Recombinant Carboxyl-terminal Fibrin-binding Domain of Human Fibronectin Expressed in Mouse L Cells*

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The carboxyl-terminal fibrin-binding domain, Fib2, of human fibronectin was expressed in mouse L cells as a fusion protein with the signal sequence of human protein C inhibitor. The recombinant Fib2 (rFib2) protein synthesized by transfected cells retained the ability to form dimers with each other or with mouse fibronectin subunits and was secreted to the medium after extensive glycosylation. Only a small fraction of the secreted protein was incorporated into the pericellular matrix. Interestingly, the secreted rFib2 protein displayed a remarkable heterogeneity upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, giving rise to a broad band corresponding to \( M_r \) of 60,000–90,000. The heterogeneity was eliminated mostly by treatment with neuraminidase and further by treatment with endo-\( \alpha \)-N-acetylgalactosaminidase. Treatment with peptide:N-glycosidase F did not alter the heterogeneity of the protein, indicating that differential sialylation of O-linked, but not N-linked, glycans is largely responsible for the apparent heterogeneity. The presence of O-linked but absence of N-linked glycans was further supported by the observations that peanut agglutinin specifically bound to the desialylated rFib2 protein, whereas neither concanavalin A nor lentil lectin bound to the protein irrespective of prior neuraminidase treatment. Since the apparent heterogeneity of the rFib2 protein was only observable with the secreted, but not the cytoplasmic form, sialylation of O-linked glycans may be essential for, or regulate as a rate-limiting step, the transit of the recombinant protein to the extracellular space.

Fibronectin (FN) is a cell adhesive protein present in the extracellular matrix and various body fluids. FN has been implicated in diverse biological processes accompanying cell adhesion and migration on the extracellular matrix, such as embryonic development, wound healing, hemostasis and thrombosis, and tumor metastasis (for reviews, see Refs. 1–4). Oncogenic transformation of cells usually results in a marked decrease of FN associated with cell periphery (5, 6). FN is synthesized by fibroblasts and other cell types as a dimer of identical or nonidentical subunits with \( M_r \) of about 250,000. Although FN is encoded by a single gene, differential splicing of the primary transcript at three specific regions results in as many as 20 different isoforms having slightly different amino acid sequences (7–13). FN has a characteristic modular structure; it consists of three homologous repeats, termed types I, II, and III (14). These repeats are assembled into a series of structural domains, each having distinct binding activities toward collagens, sulfated glycosaminoglycans, fibrin, and cell surface receptors collectively termed integrins (2, 15–17).

Despite extensive characterization of each functional domain, little has been understood about the significance of the functional cooperativity among these domains. This is mainly because the conventional methodology employed in previous studies, i.e. proteolytic dissection of the intact protein followed by isolation and characterization of each domain, could not address the functional cooperativity among distally located domains. Thus, an alternative approach has been sought (18). Using the recombinant DNA technique one can express essentially any combination of the domains as a single polypeptide and evaluate the significance of their functional cooperativity.

As a first step toward this goal, we constructed a recombinant cDNA to express the carboxyl-terminal fibrin-binding domain, Fib2, of human FN in cultured mouse cells as a fusion protein with the signal sequence of human protein C inhibitor. The recombinant Fib2 (rFib2) protein thus expressed was capable of forming dimers with each other or with endogenous mouse FN subunits and secreted to the medium after an extensive O-linked glycosylation.

