Selective Secretion of Alternatively Spliced Fibronectin Variants

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Abstract. We demonstrate that the alternatively spliced variable (V) region of fibronectin (FN) is required for secretion of FN dimers during biosynthesis. Alternative splicing of the V segment of the rat FN transcript generates three subunit variants (V120, V95, V0) that differ by the inclusion or omission of an additional 120 or 95 amino acids. We are exploring the functions of this segment by expressing variant cDNAs in normal and transformed fibroblasts. Like FN itself, the cDNA-encoded polypeptides (deminectins [DNs]) containing the V120 or V95 segment are efficiently secreted as disulfide-bonded homodimers. However, few homodimers of DNs lacking this region, V0 DNs, are secreted. V0 homodimers do form inside the cell, as demonstrated by biosynthetic analyses of dimer formation and secretion using pulse-chase and time course experiments, but these dimers seldom reach the cell surface and are probably degraded intracellularly. Coexpression of V0 and V120 subunits results in intracellular formation of three types of dimers, V0-V0, V0-V120, and V120-V120, but only the V120-containing dimers are secreted. This selective retention of V0 homodimers indicates that the V region is required for formation and secretion of native FN dimers. In an analogous in vivo situation, we show that plasma FN also lacks V0–V0 dimers and consists of V0-V+ and V+-V+ combinations.

Dissection of V region sequences by deletion mapping localizes the major site involved in DN dimer secretion to an 18-amino acid segment within V95. In addition, high levels of dimer secretion can be restored by insertion of V into a heterologous site 10 kD COOH terminal to its normal location. We discuss the potential role of intracellular protein–protein interactions in FN dimer formation.

FIBRONECTIN (FN)[1] is an integral part of the extracellular matrix, binding to cells, collagen, proteoglycans, and other macromolecules, and playing an important role in cell adhesion and spreading, cell migration, hemostasis and thrombosis, cell morphology, and cytoskeletal organization, and oncogenic transformation (for review, see Yamada, 1983; Hynes, 1986; McDonald, 1988; Ruoslahti, 1988; Mosher, 1989). Alternative splicing of the FN gene transcript results in cell type specific subunit variation (Schwarzbauer et al., 1983, 1985, 1987*b*; Kornblihtt et al., 1984, 1985; Bernard et al., 1985; Paul et al., 1986; Sekiguchi et al., 1986; Norton and Hynes, 1988; Schwarzbauer, 1989). Three sites of alternative splicing occur in FN: EIIIA, EIIIB and the variable (V) region (also known as ED-A, ED-B and IIICS, respectively). The first two are single type III repeats encoded by single exons that are included or skipped during splicing and are found in a subset of cellular FN subunits. Exon subdivision of the V region gives rise to three variants in rat and five in human by splicing within the coding sequences. The three forms in rat are called V0, V95, and V120 to denote the number of extra amino acids included in each variant.

The requirement for different combinations of alternatively spliced FN variants in any biological processes remains obscure. In an effort to understand the role of alternative splicing in FN structure and function, we are expressing cDNAs encoding the rat FN variants via retroviral vectors in cultured mammalian cells and analyzing the activities in a variety of biochemical and cell biological experiments (Schwarzbauer et al., 1987*a*). We have concentrated on the COOH-terminal half of FN encompassing the three regions of alternative splicing, the cell, heparin, and fibrin binding domains, and the cysteine residues required for interchain disulfide bond formation. FN dimers are assembled in the rough endoplasmic reticulum (Choi and Hynes, 1979) and the truncated polypeptides (deminectins [DNs]) are secreted as dimers both with the endogenous FN monomers (FN–DN heterodimers) and, in the case of V+ forms, with themselves (DN homodimers). The levels of V+ DN homodimers and heterodimers in the cell conditioned medium are proportional to the amounts of each subunit synthesized suggesting dimer assembly by random assortment of monomers (Schwarzbauer et al., 1987*a*). In mouse fibroblasts, which make relatively high levels of FN, the heterodimer concentration far exceeds the DN homodimer concentration.

We have elected to express DNs in transformed cells that make much less endogenous FN resulting in V+ DN dimers secreted at levels in excess of FN–DN heterodimers. How-

1. *Abbreviations used in this paper*: DN, deminectin; DOC, deoxycholate; FN, fibronectin; GSBP, glycosylation site binding protein; LTR, long terminal repeat; PDI, protein disulfide isomerase; V, variable region of FN.

ever, few if any V0 DN homodimers are seen in the cell conditioned medium. This could be explained in a number of ways including that the V segment is somehow required either for dimer formation or for efficient secretion of DN dimers, or that the presence of the V segment confers stability on FN dimers.

