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Characterization of FIBCD1 as an Acetyl Group-Binding Receptor That Binds Chitin

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Chitin is a highly acetylated compound and the second most abundant biopolymer in the world next to cellulose. Vertebrates are exposed to chitin both through food ingestion and when infected with parasites, and fungi and chitin modulate the immune response in different directions. We have identified a novel homotetrameric 55-kDa type II transmembrane protein encoded by the FIBCD1 gene and highly expressed in the gastrointestinal tract. The ectodomain of FIBCD1 is characterized by a coiled-coil region, a polycationic region and C-terminal fibrinogen-related domain that by disulfide linkage assembles the protein into tetramers. Functional analysis showed a high-affinity and calcium-dependent binding of acetylated components to the fibrinogen domain, and a function in endocytosis was demonstrated. Screening for ligands revealed that the FIBCD1 is a high-affinity receptor for chitin and chitin fragments. FIBCD1 may play an important role in controlling the exposure of intestine to chitin and chitin fragments, which is of great relevance for the immune defense against parasites and fungi and for immune response modulation. The Journal of Immunology, 2009, 183: 3800–3809.

Chitin is a linear homopolymer of β-1,4-linked N-acetylglucosamine, which next to cellulose is the most abundant known biopolymer (1). Chitin is an important structural component in the cell wall of most fungi (2), in the eggshell of parasitic nematodes (3), and in the exoskeleton of all types of arthropods, as well as in the cuticle of the epidermis and the trachea and the lining of the gut of many insects (4). Vertebrates are therefore exposed to chitins through ingested food or when infected with nematodes or fungi.

In plants, chitin and its fragments, chitin oligosaccharides or N-acetylchitoioigosaccharides, are recognized as the typical fungal pathogen-associated molecular pattern (PAMP) that triggers various defense responses. These include cell surface chitin recognition receptors like CEBIP (5) and receptor-like kinases like CERK1 that elicit MARK activation, reactive oxygen species generation, and gene expression upon activation with chitin (6). Chitin has recently been identified as a PAMP that modulates the allergic response in mice (7). Chitin induces an immune response characterized by infiltration of cells that express IL-4 and IL-13 including Th2 cells, eosinophils and basophiles (7), a response that typically is seen associated with allergic and parasitic worm immune response. Vertebrates lack the ability to produce chitin, but despite this they do express highly conserved chitinases (8). The acidic mammalian chitinase (AMCase) is expressed mainly in the salivary glands and by the stomach (9), whereas the chitotriosidase is expressed by tissue macrophages. Both are endo-β-1,4-N-acetylglucosaminidases that are believed to be involved in food digestion and immunity (10). The human chitotriosidase was shown to have a fungistatic effect (11), and the AMCase has been linked to the pathophysiology of asthma (12). Soluble mammalian chitin-binding proteins are known. The C-type lectin RegIIIg (or HIP/PAP) secreted by Paneth cells is a common pattern recognition molecule for chitin and peptidoglycan that is induced by symbiotic bacteria (13). However, a classical chitin receptor has not yet been identified in vertebrates (14). Here we report the identification a type II membrane protein expressed apically on enterocytes. The protein binds acetylated components including chitin and directs ligands for endocytosis. This receptor is encoded by the FIBCD1 gene and conserved in all the main vertebrate species.

Materials and Methods

Buffers

TBS (140 mM NaCl, 10 mM Tris-Cl, pH 7.4); TBS/Tw (TBS containing 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate); Merck-Schuchardt); PBS (137 mM NaCl, 3 mM KCl, 8 mM NaHPO4, 1.5 mM KH2PO4, pH 7.4); coating buffer (60 mM Na2CO3, 35 mM NaHCO3, 0.02% (w/v) NaN3, pH 9.6); substrate buffer (100 mM Tris-HCl, 5 mM MgCl2, 100 mM NaCl, pH 9.5).