EXPERIMENTAL PROCEDURES

Materials—DNA modifying and restriction enzymes were obtained from Takara Shuzo (Kyoto, Japan). [\( ^{32} \)P]Methionine, GeneScreen Plus, and a DNA random primer extension kit were purchased from Du Pont-New England Nuclear. \([\alpha ^{32} P]dCTP\) from Amersham Japan (Tokyo, Japan), and G418 (Geneticin), N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, thermolysin from Sigma. Endo-\( \alpha \)-N-acetylgalactosaminidase from Alcaligenes sp. was obtained from Seikagaku Kogyo (Tokyo, Japan), neuraminidase from Arthrobacter ureafaciens was from Nacalai Tesque (Kyoto, Japan), and peptide-N-glycosidase F from Flavobacterium meningosepticum from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Peroxidase-conjugated lectins were purchased from Seikagaku Kogyo. The eukaryotic expression vector pBacCAT9 and pKOneo were kindly provided by Dr. Norman Davidson (California Institute of Technology, Pasadena, CA) and Dr. Allen Senear (Fred Hutchinson Cancer Research Center, Seattle, WA), respectively. The cDNA encoding the signal sequence of human protein C inhibitor, PCI-GmplO, was a generous gift of Dr. Norman Davidson (California Institute of Technology, Pasadena, CA) and Dr. Allen Senear (Fred Hutchinson Cancer Research Center, Seattle, WA), respectively. The cDNA encoding the signal sequence of human protein C inhibitor, PCI-GmplO, was a generous gift of Dr. Norman Davidson (California Institute of Technology, Pasadena, CA) and Dr. Allen Senear (Fred Hutchinson Cancer Research Center, Seattle, WA), respectively.

The carboxyl-terminal fibrin-binding domain of FN, Fib2, recombinant Fib2, IIIC-5, type III-connecting segment, C1, C2, C3, and C4, cyclic- \( \alpha \)-aminovaleric acid sulfoyl acid, SDF-PAGE, sodium dodecyl sulfate-polycrylamide gel electrophoresis.

401
Expression of a Recombinant Fibronectin Domain in Mouse Cells

Masahiko Katayama (Takara Shuzo Co., Ltd.) and Chiaki Hoshino-Ikeda (National Institute for Basic Biology, Okazaki, Japan)

The recombinant Fib2 domain was constructed by replacing the chimerical acrylamide transferase gene of pl3CAT9 (20) with the cDNAs encoding the signal sequence of human protein C inhibitor, a putative N-linked glycosylation site, and the Fib2 domain (see also Fig. 1). The 17-base pair fragment of the cDNA Pti-C1 was used to clone the entire IIICS region of human FN into the plasmid pUC19, and isolated as a HindIII/EcoRI fragment. This cDNA sequence was ligated in tandem with the HindIII/EcoRI-cleaved fragment of the plasmid pUC19, yielding the Fib2 expression vector pAISF21.

DNA Transfection and Selection of Stable Transformants-Mouse L cells were used as the recipient for DNA transfection. Transfection of the cDNA was performed using the calcium phosphate precipitation technique as described by Chen and Okayama (21). Calcium phosphate-DNA solution containing 10 μg of pAISF21 and 1 μg of pKQone was used to transfect 2 × 10⁶ cells grown in a 6-cm dish. Stably transformed cells were selected in the growth medium containing 0.8 mg/ml of G418. Routinely, several hundred colonies of transformants were obtained per transfection. Randomly selected colonies were individually expanded, assayed for the expression of the recombinant Fib2 protein by Northern blot analysis (19), and the isolate expressing the highest level of the mRNA was subjected to the second round of cloning by the limiting dilution method.

NORTHERN BLOT ANALYSIS—RNAs were extracted from cells by the guanidine/ChCl method (22). After glyoxalation, RNAs were separated on a 1% agarose gel and transferred to a GeneScreen Plus membrane. The membrane was then hybridized with the 32P-labeled probe DNA prepared by the random primer extension method (23).

Pulse-Chase Experiments and Immune Precipitation—Cells grown to confluence in a 6-cm culture dish were preincubated in methionine-free minimum essential medium for 1 h, then pulse-labeled with [35S]methionine at 50 μCi/ml for 1 h. After labeling, unlabeled methionine was added to the medium at 30 μg/ml for chase. At indicated time points, the medium was harvested, clarified by centrifugation at 10,000 × g, and incubated with monoclonal anti-human FN antibody immobilized on Sepharose CL-4B (9). The cell layers were rinsed with phosphate-buffered saline, and scraped off in 20 mM Tris/HCl (pH 7.6) containing 2% sodium deoxycholate, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was homogenized by repeated passages through a 27-gauge needle and incubated with the monoclonal antibody immobilized on Sepharose beads overnight at 4°C. The beads were washed three times with phosphate-buffered saline and then boiled in the sample treatment buffer containing 2% SDS and 5 mM 2-mercaptoethanol. The solubilized proteins were analyzed by SDS-PAGE and subsequent fluorography (24). The radioactive association with protein bands of interest was quantified with a Fuji Bio-Image Analyzer BA100 using an erasable phosphor imaging plate (25).

