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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* (r*Fib2*) 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 r*Fib2* 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-α-*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 r*Fib2* protein, whereas neither concanavalin A nor lentil lectin bound to the protein irrespective of prior neuraminidase treatment. Since the apparent heterogeneity of the r*Fib2* 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)[1] 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* (r*Fib2*) 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). [^{35}S]Methionine, GeneScreen *Plus*, and a DNA random primer extension kit were purchased from Du Pont-New England Nuclear, [α^{32}P]dCTP from Amersham Japan (Tokyo, Japan), and G418 (Geneticin), *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, thermolysin from Sigma. Endo-α-*N*-acetylgalactosaminidase from *Alcarigenes* sp. was obtained from Seikagaku Kogyo (Tokyo, Japan), neuraminidase from *Arthrobactor 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 pBactCAT9 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-6mp10, was a generous gift of Dr. Koji Suzuki (Institute for Enzyme Research, University of Tokushima, Tokushima, Japan). Monoclonal antibodies FN9-1 and FN8-12 as well as their derivatives immobilized on Sepharose CL4B were

[1] The abbreviations used are: FN, fibronectin; *Fib2*, the carboxyl-terminal fibrin-binding domain of FN; r*Fib2*, recombinant *Fib2*; IIICS, type III-connecting segment; CAPS, cyclohexylaminopropane sulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

generous gifts of Masahiko Katayama (Takara Shuzo Co., Ltd.). Fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG were obtained from Cappel Worthington Biochemicals (Malvern, PA).

Cells and Cell Culture—Mouse L cells were kindly provided by Dr. Masahiro Ishiura (National Institute for Basic Biology, Okazaki, Japan) and SV40-transformed human fibroblasts WI-38VA13 were from Dr. Hiroshi Mizusawa (Japanese Cancer Research Resources Bank, Tokyo, Japan). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO).

DNA Constructions—The cDNA clone pHCF22 encoding the entire IIICS region of human FN was prepared by oligonucleotide-primed cDNA synthesis (19) using the poly(A)$^+$ RNA isolated from SV40-transformed human fibroblast WI-38VA13 as a template. The 699-base pair *SacI-ApaI* fragment of pHCF22 and the 1429-base pair *ApaI-HindIII* fragment of pLF2 (9) were cloned together into the *SacI/HindIII*-cleaved pUC118, yielding the plasmid pHCF2D which encodes the entire IIICS region and the *Fib2* domain.

A plasmid vector pAISF21 for the expression of the human *Fib2* domain was constructed by replacing the chloramphenicol acetyltransferase gene of pBactCAT9 (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). A 127-base pair fragment of the cDNA PCI-6mp10 encoding the signal sequence of human protein C inhibitor was excised by double digestion with *HindIII* and *SmaI*, subcloned in the *HindIII/SmaI*-cleaved pUC19, and isolated as a *HindIII-EcoRI* fragment. The cDNA sequence encoding a putative *N*-linked glycosylation site of human FN was isolated from pLF5 (9) as a 170-base pair *EcoRI-PvuII* fragment (see Fig. 1), subcloned into pUC18, and isolated as a *EcoRI-BamHI* fragment. The cDNA encoding most of the IIICS region and its downstream amino acid sequence of human FN was excised from pHCF2D as a 1591-base pair *BglII-ScaI* fragment. These three fragments were ligated in tandem with the *HindIII/HpaI*-cleaved pBactCAT9, 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 cells 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 pKOneo was used to transfect 2 × 10^5 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* mRNA 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/CsCl method (22). After glyoxalation, RNAs were separated by electrophoresis on a 1% agarose gel and transferred to a GeneScreen *Plus* membrane. The membrane was then hybridized with the ^{32}P-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 [^{35}S] 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 CL4B. 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% 2-mercaptoethanol. The solubilized proteins were analyzed by SDS-PAGE and subsequent fluorography (24). The relative radioactivity associated with protein bands of interest was quantified with a Fujix 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 used were fluorescein-conju-

gated 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 Axiophot and photographed on Kodak Tri-X films.