Cloning of full-length, ectodomain, and fibrinogen-related domain of FIBCD1

The various FIBCD1 constructs were generated by PCR using Phusion DNA polymerase (Finnzymes) and using as template IMAGE clone ID 4811679 (GenBank accession number BC032953; http://www.ncbi.nlm.nih.gov/GenBank/). Full-length FIBCD1 was generated with the primers: 5’-GTCTCTGCGCGAGAGATGTT-3’ and 5’-GTCTAGCGCTCTCCCGGACC-3’. The V5-His-tagged ectodomain of FIBCD1, encoding aa 54 – 461, was generated with the primers: 5’-GCCACCCACCCACAGCGCC-3’ and 5’-GGGTCCTCCCGGACC-3’. The fibrinogen-related domain of FIBCD1 was generated with the primers: 5’-CTGATACCCGCACTGGCGTCCTC-3’ and 5’-CCCTGAAGTTCTAGGCGCTCTCCCGGACC-3’.

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PCR products were cloned into the expression vectors pSecTag/FRT/V5-His TOPO TA (FIBCD1 ectodomain), pcDNA/FRT/V5-His TOPO TA (FIBCD1 full-length), both vectors from Invitrogen), or into the pNT-Bac vector (the fibrinogen-related domain of FIBCD1), which was kindly donated by Nicole M. Thielens (Institut de Biologie Structurale J.-P. Ebel, Grenoble, France) using standard molecular biology techniques. The inserts were sequenced in their entirety.

Generation of HEK293 and Chinese hamster ovary (CHO) cells expressing recombinant forms of FIBCD1
To generate cells expressing recombinant forms of the FIBCD1, the Fbp-In system (Invitrogen) was used as described (15) except that HEK293 cells were selected for stable integration of an FIBCD1-containing construct using 150 μg/ml hygromycin B (Invitrogen).

Expression of the fibrinogen-related domain of FIBCD1 in insect cells
The pNT-Bac vector containing the fibrinogen-related domain was expressed in using the baculovirus expression system in sf9 cells essentially as described by Rossi et al. (16).

Purification of the V5-His-tagged FIBCD1 ectodomain
Purification was performed using a HiTrap Chelating HP (Amersham Pharmacia Biotech) column as described (15).

SDS-PAGE and Western blotting
Proteins were separated on 4–12% polyacrylamide gradient gels in a discontinuous buffer system and blotted onto polyvinylidene difluoride membrane (Immobilon P; Millipore). The membrane was incubated with 0.5 μg/ml monoclonal mouse anti-V5 Ab or 0.5 μg/ml HG-HYB-12-2 followed by alkaline phosphatase-coupled rabbit anti-mouse IgG (Sigma-Aldrich) diluted 1/2000 in TBS/Tw (50 mM NaCl, 10 mM Tris-HCl, 0.02% (w/v) Na₃PO₄, 0.05% (v/v) pH 7.4 Tween 20). The membrane was washed and developed as described (15). Western blotting was also performed using enhanced chemiluminescence (GE Healthcare) for development according to the manufacturer’s recommendations. Silver staining was performed essentially as described (17).

Chemical cross-linking of FIBCD1
The V5-His-tagged FIBCD1 ectodomain was cross-linked using bis(sulfosuccinimidyl)suberate (BS3; Pierce Biotechnology). Briefly, BS3 was added to the recombinant V5-His-tagged FIBCD1 ectodomain fractions in a 10- to 320-molar excess and incubated at room temperature for 30 min; then the reactions were stopped by the addition of 0.1 M Tris-HCl buffer, pH 7.4. The cross-linked samples were reduced and analyzed by SDS-PAGE and Western blotting.

Deglycosylation of FIBCD1
The presence of N-linked saccharides on the V5-His FIBCD1 ectodomain that was expressed in HEK293 cells was demonstrated by enzymatic digestion as described (15).

FACS analysis
CHO cells expressing full-length FIBCD1 were incubated with 10 μg/ml anti-FIBCD1 (HG-HYB-12-1) Ab in FACS buffer (PBS, 1% BSA, and 0.05% azide) for 30 min at 4°C, washed twice in FACS buffer, incubated with FITC-conjugated F(ab)₂ goat anti-mouse (DakoCytomation) for 30 min, and then washed three times with FACS buffer before analysis.