Indirect Immunofluorescence Staining—Cells were grown on coverslips to confluence and immunostained with either polyclonal or monoclonal anti-human FN antibodies at appropriate dilutions without fixation. The secondary antibodies were fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG, respectively. The immunostained cells were fixed with 3% paraformaldehyde and examined with a Zeiss epifluorescence microscope Axioshot and photographed on Kodak Tri-X films.

Isolation of the Recombinant Fib2 (rFib2) Protein—The rFib2 protein secreted by stable transformants was isolated from conditioned medium by immunoaffinity chromatography using monoclonal antibody FIB-21 immobilized on Sepharose CL-4B. All manipulations were conducted at 4°C. The conditioned medium was harvested and clarified by centrifugation at 170,000 x g at 4°C. The supernatant was supplemented with EDTA (5 μM) and phenylmethylsulfonyl fluoride (1 mM) and then applied to the antibody column (bed volume, 2 ml). The column was washed with phosphate-buffered saline and the bound proteins were eluted with 0.2 M glycine/HCl buffer (pH 2.8), dialyzed against 2 mM CAPS buffer (pH 10.5) containing 0.5 mM EDTA, and lyophilized.

Enzyme Digestion of the rFib2 Protein—Limited proteolysis of the rFib2 protein with trypsin and thermolysin was carried out as described previously (26). Treatment with glycosidases was performed as follows: the recombinant protein (20 μg) was treated with (a) 50 milliunits of neuraminidase in 0.1 mM sodium acetate buffer (pH 5.0) at 37°C for 4 h, (b) 0.5 milliunits of peptide N-glycosidase F in 50 mM Tris/HCl (pH 9.0) containing 20 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% SDS, and 0.65% Triton X-100, at 37°C for 60 h, or (c) 10 milliunits of endo-a-N-acetylgalactosaminidase in 50 mM sodium citrate (pH 4.2) containing 1 mM EDTA at 37°C for 15 min. In some experiments, the rFib2 protein was first treated with neuraminidase, dialyzed against water, and then digested with peptide N-glycosidase F or endo-a-N-acetylgalactosaminidase under the conditions specified above.

SDS-PAGE, Immunoblotting, and Lectin Blotting—SDS-PAGE was performed as described by Laemmli (27). Proteins separated on the gels were transferred to nitrocellulose membrane (28) and then stained with antibodies as described (29) or with peroxidase-lectin conjugates as described by Kijimoto-Ochiai et al. (30). The bound peroxidase-lectin conjugates were visualized with 4-chloro-1-naphthol.

RESULTS

Isolation of Stable Transformants Expressing the rFib2 Protein—The plasmid pAISF21 was constructed to express in the mouse L cells the entire carboxyl-terminal Fib2 domain of human FN, including most of the preceding IIICS region, as a fusion protein with the signal sequence of human protein C inhibitor (Fig. 1). A short cDNA sequence encoding a putative N-linked glycosylation site in the cell-binding domain was inserted in frame between those encoding the signal sequence and the rFib2 domain. The expression of the recombinant cDNA was driven by chicken β-actin promoter in conjunction with the 32P-labeled probe DNA prepared by the random primer extension method (23).

Pulse-Chase Experiments and Immune Precipitation—Cells grown to confluence in a 6-cm culture dish were preincubated in methionine-free minimum essential medium for 1 h, then pulse-labeled with [35S]methionine at 50 μCi/ml for 1 h. After labeling, unlabeled methionine was added to the medium at 30 μg/ml for chase. At indicated time points, the medium was harvested, clarified by centrifugation at 10,000 x g, and incubated with monoclonal anti-human FN antibody immobilized on Sepharose CL-4B (9). The cell layers were rinsed with phosphate-buffered saline, and scraped off in 20 mM Tris/HCl (pH 7.6) containing 2% sodium deoxycholate, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was homogenized by repeated passages through a 27-gauge needle and incubated with the monoclonal antibody immobilized on Sepharose beads overnight at 4°C. The beads were washed three times with phosphate-buffered saline and then boiled in the sample treatment buffer containing 2% SDS and 5 mM 2-mercaptoethanol. The solubilized proteins were analyzed by SDS-PAGE and subsequent fluorography (24). The radioactive association with protein bands of interest was quantified with a Fuji Bio-Image Analyzer BA100 using an erasable phosphor imaging plate (25).