Isolation of the Recombinant Fib2 (rFib2) Protein—The rFib2 protein secreted by stable transformants were isolated from conditioned medium by immunoaffinity chromatography using monoclonal antibody FN8-12 immobilized on Sepharose CL4B. All manipulations were conducted at 4 °C. The conditioned medium was harvested and clarified by centrifugation at 1700 × g at 4 °C. The supernatant was supplemented with EDTA (5 mM) 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 M 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 0.85% Triton X-100, at 37 °C for 60 h, or (*c*) 10 milliunits of endo-α-*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-α-*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-naphtol.

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 *Fib2* domain. The expression of the recombinant cDNA was driven by chicken β-actin promoter in conjunction
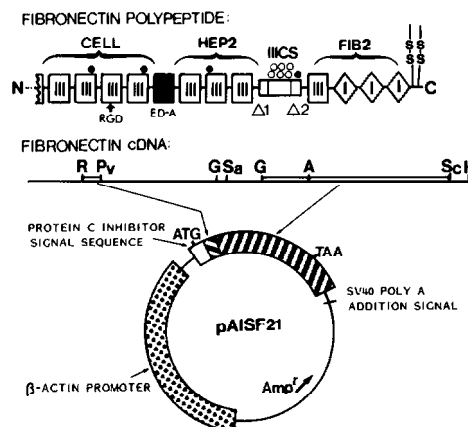
FIG. 1. **Construction of the *Fib2* expression vector pAISF21.** A model for polypeptide structure of human FN as well as the corresponding cDNA restriction map are diagrammed above the plasmid construction. *Closed* and *open circles* shown in the model for the polypeptide structure represent the putative sites for *N*- and *O*-linked glycosylation (31), respectively. Abbreviations used for the restriction sites are: *A, ApaI; G, BglII; H, HindIII; Pv, PvuII; R, EcoRI; Sa, SacI; Sc, ScaI.*

with SV40 polyadenylation signal (20).

Cotransfection of 2×10^5 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 r*Fib2* 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 r*Fib2* 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 r*Fib2* 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 r*Fib2* protein, the cells were pulse-labeled with [^{35}S]methionine and the appearance of the r*Fib2* protein in the cytoplasm and in the medium was followed by immunoprecipitation with monoclonal anti-human *Fib2* antibody FN8-12 immobilized on Sepharose CL4B (Fig. 3). After 2 h of chase, three proteins with apparent M_r of 66,000, 54,000, and 51,000 were immunoprecipitated from the detergent lysate of the cells (Fig. 3*B*, *lane 2*), whereas heterogeneous proteins migrating at 60–90-kDa region on the gel were precipitated from the conditioned medium (Fig. 3*B*, *lane 4*). Control antibody FN9-1, which is directed to the aminoterminal domain of human FN, did not immunoprecipitate these proteins either from the cell lysate or from the medium (Fig. 3*B*, *lanes 1* and *3*). None of these proteins were precipitated from the detergent lysate of the nonexpressor L/205 with FN8-12 (Fig. 3*A*, *lanes 2* and *4*), confirming the specificity of the immunoprecipitation with this antibody. Since the molecular mass of the r*Fib2* protein calculated from the cDNA sequence is 49 kDa after removal of the signal sequence, these proteins immunoprecipitable with FN8-12 are likely to be differentially glycosylated forms of the r*Fib2* protein. The significant difference in the electrophoretic mobility between the r*Fib2* proteins precipitated from the cell lysate and from the medium is likely due to the sialylation of *O*-linked glycans occurring during secretion of the recombinant protein (see below).