In this report, we describe our analyses of the involvement of the V region of alternative splicing in intracellular FN dimer formation and secretion. V0 DN homodimers are not found in the cell medium, even though V0 dimers do form inside the cell. The truncated V0 subunits can be "rescued" by expressing both V0 and V120 DNs in the same cell, in which case V0-V120 mixed dimers are secreted. Our analyses of purified rat plasma FN demonstrate that V0 homodimers are absent from this population as well. We have further characterized the sequences involved in this process by deletion mapping of the V region. From these data we discuss FN dimer formation and secretion based on intracellular interactions during processing and transport.

Materials and Methods

Cell Culture

ψ2 and SVT2 (SV40-transformed 3T3 provided by A Levine lab) cell lines were grown in DME supplemented with 10% calf serum (Hyclone Laboratories, Logan, Utah). ψ2 cells were transfected with retroviral vectors containing various FN cDNAs and SVT2 cells were infected with recombinant retroviruses produced either transiently or stably by the transfected ψ2 cells as previously described (Schwarzbauer et al., 1987*a*). Transfectants and infectants were selected for resistance to either Geneticin (Gibco Laboratories, Grand Island, NY) or histidinol (Sigma Chemical Co., St. Louis, MO) depending on which selectable marker, *neo* (Davies and Jiminez, 1980) or *hisD* (Hartman and Mulligan, 1988), was present in the vector. Usually 6–12 colonies from each infection were cloned and screened for levels of cDNA-encoded polypeptide production. To produce cells secreting two different DNs, cells were first infected with one recombinant virus and selected for drug resistance followed by infection with the second recombinant virus and cloned after selection for double resistance.

FN and Antibody Preparation

FN was purified from rat plasma by gelatin-Affigel affinity chromatography (Engvall and Ruoslahti, 1977). For preparation of anti-FN antisera, FN was further purified by ion exchange chromatography using a Mono Q HR5-5 column (Pharmacia Fine Chemicals, Piscataway, NJ; LKB Instruments Inc., Bromma, Sweden) with an LKB HPLC system. FN was applied to the column in 20 mM Tris-HCl, pH 8.3, 50 mM NaCl, and eluted at ~0.25 M NaCl in a linear gradient of .05–1.0 M NaCl.

New Zealand white rabbits were immunized with 0.1 mg of FN in complete Freund's adjuvant (Gibco Laboratories) and boosted after 2 wk with another 0.1 mg in incomplete Freund's (Mautner and Hynes, 1977). Immune bleeds were collected weekly beginning 2 wk after the boost. Individual bleeds of similar titer were pooled for use in immunoprecipitations and immunoblotting. The amount of antiserum needed to immunoprecipitate all FN from a given quantity of cell conditioned medium was determined empirically and all immunoprecipitations were carried out in antibody excess.

Metabolic Labeling and Immunoprecipitations

Cells were metabolically labeled with [^{35}S]methionine (trans-label, ICN Radiochemicals, Irvine, CA) at 25 μCi/ml for 24 h. Labeled media were collected, cells and debris were removed by centrifugation, and EDTA and PMSF were added to 10 and 2 mM, respectively, as protease inhibitors. Short term labelings were performed as described by Choi and Hynes (1979). For determination of the rate of secretion of FN and DNs, cells were labeled continuously with 200 μCi [^{35}S]methionine/ml of medium and aliquots were removed at selected time points and processed as above but with two additional inhibitors, iodoacetic acid and *N*-ethylmaleimide at 1 mM each. In pulse-chase experiments, monolayers were pulsed with methionine-free medium containing 200 μCi [^{35}S]methionine/ml for 10 min followed by a chase with medium plus excess cold methionine for times from 10 to 60 min. Cells were rinsed with PBS followed by lysis with cold 2% deoxycholate (DOC) in 50 mM Tris-HCl, pH 8.8, 2 mM EDTA, plus inhibitors as above. Cell lysates were put through a 26G hypodermic needle attached to a 1-cm^3 syringe to shear DNA and spun to remove DOC insolubles.

^{35}S-labeled FN and DNs were immunoprecipitated in the presence of 0.5% NP40/0.5% DOC/0.1% SDS using a rabbit anti-rat pFN polyclonal antiserum followed by Protein A-agarose beads (Repligen Corp., Cambridge, MA). Beads were washed and proteins were eluted by boiling in electrophoresis sample buffer without reducing agent. Immunoprecipitation of 0.1 μg of purified rat pFN with anti-V95 antiserum was carried out as described above. However, the antibody titer was insufficient to bring down all of the FN, so an aliquot of the supernatant was taken through two more rounds of immunoprecipitation by addition of more anti-V95 and Protein A beads.

