molecules	Surfaceome
Surface Extract	2332	1903	453	98 ^a (32 ^b /66 ^c)
Membrane Extract	1467	949	291	87 (32/55)

The spectra and peptide data from the proteomic analysis of each extract are presented. The data include intracellular and extracellular components in the “Total”. Only plasma membrane and ECM molecules are included in the “Surfaceome”, with the # of surfaceome molecules and the # of shared and unique molecules (between the 2 extracts) presented.

^a total # of surface (ECM or PM) proteins in the extract

^b # of surface proteins in both surface and membrane extract

^c # of surface proteins unique to the extract

Table 2

Surface- and plasma membrane-associated proteins of the developing mouse FDL tendon

Protein name	Accession #	Molecular weight (Da)	Unique peptides	% sequence coverage
Proteins Identified in Surface-associated Extract Only				
Binding				
Biglycan (Bone/cartilage proteoglycan I) (PG-S1)	PGS1_MOUSE	41,637.90	7	25.80%
Calreticulin	CALR_MOUSE	47,978.10	1	2.88%
Cartilage oligomeric matrix protein	COMP_MOUSE	82,331.70	1	1.32%
Decorin	PGS2_MOUSE	39,792.80	6	20.90%
Dermcidin; AltName: Preproteolysin	DCD_MOUSE	11,266.10	2	12.70%
Development and differentiation-enhancing factor 2	DDEF2_MOUSE	106,789.60	1	0.84%
Fibromodulin	FMOD_MOUSE	43,038.00	3	10.60%
Galectin-7	LEG7_HUMAN	15,056.70	2	18.40%
Glypican-4	GPC4_MOUSE	62,569.20	1	3.23%
Keratocan	KERA_MOUSE	40,387.90	7	25.10%
Lumican	LUM_MOUSE	38,249.40	3	13.30%
Matrilin-2	MATN2_MOUSE	106,761.30	4	6.38%
Mimecan	MIME_MOUSE	33,996.20	5	18.10%
Periostin	POSTN_MOUSE	93,129.20	6	12.10%
Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A)	S10A8_MOUSE	10,816.90	2	23.70%
Tenascin	TENA_MOUSE	231,787.60	4	4.08%
Tetranectin	TETN_MOUSE	22,240.00	2	12.90%
Transforming growth factor-beta-induced protein ig-h3	BGH3_MOUSE	74,581.00	7	17.40%
Catalytic Activity				
A disintegrin and metalloproteinase with thrombospondin motifs 1	ATS1_MOUSE	105,823.40	1	1.03%
Cartilage-associated protein	CRTAP_MOUSE	46,149.20	2	4.25%
Cationic trypsin	TRY1_BOVIN	25,767.20	4	23.20%
EH domain-containing protein 1; AltName: Testilin	EHD1_MOUSE	60,611.10	6	18.70%
EH domain-containing protein 4	EHD4_MOUSE	61,159.60	2	5.55%
Lysyl oxidase homolog 1 precursor (Lysyl oxidase-like protein 1) (LOL)	LOXL1_MOUSE	63,090.70	2	4.53%
Tolloid-like protein 1	TLL1_MOUSE	114,516.80	1	0.79%
Enzyme Regulation				
Alpha-1-antitrypsin 1-1	A1AT1_MOUSE	45,985.70	1	5.33%
Fibronectin	FINC_MOUSE	272,464.80	9	7.47%
Procollagen C-endopeptidase enhancer 1	PCOC1_MOUSE	50,150.10	5	15.40%
Serpin B3 (Squamous cell carcinoma antigen 1) (SCCA-1)	SPB3_MOUSE	44,547.90	2	7.95%
Signal Transduction				
Alpha-2-macroglobulin receptor-associated protein	AMRP_MOUSE	42,198.80	2	7.22%