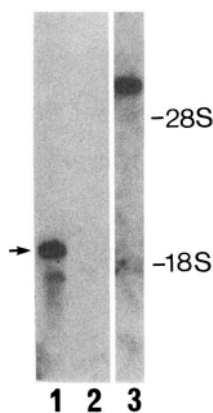It should be noted that, besides these proteins, the endog-
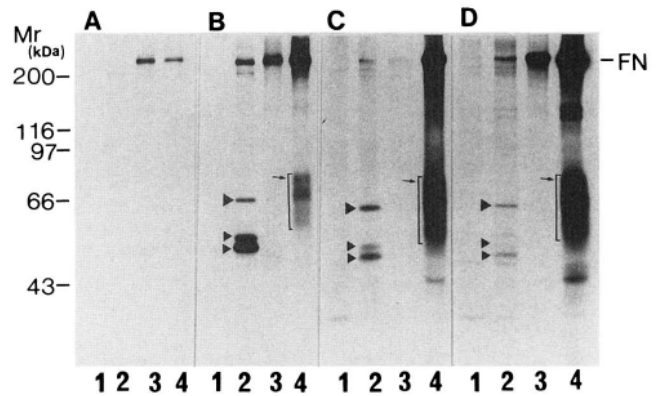
FIG. 3. **Pulse-chase analysis of the r*Fib2* protein expressed in transformed L cells.** L/205 (*A*) and L/201 (*B–D*) cells were pulse-labeled with [^{35}S]methionine and then chased with nonlabeled methionine as described under "Experimental Procedures." After 2 h (*A* and *B*), 7 h (*C*), and 24 h (*D*) of chase, the radiolabeled r*Fib2* protein was immunoprecipitated from the detergent lysate of the cells (*lanes 1* and *2*) or from the conditioned medium (*lanes 3* and *4*) with monoclonal antibody FN9-1 (*lanes 1* and *3*) or FN8-12 (*lanes 2* and *4*). The positions of the cell-associated form (*arrowheads*) and secreted form (*brackets*) of the r*Fib2* proteins are indicated. Shown in the *left margin* are the positions of the molecular weight standards.
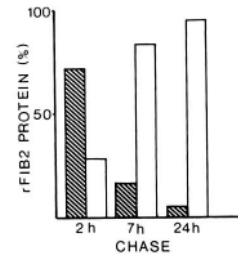
FIG. 4. **Transit of the pulse-labeled r*Fib2* protein.** The percentages of the cell-associated (*hatched column*) and secreted (*open column*) r*Fib2* proteins after the indicated periods of chase were determined by quantifying the radioactivities associated with the 51-, 54-, and 66-kDa (*cell-associated*) and 60–90-kDa (*secreted*) protein bands marked in the Fig. 3.

enous mouse FN was also immunoprecipitated with FN8-12 (Fig. 3*B*, *lanes 2* and *4*). This is mainly due to the presence of the heterodimer consisting of the r*Fib2* protein linked to mouse FN subunits (see below), although mouse FN alone appears to cross-react weakly with FN8-12 and FN9-1 (Fig. 3*A*, *lanes 3* and *4*).

Quantitation of the relative radioactivity associated with the r*Fib2* proteins recovered from the cell lysate and from the medium indicated that nearly 70% of the pulse-labeled r*Fib2* protein was recovered in the cell lysate and 30% in the medium after 2 h of the chase (Figs. 3*B* 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 cytoplasmic form of the r*Fib2* protein. After 24 h of chase, more than 95% of the pulse-labeled r*Fib2* protein was secreted to the medium (Fig. 4).

Although most, if not all, of the r*Fib2* 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. 5*A*), although the matrix of L/205 cells was not (Fig. 5*B*). The matrix of neither L/201