In some cases, FN and DNs were partially purified from conditioned medium by batchwise heparin-agarose (Sigma Chemical Co.) affinity chromatography, washed with PBS, and eluted as for immunoprecipitations.

Polyacrylamide Gel Electrophoresis and Immunoblotting

Protein samples with or without reduction were electrophoresed along side prestained molecular weight standards (Sigma Chemical Co.) through SDS polyacrylamide gels (Laemmli, 1970), fixed, treated with Enhance (New England Nuclear, Boston, MA), and exposed to film (X-omat; Eastman Kodak Co., Rochester, NY) at −80°C for 12 h–3 wk depending on the experiment. For analysis by two-dimensional gel electrophoresis, nonreduced samples were run in SDS–5% polyacrylamide tube gels, reduced in situ with 10% β-mercaptoethanol, and electrophoresed through SDS-6% polyacrylamide gels. Unlabeled samples were visualized by silver staining (Merril et al., 1984).

Fluorographic signals from one dimensional gels were quantitated by densitometry using a video densitometer (620; Bio-Rad Laboratories, Cambridge, MA). Alternatively, bands were cut out of dried, Enhanced gels and counted in Ecoscint (National Diagnostics, Inc., Somerville, NJ) in a scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

For antibody detection after electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) using the Mini-Protean II transfer apparatus (Bio-Rad Laboratories, Cambridge, MA) and following the protocol suggested by the manufacturer. Filters were blocked and probed with antibodies as described by Paul et al. (1986) using an anti-rat pFN polyclonal antiserum at a dilution of 1:500. Bound antibody was detected using biotinylated goat anti-rabbit second antibody, streptavidin horseradish peroxidase, and 4-CN substrate (Bethesda Research Laboratories, Gaithersburg, MD).

Retroviral Vectors and cDNA Constructions and Deletions

The retroviral vector, pLP, used throughout these studies is a modification of pDOP (Schwarzbauer et al., 1987*a*) in which the pBR322 origin of replication has been moved to the 3′ end of the *neo* gene, the retroviral 5′ splice site has been deleted, and the pBR322 plasmid backbone has been deleted leaving only polyoma early region sequences outside of the long terminal repeats (LTRs). Two forms of pLP have been constructed that differ only in the encoded pre/pro sequences: pLPP with the parathyroid hormone signal sequence cDNA preceding the cloning site and pLPS with FN cDNA encoding the signal sequence plus the first 18 amino acids of mature FN. Both of these vectors were used in the expression experiments but only data from pLPS constructions are presented. The rat FN cDNAs that were inserted into pLPS had their 5′ termini in the middle of type III-8 (Fig. 1 *A*) and extended to the COOH terminus. All were minus EIIIA and plus or minus the V region as indicated.

Some of the experiments required coexpression of two different cDNAs. To obtain retrovirally infected cells carrying two different proviruses, a second selectable marker was used, the bacterial *his D* gene (Hartman and Mulligan, 1988). The vector, pGEP-His, was constructed by replacing the *neo*-pBR322 origin cassette in pLPP with the *his D* gene. For propagation in bacteria, the polyoma early region sequences outside of the LTRs were substituted with the *amp* gene and origin of replication sequences from pGEM (Promega Biotec, Madison, WI).