Protein name	Accession #	Molecular weight (Da)	Unique peptides	% sequence coverage
Cardiotrophin-like cytokine factor 1	CLCF1_MOUSE	25,244.10	1	3.56%
Chondroitin sulfate proteoglycan 4	CSPG4_MOUSE	252,384.10	3	2.23%
Collagen alpha-3(IV) chain ^{Also Structural}	CO4A3_MOUSE	161,713.00	1	0.54%
Guanine nucleotide-binding protein G(i), alpha-2 subunit ^{Also Catalytic}	GNAI2_MOUSE	40,454.10	5	22.80%
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GBB2_MOUSE	37,313.80	2	6.76%
Insulin-like growth factor I	IGF1_MOUSE	17,075.80	1	13.10%
Integrin beta-7	ITB7_MOUSE	87,391.70	1	1.86%
Inversin	INVS_MOUSE	117,112.30	1	3.58%
Lysosome-associated membrane glycoprotein 2	LAMP2_MOUSE	45,629.30	2	4.34%
Macrophage mannose receptor 2	MRC2_HUMAN	166,637.40	2	1.83%
Platelet glycoprotein 4	CD36_MOUSE	52,681.00	2	5.72%
Transmembrane emp24 domain-containing protein 9	TMED9_MOUSE	24,991.90	2	9.35%
Structural Molecules				
Aggrecan core protein	PGCA_MOUSE	221,987.00	2	1.50%
Alpha-crystallin B chain	CRYAB_MOUSE	20,051.40	2	22.30%
Annexin A4	ANXA4_MOUSE	35,973.90	5	21.00%
Basement membrane-specific heparan sulfate proteoglycan core protein	PGBM_MOUSE	398,260.20	2	0.94%
Catenin alpha-1	CTNA1_MOUSE	100,089.90	2	5.96%
Clathrin heavy chain 1; AltName: CLH-17	CLH1_MOUSE	191,600.90	22	22.10%
Collagen alpha-1(V) chain	CO5A1_MOUSE	183,662.00	2	1.09%
Collagen alpha-1(XII) chain	COCA1_MOUSE	340,194.60	55	27.90%
Collagen alpha-2(I) chain	CO1A2_MOUSE	129,539.30	10	10.10%
Collagen alpha-2(XI) chain	COBA2_MOUSE	171,516.30	1	1.27%
Collagen alpha-6(VI) chain	CO6A6_MOUSE	246,308.00	3	2.38%
Moesin	MOES_MOUSE	67,750.80	2	3.29%
Myelin P0 protein	MYP0_MOUSE	27,604.50	2	7.66%
Radixin	RADI_HUMAN	68,547.50	4	16.30%
Syndecan-1 (CD138)	SDC1_MOUSE	32,885.80	1	3.22%
Talin-1	TLN1_MOUSE	269,813.30	3	2.36%
Thrombospondin-3	TSP3_MOUSE	103,953.90	2	3.77%
Transporters				
Apolipoprotein A-I	APOA1_MOUSE	30,569.40	2	8.33%
BTB/POZ domain-containing protein KCTD12	KCD12_MOUSE	35,875.00	5	19.00%
Cation-independent mannose-6-phosphate receptor	MPRI_MOUSE	273,795.00	5	2.13%
Plasma membrane calcium-transporting ATPase 1 (PMCA1) ^{Also Catalytic}	AT2B1_MOUSE	138,741.20	2	2.70%
Serum albumin	ALBU_MOUSE	68,674.90	18	40.60%
Sodium/potassium-transporting ATPase subunit alpha-1 ^{Also Catalytic}	AT1A1_MOUSE	112,881.50	4	5.77%

Protein name	Accession #	Molecular weight (Da)	Unique peptides	% sequence coverage
Voltage-dependent anion-selective channel protein 2	VDAC2_MOUSE	31,715.60	3	11.90%
Proteins Identified in Membrane Extract Only				
Binding				
Adipocyte plasma membrane-associated protein	APMAP_MOUSE	46,417.40	1	2.89%
CD166 antigen	CD166_MOUSE	65,075.40	3	7.20%
Flotillin-1	FLOT1_MOUSE	47,494.80	1	2.57%
Immunoglobulin superfamily containing leucine-rich repeat protein	ISLR_MOUSE	45,597.80	1	2.34%
Junction plakoglobin	PLAK_MOUSE	81,783.90	1	1.88%
Kin of IRRE-like protein 1	KIRR1_MOUSE	87,159.10	1	2.28%
Kin of IRRE-like protein 2	KIRR2_MOUSE	74,510.30	1	2.00%
Neuromodulin	NEUM_MOUSE	23,614.30	1	7.49%
Protocadherin Fat 4	FAT4_MOUSE	540,317.00	1	0.36%
Protocadherin-15	PCD15_MOUSE	214,801.50	1	0.62%
Proteolipid protein 2	PLP2_MOUSE	16,590.60	1	7.89%
Sarcolemmal membrane-associated protein	SLMAP_MOUSE	96,917.20	1	3.31%
SPARC	SPRC_MOUSE	34,431.70	1	5.30%
Transmembrane protein 131	TM131_MOUSE	200,497.40	1	1.09%
UPF0577 protein KIAA1324	K1324_MOUSE	110,664.10	1	1.09%
Vascular cell adhesion protein 1	VCAM1_MOUSE	81,300.00	3	5.82%
Catalytic Activity				
Alcohol dehydrogenase A88	AK1A1_MOUSE	36,569.20	1	2.46%
Carboxypeptidase M	CBPM_MOUSE	50,539.10	1	2.03%
Matrix metalloproteinase-25	MMP25_MOUSE	68,479.30	1	2.93%
Neprilysin	NEP_MOUSE	85,687.00	2	2.80%
Nicastrin	NICA_MOUSE	78,473.00	2	2.97%
Prolyl 3-hydroxylase 1	P3H1_MOUSE	83,634.50	2	2.98%
Testisin	TEST_MOUSE	36,157.50	1	6.79%
Enzyme Regulation				
Caveolin-1	CAV1_MOUSE	20,521.40	3	24.20%
Rab GDP dissociation inhibitor beta	GDIB_MOUSE	50,521.10	4	11.50%
Thy-1 membrane glycoprotein (CD90)	THY1_MOUSE	18,062.50	2	13.60%
Signal Transduction				
5-hydroxytryptamine receptor 2A	5HT2A_MOUSE	52,825.40	1	5.31%
CD44 antigen; Alt Name: Hyaluronan Receptor	CD44_MOUSE	85,598.60	1	1.54%
Epidermal growth factor receptor	EGFR_MOUSE	134,836.90	1	1.82%
Glutamate receptor 1	GRIA1_MOUSE	100,616.80	1	2.22%
Inositol 1,4,5-triphosphate receptor-interacting protein	IPRI_MOUSE	63,221.40	1	2.34%
Insulin receptor-related protein	INSRR_MOUSE	144,729.00	2	2.38%
Integrin alpha-1	ITA1_MOUSE	130,792.70	1	0.68%
Integrin alpha-5	ITA5_MOUSE	115,040.60	3	4.18%