FIG. 2. **Northern blot analysis of the r*Fib2* mRNA.** Total RNA from stable transformants (20 µg) and from human fibroblast WI-38VA13 (5 µg) 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.
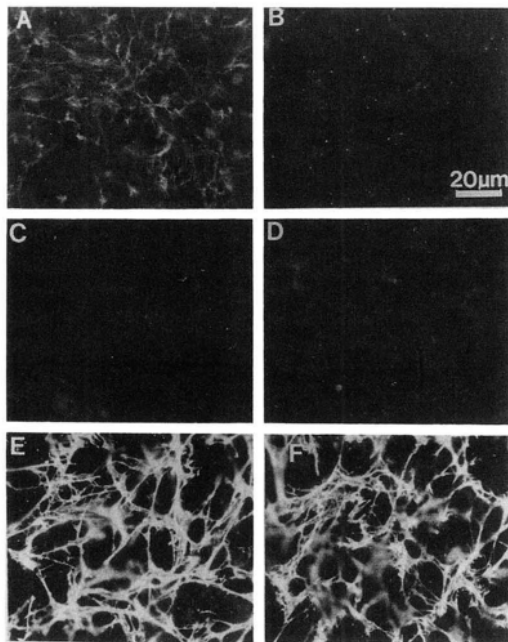
FIG. 5. **Indirect immunofluorescence staining of transformed L cells.** Confluent monolayers of L/201 (*A*, *C*, and *E*) and L/205 (*B*, *D*, and *F*) cells were stained with monoclonal antibodies FN8-12 (*A* and *B*) and FN9-1 (*C* and *D*) or with polyclonal anti-human FN antibodies.

FIG. 6. **Immunoblot analysis of the purified r*Fib2* protein.** The purified r*Fib2* protein was analyzed by SDS-PAGE under the reducing (*right*) or nonreducing (*left*) condition followed by immunoblotting with FN8-12. Proteins transferred to nitrocellulose membrane were first stained with fast green (*lanes 1* and *2*) and then immunostained with FN8-12 (*lanes 3* and *4*). *Lanes 1* and *3*, human plasma FN (5 µg); *lanes 2* and *4*, the purified r*Fib2* protein (5 µg). The positions of the heterodimer (*arrow*), homodimer (*open arrowhead*), and monomer (*closed arrowhead*) of the r*Fib2* protein are indicated in the *margin*.

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 which cross-react with mouse FNs (Fig. 5, *E* and *F*).

Characterization of the rFib2 Protein Secreted to the Medium—The r*Fib2* protein secreted to the conditioned medium was isolated by immunoaffinity chromatography for further biochemical characterization. SDS-PAGE of the purified r*Fib2* protein under nonreducing conditions followed by immunoblot analysis with FN8-12 identified three forms of the r*Fib2* 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 r*Fib2* protein linked to an intact mouse FN subunit. Other two forms of the r*Fib2* protein gave broad bands corresponding to M_r of 100,000–160,000 and 60,000–90,000. These forms are likely to represent homodimers and monomers of the r*Fib2* protein, respectively. Upon reduction with 2-mercaptoethanol, the r*Fib2* protein gave only the band corresponding to the monomer (Fig. 6, *right*, *lane 4*). Quantitation of the relative radioactivities of these three forms identified under nonreducing conditions indicate that the heterodimer, homodimer, and monomer comprise 26, 44, and 30%, respectively, of the total r*Fib2* protein secreted to the medium. These results indicate that the r*Fib2* protein retains the ability to recognize each other for specific dimerization of FN subunits.