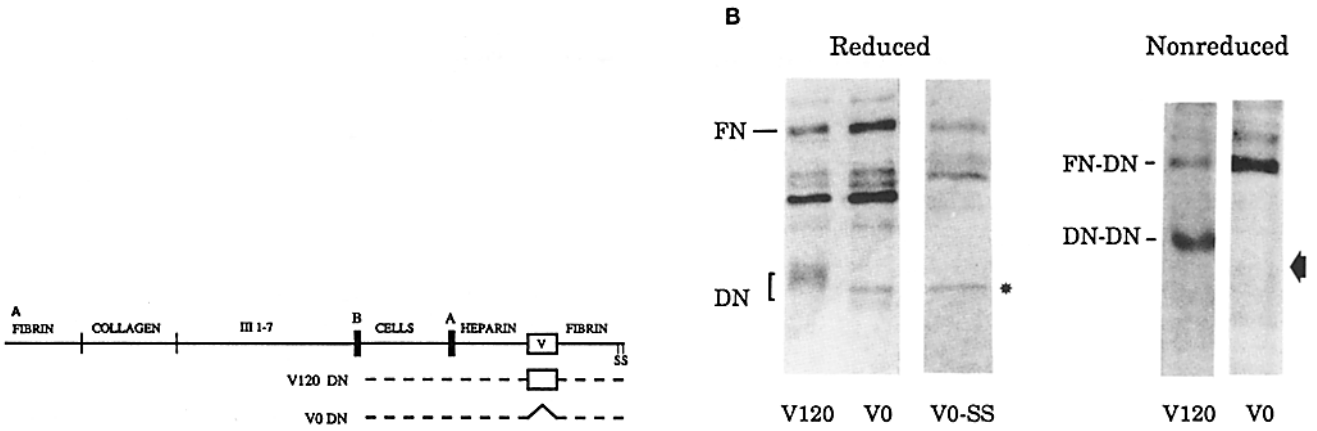

Figure 1. A, Schematic of the domain structure of FN and DNs. The domains for binding to fibrin, collagen, cells, and heparin are labeled. A pair of COOH-terminal cysteines form the interchain disulfides (*SS*) in the dimer. The two sites of alternative splicing, EIIIB and EIIIA (*thick bars*, *B* and *A*, respectively), are located on either side of the cell binding region. Between the collagen domain and EIIIB are type III repeats 1–7. The amino-termini of V0 and V120 DNs lie in the middle of type III-8, immediately COOH-terminal to EIIIB. These DNs span the COOH-terminal half of FN, and are EIIIA minus and V120 plus or minus (*V0*) as indicated. *B*, Analysis of secreted DNs. FN and DN were purified from labeled media of SVT2 infectants by batchwise heparin–agarose affinity chromatography and electrophoresed reduced to separate monomers or nonreduced for dimers. Identities of FN and DNs were confirmed by immunoprecipitation (not shown). (*Reduced*) FN, V120, and V0 DN monomers are indicated as are the V0–SS polypeptides (*) that lack the COOH-terminal interchain disulfides and are secreted as monomers. V120 DNs migrate as a diffuse band at ~120 kD and V0 DNs appear as a doublet between 100 and 110 kD. (*Nonreduced*) V120 homodimer levels (DN–DN) exceed heterodimers (FN–DN) while only the heterodimer form is present in the V0 sample. The position where V0 DN homodimers would be located, if present, is marked (*arrow*). Prestained molecular mass standards of 180, 116, 84, 58, and 48 kD were included on all gels (not shown).

All deletions within the V segment and of the COOH-terminal cysteines were made by using conveniently located restriction endonuclease sites. Enzymes were from New England Biolabs (Beverly, MA) or Boehringer Mannheim Diagnostics, Inc. (Houston, TX). The region encoding the cysteines involved in interchain disulfide bonding was removed by cutting the rat FN cDNA with Acc I (at position 7330) and Nla III (7390), the ends were blunted with Klenow fragment of *Escherichia coli* DNA polymerase, and religated to delete a 20-amino acid piece. Sites within and adjacent to the V segment that were used to create deletions include Bgl II (at position 56 within the 360 bases of V), Sau3A I (176), Nla III (192), Fnu4H I (220), Hae III (240), Acc I (308), Hpa II (333), and Alu I 3′ of V (369). Deletions called 18, 20J, 39, 40, and 93 spanned regions from 192–240, 308–369, 220–333, 56–176, and 56–333, respectively. To insert the V region into a heterologous site, a fragment from a BstE II site at position 18 to Hpa II at 333 was ligated into cDNA linearized with Hinc II and Stu I which cut between sequences encoding type III-15 and type I-10 (Schwarzbauer et al., 1983). All deletions were characterized by restriction mapping and in most cases by subcloning into one of the pGEM vectors (Promega Biotec) and sequencing across the junctions using Sequenase (United States Biochemical Corp., Cleveland, OH).

Results

Dimer Formation by V0 and V120 Subunit Variants

The FN domain structure and the truncated DNs are illustrated in Fig. 1 *A*. DN cDNAs encode the rat FN signal and pro sequences and the first 18 amino acids of the mature protein connected to the COOH-terminal half of FN from the middle of type III-8 to the end. The cDNAs were inserted into a retroviral vector, pLPS, and transfected into the $\psi 2$ packaging cell line to produce recombinant retroviruses. These viruses were then used to infect SVT2 cells (SV40-transformed 3T3 cells) and infected, G418-resistant clones were screened for high level production of DNs. The expressed polypeptides span the domains for binding to cells, heparin, and fibrin, two regions of alternative splicing, EIIIA and V, and the cysteines necessary for interchain disulfide bond formation. These DNs possess many of the functions inherent in FN such as heparin binding activity (Fig. 1 *B*), cell attachment activity (our unpublished observations), and disulfide-bonded dimer formation both with themselves (DN homodimers) and with the endogenous FN monomers produced by the cells expressing the cDNAs (FN–DN heterodimers). The heterodimers are incorporated into the extracellular matrix (Schwarzbauer et al., 1987*a*).