Confocal imaging
CHO FIBCD1/CHO cells were seeded at a density of 4 × 10⁵ cells/well 48 h before experiment. For cell surface staining, cells were incubated with 20 μg/ml monoclonal mouse anti-FIBCD1 (HG-HYB-12-1) for 1.5 h at 4°C. After wash, the cells were incubated with Alexa Fluor 488 F(ab)₂ goat anti-mouse IgG (Invitrogen) at 4°C. For internalization experiments, the cells were incubated with 20 μg/ml Alexa Fluor 488-labeled acetylated BSA at 37°C for 2 h. Finally, the cells were washed twice in PBS and fixed in 4% paraformaldehyde followed by staining with 4′,6′-diamidino-2-phenylindole nucleic acid stain (Invitrogen) and mounted using ProLong Gold antifade reagent (Invitrogen). The fluorescence was visualized with an Olympus FV1000 microscope.

Production of chicken anti-FIBCD1 Abs
The immunizations and the purification were done by David’s Biotechnology. Briefly, 1 hen (White Leghorn) was immunized three times with ~30 μg of FIBCD1-V5-His ectodomain with a 2-wk interval. The collection of the first eggs started 43 days after the first immunization. The purification of IgY polyclonal Abs was performed using a stepwise salting out method.

Production of anti-FIBCD1 mAbs
BALB/c mice were immunized for the production of mAbs against the FIBCD1 ectodomain. The mice were immunized five times with ~5 μg of recombinant FIBCD1 ectodomain with 2 wk between each immunization. The mice were boosted three times with 10 μg of FIBCD1 ectodomain protein diluted in 200 μl of PBS by injection into the dorsal tail vein. B cell hybridomas were produced by fusion between myeloma cells (American Type Culture Collection; CRL-2016; Sp2/0Ag14) and BAF3 cells. The hybridomas were expanded by immunofluorescence using a fluorescence activated cell sorter (FACS) and the supernatants were tested for FIBCD1-binding activity. The hybridomas were cloned by limiting dilution and plated into 96-well plates using 0.5 ml of per well of RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate. The hybridomas were cultured in RPMI 1640 medium with 10% FCS and the medium was changed every other day. The selection was performed using 1.56, 0.78, and 0.39 mM in TBS/Tw (5 mM CaCl₂) before chromatography. Culture supernatant from FIBCD1-FRCD domain, which was kindly donated by Nicole M. Thielens (Institut de Biologie Structurale J.-P. Ebel, Grenoble, France) using standard molecular biology techniques. The inserts were sequenced in their entirety.

Immunohistochemistry
Normal human tissues were obtained from the tissue bank at the Department of Pathology, Odense University Hospital (Odense, Denmark). The tissues were fixed in 4% formalin in PBS for 24 h and then conventionally dehydrated and embedded in paraffin. A biotin-streptavidin detection procedure was used. The tissue sections were dewaxed and rehydrated by antigen retrieval as described (19). The sections were incubated with 0.3% H₂O₂, followed by equilibration in TBS (50 mM NaCl, 10 mM Tris-HCl, pH 7.4) for 1 h. The sections were blocked with 5% normal goat serum in TBS/Tw (50 mM NaCl, 10 mM Tris-HCl, pH 7.4) for 30 min. The sections were then incubated with primary Abs overnight at 4°C in a humid chamber. The primary Abs were detected with the appropriate secondary anti-mouse or anti-rabbit IgG (horseradish peroxidase labeled) Abs (Sigma-Aldrich) diluted 1/2000 in TBS/Tw (50 mM NaCl, 10 mM Tris-HCl, pH 7.4) for 30 min. The sections were washed three times with TBS/Tw and developed using an enhanced chemiluminescence kit (GE Healthcare) for visualization of the antigen localization. The sections were counterstained with hematoxylin.

Purification of the nontagged FIBCD1 ectodomain and FIBCD1 fibrinogen-related domain on an N-acetylated immobilized resin
Toyopearl AF-Amino-650M resin (Tosoh; 5 ml) was washed twice with distilled water and mixed with 4 ml of 0.2 M sodium acetate and 2 ml of acetic anhydride and then incubated on ice for 30 min. After incubation, 2 ml of acetic anhydride were added to the mixture, and the incubation was continued for a further 30 min as described (19). The resin was washed four times with distilled water and 1 M NaOH followed by washing with TBS/Tw (0.5 M NaCl and 5 mM CaCl₂) before chromatography.