Evidence that the heterogeneous 60–90-kDa proteins are indeed the secreted form of the r*Fib2* protein was also obtained by limited proteolysis with trypsin or thermolysin. Based on our previous studies (15, 26, 29), the *Fib2* domain of the recombinant protein is expected to yield 43- and 22-kDa fragments upon trypsin and thermolysin digestion, respectively (Fig. 7*A*). A mild trypsin digestion of human cellular FN released the *Fib2* domain as 43- and 37-kDa fragments (Fig. 7*B*, *center*, *lane 2*), of which only the former fragment contained the alternatively spliced IIICS/Δ_2 peptide segment (Fig. 7*B*, *right*, *lane 2*). Trypsin digestion of the
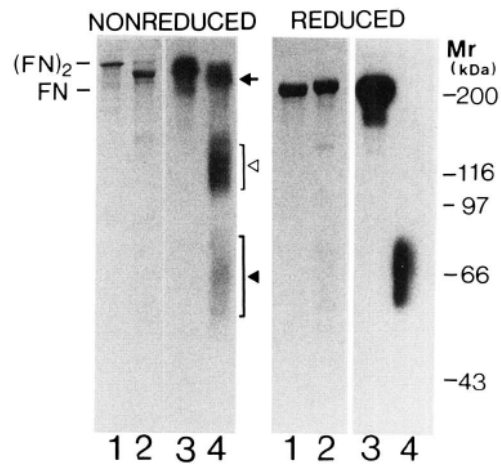
purified r*Fib2* protein, however, yielded only the 43-kDa fragment immunoreactive with both FN8-12 (Fig. 7*B*, *center*, *lane 5*) and anti-IIICS/Δ_2 antibodies (Fig. 7*B*, *right*, *lane 5*), being consistent with the presence of the IIICS/Δ_2 sequence within the recombinant protein. Furthermore, the *Fib2* domain of the purified protein was released with thermolysin as a 22-kDa fragment (Fig. 7*B*, *center*, *lane 6*), as was the case with intact cellular FN (Fig. 7*B*, *center*, *lane 3*). It should be also noted that both the 43-kDa tryptic and 22-kDa thermolysin fragments derived from the r*Fib2* protein gave apparently homogeneous bands upon SDS-PAGE. These results indicate that the apparent heterogeneity of the secreted form of the r*Fib2* 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 r*Fib2* 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. 7*A*). In order to examine this possibility, the purified r*Fib2* 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_r 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 r*Fib2* 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 r*Fib2* protein when applied without prior neuraminidase treatment (Fig. 8,
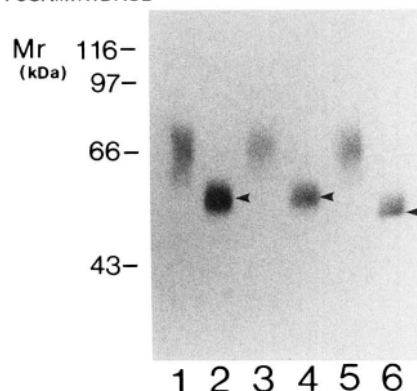
A

B
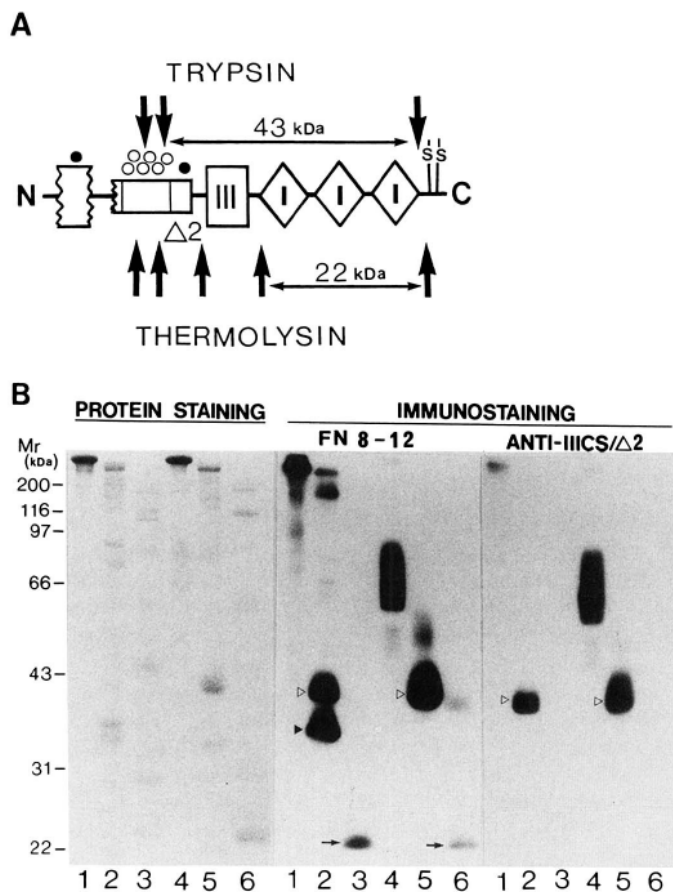
FIG. 7. **Limited proteolysis of the r*Fib2* protein.** *A*, a schematic model for the polypeptide structure of the r*Fib2* protein. The putative cleavage sites for trypsin and thermolysin (Refs. 15, 26, 41, and 56–58) are indicated by *arrows*. *Closed* and *open circles* represent the putative *N*-linked and *O*-linked glycosylation sites, respectively. *B*, Immunoblot analysis of the proteolytic digests of intact cellular FN and the r*Fib2* protein. Human cellular FN (*lanes 1–3*) and the purified r*Fib2* 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/Δ2 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 r*Fib2* protein.** The r*Fib2* 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 r*Fib2* proteins with or without subsequent treatment with other glycosidases are pointed out by *arrowheads*.