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with SV40 polyadenylation signal (20).

Co-transfection of \( 2 \times 10^5 \) L cells with pAISF21 and pKOneo and subsequent selection with G418 yielded several hundred colonies of stable transformants. Among those randomly picked up and expanded, the transformant expressing the highest level of the rFib2 mRNA was selected by Northern blot analysis and subjected to the second round of cloning by limiting dilution, resulting in the isolation of the stable transformant L/201. Northern blot analysis demonstrated that a 2.1-kilobase rFib2 mRNA was expressed in L/201 cells (Fig. 2, lane 1) but not in another G418-resistant clone L/205 (Fig. 2, lane 2). The size of the rFib2 mRNA was in good agreement with that expected from the nucleotide sequence of the cDNA insert. Southern hybridization analysis indicated that multiple copies of pAISF21 were integrated per haploid DNA in the clone L/201, whereas the clone L/205 contained a single integrated copy of the plasmid (data not shown).

Synthesis and Secretion of the rFib2 Protein by L/201 Cells—In order to examine whether L/201 cells synthesize and secrete the rFib2 protein, the cells were pulse-labeled with \[^{35}S\]methionine and the appearance of the rFib2 protein in the cytoplasm and in the medium was followed by immunoprecipitation with monoclonal anti-human FN, did not immunoprecipitate from the conditioned medium (Fig. 3B, lane 4). The positions of the cell-associated form (arrowheads) and secreted form (brackets) of the rFib2 proteins are indicated. Shown in the left margin are the positions of the molecular weight standards.

Northern blot analysis of the rFib2 mRNA. Total RNA from stable transformants (20 \( \mu \)g) and from human fibroblast WI-38VA13 (5 \( \mu \)g) was hybridized with a 1.2-kilobase human FN cDNA encoding the Fib2 domain. Lane 1, RNA from L/201; lane 2, RNA from L/205; lane 3, RNA from WI-38VA13.

Fig. 2. Northern blot analysis of the rFib2 mRNA. Total RNA from stable transformants (20 \( \mu \)g) and from human fibroblast WI-38VA13 (5 \( \mu \)g) was hybridized with a 1.2-kilobase human FN cDNA encoding the Fib2 domain. Lane 1, RNA from L/201; lane 2, RNA from L/205; lane 3, RNA from WI-38VA13.

Expression of a Recombinant Fibronectin Domain in Mouse Cells 403

Quantitation of the relative radioactivity associated with the rFib2 proteins recovered from the cell lysate and from the medium indicated that nearly 70% of the pulse-labeled rFib2 protein was recovered in the cell lysate and 30% in the medium after 2 h of the chase (Figs. 3B and 4). With increasing the chase period, the relative amounts of the cell-associated 51-, 54-, and 66-kDa (cell-associated) and 60-90-kDa (secreted) protein bands marked in the Fig. 3.

phenomenous mouse FN was also immunoprecipitated with FN8-12 (Fig. 3B, lanes 2 and 4). This is mainly due to the presence of the heterodimer consisting of the rFib2 protein linked to mouse FN subunits (see below), although mouse FN alone appears to cross-react weakly with FN8-12 and FN9-1 (Fig. 3A, lanes 3 and 4).

Quantitation of the relative radioactivity associated with the rFib2 proteins recovered from the cell lysate and from the medium indicated that nearly 70% of the pulse-labeled rFib2 protein was recovered in the cell lysate and 30% in the medium after 2 h of the chase (Figs. 3B and 4). With increasing the chase period, the relative amounts of the cell-associated 51-, 54-, and 66-kDa proteins versus those of the extracellular 60-90-kDa proteins decreased rapidly (Fig. 3, C and D), indicating that those proteins in the cell lysate represent the zymogenic form of the rFib2 protein. After 24 h of the chase, more than 95% of the pulse-labeled rFib2 protein was secreted to the medium (Fig. 4).