When V120 cDNAs are expressed in SVT2 cells, which make very little of their own FN, the DN levels exceed endogenous FN levels and the DN homodimers predominate in the cell conditioned medium (Fig. 1 *B*). V0 DNs can also be seen in reduced samples where dimers are separated into monomer components, although at molar levels less than that of the endogenous FN (Fig. 1 *B, Reduced*). Also shown are V0-SS DNs that are secreted as monomers because of deletion of the cysteines that form the interchain disulfide bonds (see below). The comparison of dimer forms from V120- and V0-secreting cells emphasizes the disproportionately small quantities or, in many cases, complete lack of V0 DN homodimers, although V0 DNs are secreted as heterodimers (Fig. 1 *B, Nonreduced*).

Biosynthesis of FN Dimers

The lack of V0 homodimers in culture medium raises the question, are V0 DNs able to form dimers with each other? To answer this, we carried out pulse-chase labeling experiments and isolated the intracellular FN and DN dimers by immunoprecipitation. Fig. 2 shows the fluorographs of two dimensional nonreduced/reduced SDS-polyacrylamide gels of the DOC-soluble fraction of pulse-labeled cells. After a 10-min pulse and a 10-min chase period, FN–DN heterodimers are present in all cells. More importantly, homodimers of V0 DNs occur at relatively high levels (*A*). In cells simultaneously expressing both V0 and V120 DNs (*B*), spots representing V120 homodimers, V0-V120 mixed dimers, and

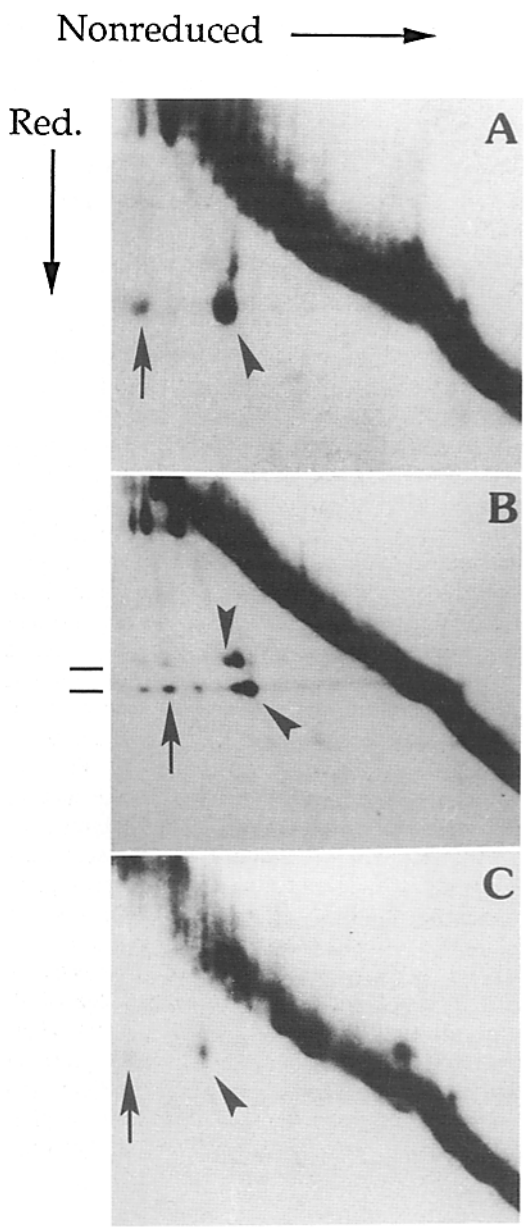

Figure 2. Intracellular dimers. Cells were pulse labeled with [^{35}S]-methionine for 10 min followed by a 10-min chase, lysed in DOC buffer, and immunoprecipitated as described in the Materials and Methods. Immunoprecipitates from V0 (*A*), V0+V120 (*B*), and V120 (*C*) cells were electrophoresed nonreduced in the first dimension and reduced through the second dimension gel to separate dimer subunits. DNs from heterodimers (*arrows*) and homodimers (*arrowheads*) are present in all three samples. V120 and V0 DNs are indicated by ticks at the left (*B*). Subunits corresponding to V0–V120 mixed dimers can be seen in the sample from double infectants (*B*). Immunoprecipitates of DOC lysates usually contained nonspecifically associated proteins which form the diagonal in these fluorographs.