FIG. 9. **Lectin-blot analysis of the r*Fib2* protein.** The purified r*Fib2* protein (5 μg) 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.
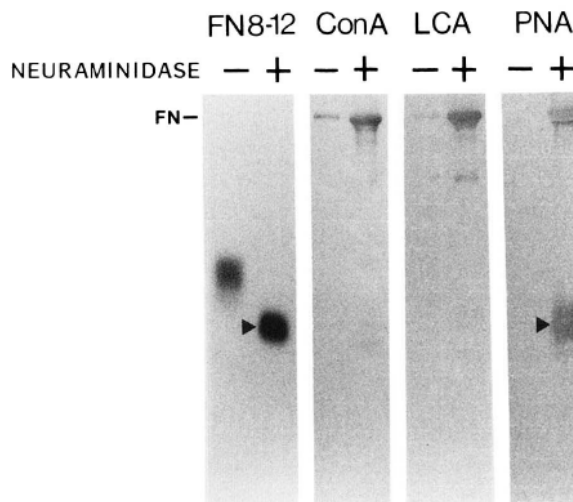
lane 5), this is probably due to the inability of the enzyme to cleave off the sialylated *O*-glycans (32).

The presence of *O*-linked, but absence of *N*-linked, glycans was further supported by the lectin blotting analysis (Fig. 9). Peanut agglutinin, a lectin that binds to desialylated *O*-linked glycans, stained the r*Fib2* protein only after neuraminidase treatment. In contrast, concanavalin A and lentil lectin, both of which preferentially bind to *N*-glycans, failed to stain the r*Fib2* protein irrespective of prior neuraminidase treatment, although both lectins could bind to mouse FN. The binding specificity of these peroxidase-conjugated lectins was confirmed by inhibition of the staining with 0.2 M lactose (data not shown). Taken together with the results obtained using various proteases and glycosidases, these results indicate that the unexpected heterogeneity of the secreted form of the r*Fib2* protein is mostly, if not totally, due to the differential sialylation of *O*-linked glycans localized at the IIICS region.

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 r*Fib2* 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 r*Fib2* 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 r*Fib2*

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 "deminectin," 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 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 r*Fib2* 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 r*Fib2* protein described in the present study when compared with the longer recombinant protein or intact subunits.

The r*Fib2* protein secreted by the transformed cells displayed remarkable heterogeneity upon SDS-PAGE. Although the molecular mass of the processed r*Fib2* 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 r*Fib2* 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 r*Fib2* protein was mostly abolished by neuraminidase treatment. Sialic acid residues causing the heterogeneity of the protein seems to be carried by *O*-linked but not by *N*-linked glycans, since peptide:*N*-glycosidase F treatment did not alter the broad banding pattern of the protein. In support of this conclusion, neither concanavalin A nor lentil lectin bound to the r*Fib2* protein irrespective of prior neuraminidase treatment, indicating that *N*-linked glycans are essentially, if not absolutely, absent in the recombinant protein.