Characterization of the ligand-binding properties of FIBCD1
An ELISA system was used to evaluate the ability of various acetylated and nonacetylated compounds to inhibit the binding of recombinant FIBCD1 ectodomain and acetylated BSA (Sigma-Aldrich). Microtiter plates (NUNC Maxisorp) were coated with acetylated BSA (Sigma-Aldrich) or BSA (Sigma-Aldrich) and blocked with TBS/Tw before being incubated with FIBCD1 ectodomain samples diluted in TBS/Tw (5 mM CaCl₂). The plate was washed with TBS/Tw (5 mM CaCl₂) and incubated for 1 h with alkaline phosphatase-labeled anti-chicken IgY (whole molecule; Sigma-Aldrich) diluted 1/1500 in TBS/Tw (5 mM CaCl₂). The plate was washed with TBS/Tw (5 mM CaCl₂) and incubated for 1 h with keyhole limpet hemocyanin (Sigma-Aldrich) for 1 h followed by washing. The wells were incubated for 2 h at room temperature with chicken anti-FIBCD1 Abs diluted 1/1000 in TBS/Tw (5 mM CaCl₂). The plate was washed with TBS/Tw (5 mM CaCl₂) and incubated for 1 h with alkaline phosphatase-labeled anti-chicken IgY (whole molecule; Sigma-Aldrich) diluted 1/1500 in TBS/Tw (5 mM CaCl₂) followed by washing and developed with 1 mg/ml nitrophenylphosphate disodium salt (Boehringer Mannheim) in substrate buffer. The specificity of the binding to acetylated BSA was assayed by inhibition with recombinant and nonacetylated compounds including glucose, L-alanine, L-malic acid, L-aspartic acid, L-glutamic acid, L-asparagine, L-serine, L-threonine, L-histidine, L-glutamine, L-proline, L-lysine, L-isoleucine, L-leucine, L-valine, and L-phenylalanine. The inhibition was performed using a stepwise salting out method.

Production of chicken anti-FIBCD1 Abs
The immunizations and the purification were done by David’s Biotechnology. Briefly, 1 hen (White Leghorn) was immunized three times with ~30 μg of FIBCD1-V5-His ectodomain with a 2-wk interval. The collection of the first eggs started 43 days after the first immunization. The purification of IgY polyclonal Abs was performed using a stepwise salting out method. The epitope was therefore located between the TM region and the FRCD domain, excluding cross-reactivity with the ficolins.
For the PAMPs-binding assay, microtiter plates were coated with LPS O55:B5 (Sigma-Aldrich), 1 μg/ml; LPS 0111:B4 (Sigma-Aldrich), 1 μg/ml; LPS 026:B6 (Sigma-Aldrich), 1 μg/ml; lipoteichoic acid (LTA; InvivoGen), 10 μg/ml; mannan (Sigma-Aldrich), 1 μg/ml; soluble peptidoglycan (InvivoGen), 10 μg/ml; and acetylated BSA (Sigma-Aldrich), 1 μg/ml, in coating buffer for 2 h at room temperature and otherwise performed as described previously except that the fibrinogen domain of FIBCD1 produced in insect cells was used. As a positive control, surfactant protein D (SP-D) was added to the plates in parallel with the FReD of FIBCD1. SP-D bound to LPS, LTA, mannan and soluble peptidoglycan but not to acetylated BSA (data not shown).

**Pull-down assay**

Chitin from crab shells, cellulose, zymosan A, and β-1,3-glucan (all from Sigma-Aldrich) was washed three times in TBS/Tw (5 mM CaCl2) and pelleted. Twenty micrograms of the fibrinogen-related domain of FIBCD1 were added to 2 mg of each carbohydrate in a total volume of 500 μl of TBS/Tw (5 mM CaCl2). For the inhibition experiment, pelleted chitin was incubated with 20 μg of FIBCD1 in TBS/Tw (10 mM EDTA) or TBS/Tw (250 mM sodium acetate). Twenty micrograms of wheat germ agglutinin (WGA) were added to 2 mg of chitin as a positive control. Twenty micrograms of BSA were added to chitin as a negative control. Twenty micrograms of FIBCD1 without chitin as a blank control. After overnight incubation at 4°C, the pelleted carbohydrates were washed three times in TBS/Tw (5 mM CaCl2). Bound protein was eluted by boiling the pelleted carbohydrates in SDS-PAGE buffer and resolved by SDS-PAGE through 4–17% polyacrylamide gels followed by Coomassie staining.