V0 homodimers are clearly evident. Note the abundance of the intracellular V0 DN dimers. Clearly, V0 subunits are competent to form dimers with each other. However, these dimers do not appear as stable forms of DN in the conditioned medium of cells. What is their fate?

To eliminate the possibility that the dimers are secreted but rapidly degraded, we carried out a time course analysis of secreted DNs. Cells were labeled continuously with [^{35}S]-methionine and aliquots of medium were collected and analyzed at times from 30 min to 2 h. The time course data showed that the V0 dimers seen inside the cell in the pulse-chase experiments never appear at the cell surface, while V120 dimers begin to appear in the medium after ~40 min of labeling, at approximately the same time as FN dimers (data not shown). Taken together we conclude that the V0 dimers form inside the cell, probably within the rough endoplasmic reticulum, but for some reason they are not secreted and are probably degraded intracellularly.

We next tested whether the V segment is needed simply for intracellular transport to the cell surface. cDNAs encoding DNs unable to form disulfide-bonded dimers were constructed by deleting the region encoding the two COOH-terminal cysteines and were expressed in combination with V0 or V120 inserts (V0–SS and V120–SS). The cell-conditioned media were screened by heparin affinity chromatography and by immunoprecipitation for the presence of DN monomers in nonreduced samples. Both V0–SS and V120–SS DNs were secreted in monomeric form and were stable at least for the 24-h labeling period. An example of a secreted monomeric V0–SS DN is shown in Fig. 1 *B* (V120–SS data not shown). These results demonstrate that the V segment is not essential for transport of DNs from the ER through the Golgi complex and to the surface, and that the V region is not required to stabilize DNs in the cell-conditioned medium. The fact that monomeric V0 subunits are secreted but V0 dimers are not shows that the selective retention of V0 homodimers is specific to the dimer form itself.

V0-V120 Mixed Dimers Are Secreted

When V0 DNs are dimerized with full length FN subunits, they are efficiently secreted. We tested the ability of V120 DNs to replace intact FN monomers in this capacity. Cell clones already secreting V0 DN–FN heterodimers were infected with recombinant retroviruses carrying the V120+ cDNA and the bacterial *his D* gene as selectable marker (Hartman and Mulligan, 1988) and infectants were identified by growth in double selective medium, screened for expression of both V0 and V120 DNs, and analyzed for the types of dimers that were secreted. Fig. 3 *A* shows that doubly infected clones secrete two sizes of DN dimers, one minor form that comigrates with V120 homodimers and the other more abundant form migrating ahead and consisting of V0-V120 mixed dimers (see below). Heterodimer bands are also apparent in V120 and V0+V120 lanes but the V0 DN-FN band is barely visible in the parent V0 sample. The exact composition of these bands was determined by two dimensional nonreduced/reduced SDS-PAGE (Fig. 3 *B*) and clearly demonstrates the presence of V0-V120 mixed dimers and the complete absence of V0 homodimers in the culture medium. In the pulse-chase analysis (Fig. 2 *B*), the levels of V0 dimers within the cell are apparently reduced when V120 and V0 forms are synthesized concomitantly suggesting that the V120 form is titrating out the V0 DNs and thus effectively decreasing the levels of V0 dimers made. Therefore, V0 DNs can dimerize with other truncated molecules and while these mixed dimers are efficiently secreted, the V0 homodimers remain inside the cells.