Although most, if not all, of the rFib2 protein synthesized by L/201 cells was secreted to the medium, a small fraction of the protein became incorporated in the pericellular matrix upon prolonged cell culture. Indirect immunofluorescence staining with monoclonal anti-human FN antibodies demonstrated that the matrix of confluent L/201 cells was weakly immunostained with FN8-12 (Fig. 5A), although the matrix of L/205 cells was not (Fig. 5B). The matrix of neither L/201...
nor L/205 cells was stained by immunofluorescence with the control antibody FN 9-1 (Fig. 5, C and D), although both matrices were strongly stained with polyclonal anti-human FN antibodies.

Characterization of the rFib2 Protein Secreted to the Medium—The rFib2 protein secreted to the conditioned medium was isolated by immunoaffinity chromatography for further biochemical characterization. SDS-PAGE of the purified rFib2 protein under nonreducing conditions followed by immunoblot analysis with FN8-12 identified three forms of the rFib2 protein (Fig. 6, left, lane 4): one form migrated at the position between the monomer and dimer of intact mouse FN, indicating that it represents the heterodimer of the rFib2 protein linked to an intact mouse FN subunit. Other two forms of the rFib2 protein gave broad bands corresponding to 43- and 22-kDa fragments derived from the rFib2 protein. These results indicate that the apparent heterogeneity of the secreted form of the rFib2 protein would arise from posttranslational modification(s) occurring at either the amino- or carboxyl-terminal flanking region of the Fib2 domain.

A likely candidate for such modifications is glycosylation, since the rFib2 protein contains two putative sites for N-glycosylation and six sites for O-glycosylation (31), most of which could be removed by a mild trypsin digestion (see Fig. 7A). In order to examine this possibility, the purified rFib2 protein was treated with various exo- and/or endoglycosidases (Fig. 8). Treatment with neuraminidase resulted in a marked reduction of the apparent molecular mass of the protein, yielding a less heterogeneous band migrating at M, of 58,000 (Fig. 8, lane 2). It is unlikely that sialic acid residues removed by neuraminidase are derived from N-linked glycans, since the electrophoretic mobility of the protein was not affected by treatment with peptide:N-glycosidase F irrespective of prior neuraminidase digestion (Fig. 8, lanes 3 and 4). Since the sialylated N-linked glycans on the gelatin-binding domain of human plasma FN were removed with peptide:N-glycosidase F under the same conditions (data not shown), these results suggest that N-linked glycan is likely absent in the recombinant protein. In contrast, treatment of the desialylated rFib2 protein with endo-α-N-acetylgalactosaminidase, an enzyme that selectively removes desialylated O-linked glycans, resulted in a further reduction of the apparent molecular mass of the protein (Fig. 8, lane 6). Although the same enzyme failed to reduce the heterogeneity of the rFib2 protein when applied without prior neuraminidase treatment (Fig. 8, left, lane 4).
Expression of a Recombinant Fibronectin Domain in Mouse Cells

**Fig. 7.** Limited proteolysis of the rFib2 protein. A, a schematic model for the polypeptide structure of the rFib2 protein. The putative cleavage sites for trypsin and thermolysin (Refs. 15, 26, 41, and 56-58) are indicated by arrows. Closed and open circles represent the putative N-linked and O-linked glycosylation sites, respectively. B, Immunoblot analysis of the proteolytic digests of intact cellular FN and the rFib2 protein. Human cellular FN (lanes 1-3) and the purified rFib2 protein (lanes 4-6) were digested with trypsin or thermolysin and then subjected to SDS-PAGE under the reducing condition followed by transfer of the proteins to nitrocellulose membrane. The membrane was first stained with fast green (left) and then immunostained with FN8-12 (center) or polyclonal anti-IIICS/A2 antibodies (right). Lanes 1 and 4, control proteins; lanes 2 and 5, trypsin digests; lanes 3 and 6, thermolysin digests. Five μg of protein was applied to each lane. The positions of the tryptic 43-kDa (open arrowheads) and 37-kDa (closed arrowhead) fragments as well as the thermolysin 22-kDa fragment (arrows) are indicated.