**Endocytosis of 125I-labeled acetylated BSA**

Endocytosis experiments were performed with FIBCD1-expressing Flp-In CHO cells or nontransfected Flp-In CHO cells grown to confluence in 24-well plates essentially as described (20). In brief, acetylated BSA was labeled with 125I using the chloramine-T method, and triplicates of cells were incubated in Ham’s F-12 medium supplemented with 1% BSA containing 4000 cpm of 125I-labeled acetylated BSA for various time intervals at 37°C.

**Alexa Fluor 488 labeling of acetylated BSA**

Lyophilized powder of acetylated BSA was dissolved in PBS, pH 7.4, to a concentration of 10 mg/ml, and thiols for coupling were generated by reducing cystine disulfides with the reducing agent tris(2-carboxyethyl)phosphine (Sigma-Aldrich) at a concentration of 10 mM. At a concentration of 2.5 mg/ml, acetylated BSA was incubated with Alexa Fluor 488 C5 maleimide (Invitrogen) in a 1:10 molar ratio for 2 h at room temperature followed by extensive dialysis against PBS to remove free dye.

**Sequence analysis**

DNA sequence analysis, alignments, and amino acid sequence prediction were done with Lasergene software (DNASTar). Multiple sequence alignments were conducted using a ClustalW algorithm (http://www.ebi.ac.uk/ clustalw). Membrane topology and putative membrane-spanning domains were determined by Hidden Markov Model analysis software (http://www.cbs.dtu.dk/services/TMHMM/).

**Results**

**Identification and predicted domain organization of a potential acetyl group-binding protein**

We searched the National Center for Biotechnology Information Human Expressed Sequence Tag clone database using the L-ficolin cDNA sequence in a homology search for possible new acetyl group-binding molecules with membrane topology. This approach revealed a cDNA clone of 3190 bp (GenBank accession number BC032953; http://www.ncbi.nlm.nih.gov/GenBank/) that showed high homology to L-ficolin, M-ficolin, and tachylectin 5A (TL5A), and these proteins bind acetyl groups through their FReDs. Database analysis revealed that FIBCD1 is highly conserved in vertebrates from mammals to birds, amphibians, and fish (Fig. 2). Insects and worms also express membrane-bound FReDs, but in these molecules the conserved residues involved in acetyl group binding are lost.

**Structural characterization of FIBCD1**

We expressed the ectodomain of FIBCD1 as a secreted protein in the human cell line HEK293 to obtain insight into the structural organization of the receptor. The purified protein migrates corresponding to a molecular mass of ~55 kDa in the reduced state and 250 kDa in the unreduced state on SDS-PAGE (Fig. 3A). This demonstrates that FIBCD1 is assembled as a disulfide-linked homopolymeric structure. One cysteine is located in the region of potential coiled coils and two cysteines are located at the boundaries, those being involved in the interchain disulfide bridge formation is yet unknown (Fig. 1). We then did chemical cross-linking at various concentrations of BS3 of FIBCD1 ectodomain. Four distinct bands were observed corresponding to monomeric, dimeric, trimeric, and tetrameric structures, when analyzed in the reduced state by SDS-PAGE (Fig. 3B). Treatment with N-glycanase reduced the molecular mass of the ectodomain of the receptor with 2–3 kDa (Fig. 3C). FIBCD1 is predicted to be a membrane protein with type II topology. FACS analysis (Fig. 3D) and immunofluorescence confocal microscopy analysis (Fig. 3E) of CHO cells transfected with the full-length FIBCD1 cDNA showed a cell surface localization. Taken together, we conclude that FIBCD1 is a glycoprotein that forms disulfide-linked homotetramers in the plasma membrane. A model of the FIBCD1 is shown in Fig. 3F.

**Immunohistochemical localization of FIBCD1**

Immunohistochemical analysis of human tissues showed high expression of FIBCD1 in small and large intestine epithelial cells with a highly polarized localization to the apical surface corresponding to the brush border (Fig. 4, A–E) and in the ducts of the salivary glands (Fig. 4F). Weak or no reactivity was observed in stomach epithelial cells, in respiratory cells, or in urogenital epithelial cells (not shown). As a positive control, strong immunoreactivity was found in HEK293 transfected with FIBCD1 cDNA, whereas no reaction was found in nontransfected HEK293 cells (Fig. 4, G and H). The specificity of the mAb used for immunohistochemical analysis was further analyzed using Triton X-100 lysates of HEK293 and HEK293 transfected with FIBCD1 cDNA for Western blotting (Fig. 5).