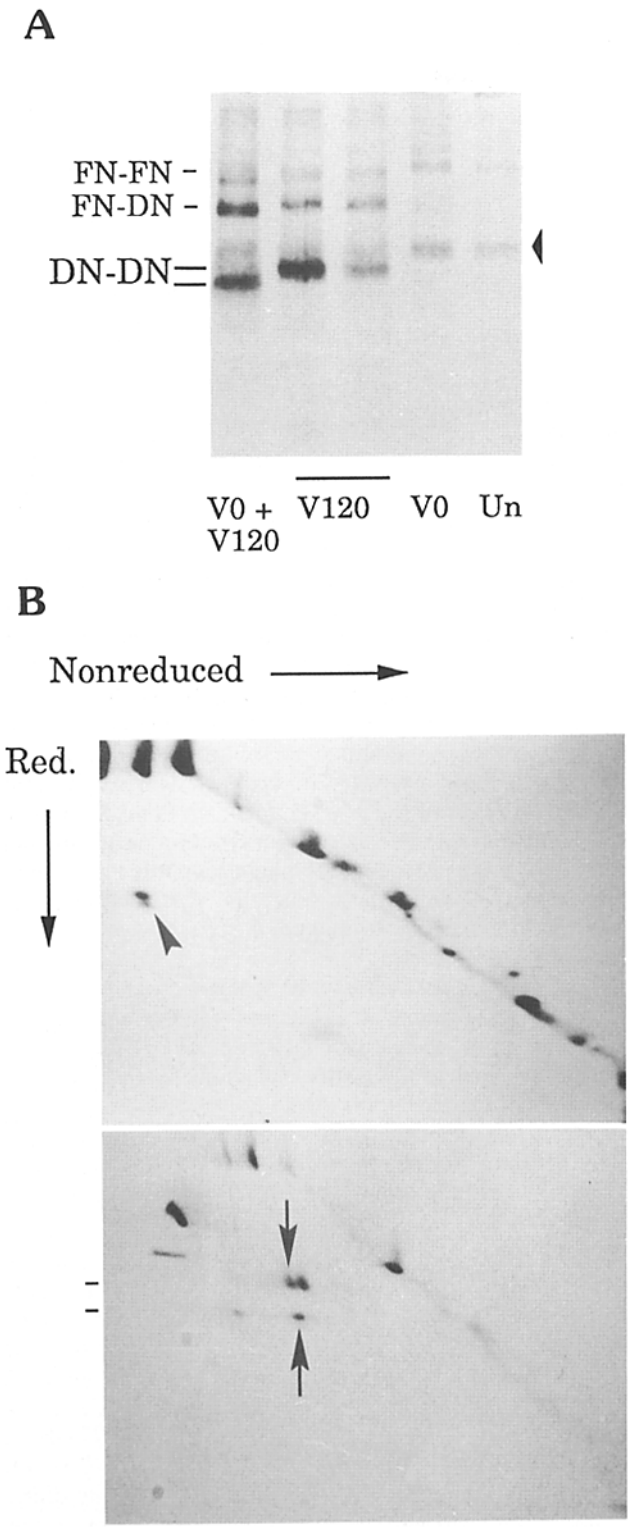

Figure 3. Secreted dimers. *A*, Immunoprecipitates of conditioned medium from cells secreting either V0, V120, or V0+V120 DNs were electrophoresed nonreduced to separate dimers. FN dimers (FN–FN) are visible in all lanes. Some FN monomers, probably resulting from degradation of dimers at the COOH terminus, can be seen in the uninfected control sample (*Un*), the V0 cell line (*V0*), and migrating just behind the DN dimers in adjacent lanes (*arrowhead*). Heterodimers (FN–DN) are obvious in V120 and V0+V120 samples but are barely visible in the V0 cell line that was used to generate the V0+V120 line. DN dimers (DN–DN, *upper line*) are the predominant dimers in the two V120 samples. The V0+V120 sample contains a faster migrating, more abundant species (DN–DN, *lower line*) consisting of V0–V120 mixed dimers (see below). *B*, Dimer components were identified by two-dimensional nonreduced/reduced SDS-PAGE. V0 cells secrete only heterodimers (*arrowhead, top*). Some heterodimers, but mostly V120 homodimers and V0-V120 mixed dimers (*upper and lower arrows*, respectively), are present in the V0+V120 medium (*bottom*). Positions of V120 and V0 DNs are indicated by ticks. Note the absence of any V0 homodimers in both samples.

The DNs are truncated forms of FN and this may affect their ability to be transported through the secretory pathway. We examined whether the same results were obtained with longer DNs. Polypeptides extending from type II-2 (in the collagen domain) and from type III-2 to the COOH terminus (see Fig. 1 *A*), ~210- and 180-kD, respectively, behaved in the same manner as the shorter DNs remaining unable to form secretable homodimers (data not shown).

Analysis of Rat Plasma FN Dimers

Rat plasma FN (pFN) is composed of ~40% V0 subunits (Schwarzbauer et al., 1985). Random assortment of FN monomers in hepatocytes, the site of synthesis of pFN (Tamkun and Hynes, 1983), would result in 16% of the dimers being V0–V0, a quantity that should be easily detected if present. To determine whether V0 homodimers exist in blood, two approaches were used to analyze the pFN dimer repertoire. First, we immunoprecipitated pFN with an anti-V95 antibody (Schwarzbauer et al., 1985) and analyzed the precipitate and the supernatant by SDS-PAGE and immunoblotting with an anti-FN polyclonal antiserum. Any dimers completely lacking V+ subunits, namely, V0 homodimers, would not be precipitated by this antibody. The antibody titer was insufficient to completely immunoprecipitate mass amounts of unlabeled pFN, but, after three rounds, we could detect no pFN remaining in the supernatant (Fig. 4 *A*). Furthermore, each immunoprecipitate contained both V+ and V0 subunits. Therefore, the vast majority of V0 subunits are apparently dimerized with V+ subunits. In the second approach, the dimers were separated into their components by two-dimensional nonreduced/reduced SDS-PAGE. V0–V0 dimers should migrate slightly ahead of V0–V+ and V+–V+ forms in the first dimension. Visualization of subunits by silver staining showed no evidence of a leading edge of V0 monomers indicative of V0 homodimers (Fig. 4 *B*). However, obvious trailing of the V+ spot indicates the presence of V+–V+ dimers. Clearly, the full spectrum of FN dimers that can be formed intracellularly is not represented in plasma. We conclude that rat pFN consists primarily of heterodimers of one V+ and one V0 subunit.