**Fig. 8.** Effect of glycosidase treatment on the heterogeneity of the rFib2 protein. The rFib2 protein was treated with neuraminidase, peptide:N-glycosidase F (P:N-glycosidase F), endo-α-N-acetylgalactosaminidase, or the combination thereof as described under "Experimental Procedures." Deglycosylated proteins (5 μg) were analyzed by immunoblotting with FN8-12 under the reduced condition. The positions of the neuraminidase-treated rFib2 proteins with or without subsequent treatment with other glycosidases are pointed out by arrowheads.

**Fig. 9.** Lectin-blot analysis of the rFib2 protein. The purified rFib2 protein (5 pg) with or without prior neuraminidase treatment was subjected to SDS-PAGE under the reducing condition, transferred to nitrocellulose membrane, and then stained with either FN8-12 or with peroxidase-lectin conjugates. Con A, concanavalin A; LCA, lentil lectin; PNA, peanut agglutinin.

**DISCUSSION**

Using the DNA transfection technique, we have established a stably transformed mouse L cells expressing the recombinant protein consisting of the signal sequence of human protein C inhibitor and the Fib2 domain of human FN. The resulting rFib2 protein retained the ability to dimerize with each other or with endogenous mouse FN subunits and was mostly secreted to the extracellular space after an extensive glycosylation. Thus, the heterologous signal sequence appears to function properly to facilitate efficient secretion of the rFib2 protein. Although a significant portion of intact FNs synthesized by a variety of cell types has been shown to be incorporated in the extracellular matrix (33-35), it was found in the present study that only a small fraction of the rFib2 protein was incorporated.
protein might be deposited in the matrix upon prolonged cell culture. The inefficient incorporation of the recombinant protein is likely due to the lack of either the amino-terminal heparin/fibrin-binding (Hep1/Fib1) or the central cell-binding (Cell) domain, which has been reported to be required for the FN matrix assembly (36, 37). Our results are consistent with the previous observation by Schwarzbauer et al. (18) that "demectin," the carboxyl-terminal half of rat FN expressed in mouse cells as a fusion protein with a heterologous signal sequence, was mostly secreted to the medium.

Oligomerization of subunits of membrane and secretory proteins is believed to occur in the endoplasmic reticulum (38-40). Although little has been understood about how the subunits of a given protein recognize each other and oligomerize through disulfide bonds, it is likely that certain structural features are generated during folding of the individual subunits that allow them to recognize each other (40). Our results show that the Fib2 domain alone can undergo specific dimerization with one another or with endogenous mouse FN subunits, suggesting that a signal for dimerization resides in the Fib2 domain or the extreme carboxyl-terminal segment where the two subunits are bridged by disulfide bonds. It should be noted, however, that about 30% of the rFib2 protein secreted by the transformed cells remained undimerized (Fig. 6). Since the carboxyl-terminal half of rat FN expressed in mouse cells is secreted as homodimers or heterodimers but not as monomers (18), affinity for the intersubunit association seems to be reduced with the rFib2 protein described in the present study when compared with the longer recombinant protein or intact subunits.

The rFib2 protein secreted by the transformed cells displayed remarkable heterogeneity upon SDS-PAGE. Although the molecular mass of the processed rFib2 polypeptide is estimated to be 49 kDa from the cDNA sequence, the protein secreted to the medium gave a broad band corresponding to 60-90 kDa under reduced conditions. Several lines of evidence indicate that unexpected behavior of the rFib2 protein on the gel is mainly due to the differential sialylation of O-linked glycans located at the IIICS region. First of all, the heterogeneity of the rFib2 protein was mostly abolished by neuraminidase treatment. Sialic acid residues causing the heterogeneity with one another or with endogenous mouse FN subunits were suggested to be a signal for dimerization resides in the Fib2 domain or the extreme carboxyl-terminal segment where the two subunits are bridged by disulfide bonds. It should be noted, however, that about 30% of the rFib2 protein secreted by the transformed cells remained undimerized (Fig. 6). Since the carboxyl-terminal half of rat FN expressed in mouse cells is secreted as homodimers or heterodimers but not as monomers (18), affinity for the intersubunit association seems to be reduced with the rFib2 protein described in the present study when compared with the longer recombinant protein or intact subunits.

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