The relative levels of the FIBCD1 mRNA were determined in 22 different human tissues by real time PCR (data not show). The mRNA was measured in virtually all tissues analyzed. No correlation was found between the mRNA levels measured by RT-PCR and the immunohistochemistry.

**FIBCD1 is a calcium-dependent acetyl group-binding molecule**

The alignment of the human ficolins with TL5A and FIBCD1 reveals that some but not all of the residues of the TL5A and L- and
FIGURE 1. The chromosome localization, genomic organization of FIBCD1, and the mRNA transcripts and deduced amino acid sequence of FIBCD1. The initiation methionine is marked as +1, potential glycosylation site. The transcription initiation site, stop codon, and polyadenylation site are boxed.
M-ficolin comprising the acetyl group-binding site are conserved in the FIBCD1 (Fig. 6). Accordingly, we attempted to purify the ectodomain of FIBCD1 by affinity chromatography on acetylated Toyopearl (19). Fig. 7A shows a Western blot of the culture supernatant from HEK293 cells expressing FIBCD1 ectodomain. Fig. 7B shows the acetate elution profile from the acetylated Toyopearl, and a silver-stained SDS-PAGE of the purified FIBCD1 ectodomain is shown in Fig. 7C. This shows that FIBCD1, like TL5A, L-ficolin, and M-ficolin, can be affinity purified using an acetate-coupled matrix.

We next tested the ability of FIBCD1 to bind acetylated molecules by an ELISA-based approach. The purified FIBCD1 ectodomain-bound acetylated BSA-coated microtiter plates in the presence of calcium. The binding was saturable and abolished by chelating of divalent ions (Fig. 7D). The binding between FIBCD1 and acetylated BSA was tested in presence of increasing concentrations of CaCl2 (Fig. 7E) and CaCl2 was substituted by other divalent cations (Fig. 7F). These experiments show that optimal binding to acetylated BSA is achieved at a calcium concentration of 0.6 mM and that manganese could substitute for calcium whereas magnesium did not.

The ligand selectivity of FIBCD1 was investigated by inhibiting the binding of FIBCD1 to acetylated BSA-coated microtiter plates (Fig. 7G). The inhibition experiments demonstrated that N-acetylated carbohydrates or amino acids, but not their corresponding nonacetylated counterparts, could inhibit the binding. Furthermore, one of the simplest compounds containing the acetyl group, sodium acetate, strongly inhibited the binding even at millimolar levels, whereas sodium propionate and sodium butyrate did not inhibit at a concentration of 50 mM. Other acetylated compounds like acetylcholine could also inhibit the binding of FIBCD1 to acetylated BSA (Table I).

FIBCD1 binds to chitin but not to other tested PAMPs

Different PAMPs were considered to be potential ligands for the FIBCD1-encoded protein. LTA and β-1,3-glucan are known ligands for L-ficolin (22) and TL5A recognize the O-Ag of LPS (23). Peptidoglycan consists of repeating GlcNAc and MurNac residues cross-linked by short peptides, a structure that is very similar to the (GlcNAc)n structure of chitin. We saw no binding to LPS, LTA, mannan, or peptidoglycan (Fig. 8, A and B). However, FIBCD1 showed clear chitin binding activity in pull-down assays.
where WGA was used as positive control (Fig. 8C). As for the binding to acetylated BSA, the binding was calcium dependent and could be inhibited by sodium acetate (Fig. 8D). Parallel pull-down experiments using equivalent masses of zymosan, cellulose (the nonacetylated counterpart to chitin), or H9252-1,3-glucan showed no binding. Together, these results imply that FIBCD1 has specific preference for chitin.