Protein name	Accession #	Molecular weight (Da)	Unique peptides	% sequence coverage
Integrin alpha-6	ITA6_MOUSE	122,133.80	3	5.04%
Integrin alpha-7	ITA7_MOUSE	129,412.20	1	1.44%
Integrin alpha-V	ITAV_MOUSE	115,262.60	4	3.93%
Integrin beta-2	ITB2_MOUSE	87,891.30	1	1.13%
Mannose-6-phosphate receptor-binding protein 1	M6PBP_MOUSE	47,242.80	1	3.20%
Netrin receptor UNC5D	UNC5D_MOUSE	106,334.50	1	1.36%
SH2 domain-containing protein 3C	SH2D3_MOUSE	94,306.20	1	1.52%
Stomatin-like protein 2	STML2_MOUSE	38,366.90	2	8.78%
Tenomodulin	TNMD_MOUSE	37,030.00	2	7.57%
Tetraspanin-31	TSN31_MOUSE	22,676.10	1	5.71%
Structural Molecules				
AnneXn A11	ANX11_MOUSE	54,095.50	2	4.97%
Transporters				
4F2 cell-surface antigen heavy chain ^{Also Catalytic}	4F2_MOUSE	58,321.20	1	2.66%
Aquaporin-1	AQP1_MOUSE	28,776.50	1	3.35%
Calcium-activated chloride channel regulator 1	CLCA1_MOUSE	100,054.70	1	2.63%
Epsin-1	EPN1_MOUSE	60,194.70	1	2.78%
Myoferlin	MYOF_MOUSE	233,311.40	2	1.27%
Receptor-transporting protein 4	RTP4_MOUSE	28,374.10	1	4.42%
Stonin-1	STON1_MOUSE	81,775.30	1	1.51%
Synaptotagmin-like protein 4	SYTL4_MOUSE	76,005.10	1	2.23%
Transient receptor potential cation channel subfamily V member 3	TRPV3_MOUSE	90,649.50	1	1.14%
Voltage-dependent calcium channel subunit alpha-2/delta-1	CA2D1_MOUSE	124,616.10	10	10.90%
Proteins Identified in Both Surface-associated and Membrane Extracts				
Binding				
Alpha-fetoprotein	FETA_MOUSE	67,320.20	<i>NB₇</i>	11.40%
Apolipoprotein A-IV	APOA4_MOUSE	45,010.80	9	27.60%
Cadherin-13	CAD13_MOUSE	78,268.50	1	1.68%
Cell surface glycoprotein MUC18	MUC18_MOUSE	71,526.20	2	4.94%
Contactin-3	CNTN3_MOUSE	113,133.70	1	0.88%
Gelsolin	GELS_MOUSE	85,923.90	4	8.72%
Hematopoietic progenitor cell antigen CD34	CD34_MOUSE	40,965.10	1	2.36%
Neural cell adhesion molecule 1	NCAM1_MOUSE	119,409.90	4	7.00%
Catalytic Activity				
Alpha-enolase	ENOA_MOUSE	47,123.70	12	33.20%
Aminopeptidase N	AMPN_MOUSE	109,636.40	10	15.80%
EH domain-containing protein 2	EHD2_MOUSE	61,048.60	2	4.79%
Enzyme Regulation				
Pigment epithelium-derived factor	PEDF_MOUSE	46,217.90	4	10.80%
Signal Transduction				