In contrast, the presence of sialylated *O*-linked glycans was evidenced by the observations that (*a*) treatment of the desialylated r*Fib2* protein with endo-α-*N*-acetylgalactosaminidase caused a small, yet significant, reduction of the apparent molecular mass of the protein and (*b*) peanut agglutinin specifically bound to the recombinant protein only after neuraminidase treatment. Involvement of *O*-linked glycans in generating the apparent heterogeneity of the r*Fib2* protein was also proven by the observation that limited proteolysis with trypsin yielded an apparently homogeneous *Fib2* fragment. Trypsin treatment has been shown to eliminate all of the *O*-linked glycans attached to the IIICS region (31, 41). Localization of the *O*-linked glycans at the IIICS region was further supported by the observation that the 43-kDa *Fib2* fragment of the r*Fib2* protein generated by mild trypsin

digestion did not contain *O*-linked glycans detectable with peanut agglutinin.[2]

Despite extensive studies on the structure and localization of *N*-linked glycans of human and hamster FNs (42–45), little attention has been paid to the occurrence and structure of *O*-linked glycans of FNs (45, 46). Skorstengaard *et al.* (47) identified one threonine-linked glycan in the IIICS region of bovine plasma FN. Matsuura *et al.* (31) demonstrated that the IIICS region of human cellular FN contains five threonine-linked and one serine-linked glycans. Since each *O*-linked glycan can carry one or two sialic acid residues, up to 12 sialic acid residues may be present in the IIICS region of the r*Fib2* protein. Thus, differential sialylation of these *O*-glycans may well explain the remarkable heterogeneity of the recombinant protein.

The IIICS region of human FN mRNA has been shown to undergo a complex pattern of alternative RNA splicing (7, 9, 13). Since the cDNA insert of pAISF21 contains the sequence encoding the IIICS/Δ_2, one might expect that differential splicing of the r*Fib2* mRNA can generate two protein isoforms with and without the IIICS/Δ_2 segment. However, tryptic digestion of the purified r*Fib2* protein revealed that only the isoform containing the IIICS/Δ_2 segment was secreted from the transformed cells, indicating that splicing machinery of mouse L cells cannot splice out the 93 nucleotides encoding the IIICS/Δ_2 segment. Alternative splicing of FN pre-mRNA has been shown to be regulated in a tissue- and cell type-specific manner (8, 48, 49). Although factors regulating alternative RNA splicing has not been characterized, the L cells may lack the *trans*-acting factor(s) that recognize the particular 93 nucleotides. Alternatively, splicing out of the 93-base segment may require an additional *cis*-acting signal that is distal to the segment and, therefore, not included in the present cDNA construct.

In striking contrast to the remarkable heterogeneity of the secreted form of the r*Fib2* protein, the proteins immunoprecipitated from the detergent lysate with FN8-12 gave three discrete bands with M_r of 51,000, 54,000, and 66,000. Unlike the secreted form, no heterogeneous protein band was detectable with the immune precipitate from the cell lysate even after prolonged chase. Given the essential role of sialylation in generating the heterogeneity of the r*Fib2* protein, these results strongly suggest that transit of the r*Fib2* protein from cytoplasm to extracellular space requires, or is regulated by, sialylation of *O*-linked glycans.

To date, the role of *N*-glycosylation in protein secretion and transit to plasma membrane has been extensively investigated using a variety of *N*-glycosylation inhibitors (50, 51). It has been reported that inhibition of the processing of *N*-linked glycans by tunicamycin (52) or swainsonine (53) would not cause any dramatic reduction of FN secretion by cultured fibroblasts. Failure of these inhibitors to block transit of FN may well be attributable to sialylation of *O*-glycans that is not affected by these inhibitors. Possible involvement of *O*-glycosylation in protein secretion has been implicated with sialomucin (54, 55). Expression of recombinant FN cDNA modified by site-directed mutagenesis will provide a model system to better understand the role of *N*-linked and *O*-linked glycosylation in the regulation of secretion of extracellular proteins.

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[2] K. Ichihara-Tanaka, unpublished observation.

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