**Uptake of 125I-labeled acetylated BSA in FIBCD1-expressing CHO cells**

FIBCD1-mediated endocytosis of 125I-labeled acetylated BSA was studied in CHO cells transfected with a FIBCD1 expression vector. Fig. 9 shows the time course of cell-associated radioactivity and TCA-soluble radioactivity (as a measurement of ligand degradation) in the medium (Fig. 9, A and B). The cell-associated radioactivity reached a plateau after 1 h of incubation, at about the same time as the TCA-soluble radioactivity increased significantly in the medium. The degradation was strongly inhibited by the weak base chloroquine and the proteinase inhibitor leupeptin (Fig. 9C), which both inhibit lysosomal proteolysis. The uptake was mediated by FIBCD1 in that no uptake was seen in nontransfected CHO cells (Fig. 9A). Furthermore, the uptake and degradation of 125I-labeled acetylated BSA were inhibited by 10 mM GlcNAc, by unlabeled acetylated BSA, and by the mAb HG-HYB-12-1, which specifically inhibits the binding between acetylated BSA and FIBCD1 (Fig. 9D). Finally, we show that CHO cells expressing recombinant FIBCD1 mediate uptake of Alexa Fluor 488-labeled acetylated BSA (Fig. 9E), whereas no uptake was seen in nontransfected CHO cells (Fig. 9F).

**Discussion**

Our in silico screen followed up by functional analysis identified the FIBCD1 as a receptor that binds acetylated structures including chitin. The fibrinogen domain was originally identified as independent folded globular domains in the C-terminal region of both the β- and γ-chain of fibrinogen.Domains homologous to the fibrinogen domain have since been identified and characterized in a growing number of nonfibrinogen proteins, where the domains are...
referred to as fibrinogen-related domains or fibrinogen-like recognition domains (FReDs) (24). Proteins that include FReDs have been identified in a large number of distantly related species including vertebrates, echinoderms, mollusks, and insects. In vertebrates, fibrinogen is involved in platelet aggregation and fibrin clot formation. The blood coagulation pathways of invertebrates, when present, are not homologous to the fibrinogen-mediated coagulation pathway of vertebrates (25), and the FReDs must have evolved for other functions. Many of the FReDs identified in invertebrates show lectin activity (26), suggesting that the primordial FReDs function may have been innate immune recognition. Others, like scabrous identified in Drosophila megalogaster, interact with Notch and play a role in embryogenesis. It is therefore likely, that FReDs have evolved to take part in diverse functions including innate immunity, embryonic development, and coagulation (27–29).

Sequence comparison indicated a potential functional relationship of FIBCD1 to TL5A and to L- and M-ficolin. We found that FIBCD1 selectively and Ca_{2+}/Mg^{2+} dependently bind acetylated monosaccharide and other acetylated components but not their nonacetylated counterparts. This suggested that the primary ligand-binding site in FIBCD1 resides in the FReD and that...
Cys245, and in M-ficolin between Asp253 and Cys254 (22), and we
speculate that the same can be the case for the homologous Asn413
and Cys414 residues in FIBCD1.

The sugar methyl group benefits from the hydrophobic environ-
ment and lies in the middle of the funnel, surrounded by the ar-
omatic site chains and in close van der Walls contact with the site
occupied by the acetyl moiety. The acetyl group specificity of TL5A was
explained by the crystal structure of TL5A in complex with GlcNAc
(32). Four aromatic site chains, Tyr210, Tyr236, Tyr248, and His220,
forms a funnel, with the methyl side chain of Ala237 at its base.
The sugar methyl group benefits from the hydrophobic en-
vironment and lies in the middle of the funnel, surrounded by the ar-
omatic site chains and in close van der Walls contact with the site
chain methyl group of Ala237. An unusual cis-peptide bond be-
tween Arg218 and Cys219 generates a sharp turn and allows the
backbone NH group of the Cys to form a tight hydrogen bond with
the carbonyl oxygen of the ligand acetamido group. The same
cis-peptide bond was found in L-ficolin (22) between Asn244 and
Cys245, and in M-ficolin between Asp253 and Cys254 (22), and we
speculate that the same can be the case for the homologous Asn413
and Cys414 residues in FIBCD1.