Protein name	Accession #	Molecular weight (Da)	Unique peptides	% sequence coverage
Basigin	BASI_MOUSE	42,426.40	6	15.40%
CD109 antigen	CD109_MOUSE	161,643.90	1	0.97%
Immunoglobulin superfamily member 8	IGSF8_MOUSE	64,991.60	1	1.64%
Integrin alpha-2	ITA2_MOUSE	128,911.10	2	2.80%
Integrin beta-1	ITB1_MOUSE	88,213.30	11	15.30%
Tumor necrosis factor receptor superfamily member 26	TNR26_MOUSE	22,690.20	1	8.82%
Structural Molecules				
Annexin A1	ANXA1_MOUSE	38,717.70	4	14.50%
Annexin A2 <i>Also an Enzyme Regulation</i>	ANXA2_MOUSE	38,660.00	13	39.80%
Annexin A5	ANXA5_MOUSE	35,736.20	10	43.60%
Annexin A6	ANXA6_MOUSE	75,872.20	23	42.30%
Collagen alpha-1(I) chain	CO1A1_MOUSE	138,014.70	5	4.40%
Collagen alpha-1(VI) chain	CO6A1_MOUSE	108,473.00	4	5.17%
Collagen alpha-1 (XIV) chain	COEA1_MOUSE	192,995.10	5	4.45%
Collagen alpha-2(VI) chain	CO6A2_MOUSE	110,317.20	2	3.00%
Ezrin	EZRI_MOUSE	69,390.80	3	11.90%
Vinculin	VINC_MOUSE	116,701.40	12	15.90%
Transporters				
Extracellular matrix protein FRAS1	FRAS1_MOUSE	442,443.20	1	0.37%
Prolow-density lipoprotein receptor-related protein 1	LRP1_MOUSE	504,709.30	1	0.46%
Serotransferrin	TRFE_MOUSE	76,706.20	10	17.40%
Voltage-dependent anion-selective channel protein 1	VDAC1_MOUSE	32,334.70	8	38.90%

The molecules identified in a proteomic analysis of the cell surface-associated (Table 1S) and membrane (Table 2S) extracts, are presented here as the P1 tendon 'surfaceome'. Classification by cellular compartment (intracellular, extracellular and plasma membrane) and by molecular function (binding, catalytic activity, enzyme regulation, signal transduction, structural molecules and transporters) are derived solely from Gene Ontology annotations as provided in NCBI and Scaffold.

NB All numbers for the shared proteins are based on data from the surface-associated extract

Table 3

Surfaceome proteins of the developing mouse FDL tendon that are involved in collagen and matrix assembly

Protein Name
Matrix Receptors**
CD44 antigen (Hyaluronan Receptor)
Integrin alpha-1
Integrin alpha-2
Integrin alpha-5
Integrin alpha-6
Integrin alpha-7
Integrin alpha-V
Integrin beta-1
Integrin beta-2
Integrin beta-7
Syndecan-1 (CD138)
Multi-domain Matrix Glycoproteins**
Fibronectin
Matrilin-2
Periostin
Tenascin
Proteoglycans
Perlecan
Biglycan
Decorin
Fibromodulin
Keratocan
Lumican
Mimecan
Collagens**
Collagen alpha-1 (I) chain
Collagen alpha-2(I) chain
Collagen alpha-1(V) chain
Collagen alpha-1(VI) chain
Collagen alpha-2(VI) chain
Collagen alpha-2(XI) chain
Collagen alpha-1(XII) chain
Collagen alpha-1(XIV) chain
Other Regulatory Proteins**
Extracellular matrix protein FRAS1
Epidermal growth factor receptor (EGFR)
Matrix metalloproteinase-25 (MT6-MMP)

Tenomodulin
Tolloid-like protein 1
Other Proteins
Annexin A1
Annexin A2
Annexin A4
Annexin A5
Basigin (Emmprin/CD147)
Cadherin-13
Cardiotrophin-like cytokine factor 1
CD109 antigen
CD166 antigen (Activated Leukocyte Cell Adhesion Molecule)
Insulin-like growth factor I
Talin-1
Thy-1 membrane glycoprotein (CD90)

Those proteins from Table 1 (surface-associated and membrane extracts combined) that we hypothesize will be crucial to collagen fibrillogenesis in tendons and to assembly of the tenocyte extracellular matrix are presented here. They are classified according to their known characteristics based on available research findings obtained from our specific Pubmed search analysis.

**

The matrix receptors, glycoproteins, collagens and matrix regulatory molecules (all in groups depicted by) of particular interest to us were confirmed via Western blots.