The hydrophobic funnel containing the sugar methyl group is
essentially conserved in FIBCD1, the main difference being Tyr248
substituted with a tryptophan (Fig. 6). The homolog-binding site
(designated S1 binding site) is also present in L- and M-ficolin. In M-ficolin, acetylated carbohydrates occupy the binding site, but
although L-ficolin binds acetylated compounds with high affinity,
no ligands were found in the hydrophobic funnel when analyzing
the crystal structure of L-ficolin (22). Site-directed mutagenesis
analysis of the FReD of FIBCD1 changing H415G or A432V ab-
rogated N-acetylated carbohydrate binding to FIBCD1 indication
that the funnel indeed is occupied in FIBCD1 (T. Thomsen and U.
Holmskov, manuscript in preparation). We speculate that phenyl-
alanine at position 262 in L-ficolin could be an essential residue for
ligand binding, but it is not conserved in either TL5A or FIBCD1. Galactose bound to S2 and the first three of four

The structure of L-ficolin revealed three additional binding sites
(S2–S4), and the acetylated ligands were found to bind to site S3
(22). The acetyl moiety of these ligands bound to the backbone
tyrosine of L-ficolin Asp133, a residue not conserved in either
TL5A or FIBCD1. Galactose bound to S2 and the first three of four

Pattern recognition receptors often enhance their selectivity for
ligands by oligomerization. The disulphide-linked ectodomain of
FIBCD1 were shown to form tetramers using cross-linking at dif-
f erent concentrations of BS3, indicating that FIBCD1 forms ho-
trimers in the plasma membrane. This arrangement of four
tetramers in the plasma membrane. This arrangement of four
tetramers in the plasma membrane.
We hypothesize that the three-dimensional arrangement of the FReD-domain represents one of the reasons for the chitin selectivity of FIBCD1. In search for a natural ligand for the receptor, we performed a pull-down experiment and found that the receptor specifically bind to chitin. The binding was Ca\(^{2+}\) dependent and could be inhibited by sodium acetate, suggesting that the binding takes place via the S1 binding site. Chitin has been shown to recruit and activate innate immune cells and induce cytokine and chemokine production via a variety of cell surface receptors (37). Phagocytosis of chitin particles has previously been shown to induce the production of IL-12, TNF-\(\alpha\), and INF-\(\gamma\) in mouse spleen cell cultures; and this cytokine production was abrogated by preincubation with soluble mannann, but the actual receptor that mediated the response was not identified (38).

The same group showed that oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in a ragweed-immunized allergic mouse model, suggesting that chitin administered this way is a Th1 adjuvant, that inhibits the allergic response (39). Chitin administered directly to the lung has the opposite effect. In this case, chitin contributed to the development of the allergic inflammatory response by inducing the accumulation of IL-4 expression innate immune cells including eosinophils and basophils; this response was unaffected by the absence of MyD88 signaling but was inhibited by treatment with AMCase (7). Recently, De Silva et al. (37) demonstrated in vitro activation of macrophages and in vivo induction of lung inflammation measured as IL-17A production. The in vitro response was dependent on chitin size and time of incubation and both in vivo and in vitro responses were TRL-2 and MyD88 dependent. It is suggested that FIBCD1 facilitates the endocytosis of acetylated mannosyl residues (37). Phagocytosis of Candida albicans is the first pattern recognition molecule in vertebrates that has been shown to bind chitin (13).

We demonstrate that FIBCD1 facilitates the endocytosis of acetylated components using \(^{125}\text{I}\)-labeled acetylated BSA as a model molecule. We found high uptake of \(^{125}\text{I}\)-labeled N-acetyl-BSA in FIBCD1-transfected CHO cells, whereas no uptake was seen in nontransfected CHO cells. The uptake was, after a lag phase, followed by the appearance of \(^{125}\text{I}\)-labeled degradation products in the medium. Degradation was inhibited by chloroquine and leupeptin, which both are known inhibitors of lysosomal proteolysis. In accordance with a receptor-mediated endocytosis, ~50% of the surface-bound radioactivity was present as TCA-soluble radioactivity after 1–2 h. FIBCD1-dependent binding and uptake of Alexa Fluor 488-labeled acetylated BSA to CHO cells further supported a role of FIBCD1 in uptake of acetylated components.

We have discovered FIBCD1 as the first membrane-bound FReD molecule in vertebrates and the first pattern recognition receptor that bind chitin and directs acetylated components for degradation in the endosome. Its prevalence at the apical part of small intestinal epithelial cells specifically point to a role in innate immunity and/or homeostasis in the intestine.

Disclosures

The authors have no financial conflict of interest.

References


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