Is There a Signal Sequence for Dimer Secretion?

As the V segment is needed for proper dimer formation/secretion of FN, one might expect to find sequences within V that confer the ability to form secretable dimers. To define such sequences, deletion analyses of the V segment and flanking regions were carried out (Fig. 5 *A*). The naturally occurring V95 alternatively spliced product has been previously shown to form dimers comparable to V120 subunits. Comparison of rat, human, and chicken sequences showed

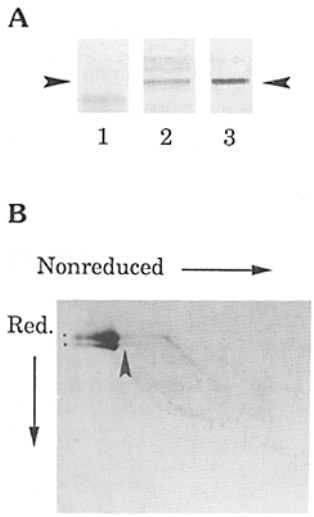

Figure 4. Purified rat pFN dimers. *A*, Rat pFN was immunoprecipitated with anti-V95 antiserum, components of the first and second precipitates (*3* and *2*) and the final supernatant (Lane *1*) were separated by 6% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-pFN antiserum. No pFN was detected in the supernatant after immunoprecipitation with the anti-V antibody. *B*, Rat pFN was analyzed by two-dimensional nonreduced/reduced SDS-PAGE. Dimers were reduced and V0 and V120 monomers were separated in the second dimension (*dots*). No leading edge of V0 subunits indicative of V0–V0 dimers can be seen (*arrowhead*).

Table I. Quantitation of Secreted Dimer Levels

	DN-DN/FN-DN
V120	2.3 ± 0.2
V0	0.3 ± 0.3
Δ20J	3.9 ± 0.6
Δ40	1.4 ± 0.1
Δ39	1.2 ± 0.3
Δ18	0.8 ± 0.3
Δ93	0.5 ± 0.1
Insert V120	2.9 ± 0.6

Quantitation of dimer levels. Relative amounts of homodimers to heterodimers (DN-DN/FN-DN) were determined by scanning densitometry of fluorographs and by counting bands excised from gels. Ratios for at least three different clones were averaged and are listed with the standard deviation. Reductions in DN homodimer levels result in a decrease in the ratio; this is most marked for Δ93.

a region in the middle of V with the highest homologous stretch (Norton and Hynes, 1988). Deletion of 40 amino acids (Δ40) containing this segment had only a moderate effect on the levels of DN dimers secreted (Table I). Alternatively, one major difference between forms carrying and lacking V is the presence of the COOH-terminal junction between the V segment and the last type III-15. Deletion of this region (Δ20J) had no negative effect on the dimers secreted (Fig. 5 *B*, Table I).

The levels of DN homodimers and FN-DN heterodimers in V120, V0, and deletion samples were quantitated and the average ratios of homodimer to heterodimer (DN-DN/FN-DN) are listed in Table I. V0-producing cell clones secreted V0 dimers at levels eightfold lower than parallel V120-expressing cells. Deletion of a large part of V95, Δ93, resulted in a significant drop in the secreted DN homodimer levels (Fig. 5), similar to the levels secreted by V0 cells (Table I). Removing smaller stretches of these 93 amino acids also reduced the levels of dimers: Δ39 had a moderate effect while deleting the central 18 amino acids surprisingly caused the largest reduction in secreted homodimers (Fig. 5, Table I). So by dissection of the V95 segment, the major effect on homodimer levels is localized to an 18-amino acid site indicating that these residues play an important role in FN dimer formation/secretion but also suggesting that cooperative effects of more than one segment of the V region are involved in this process.

We also tested the ability of the V region to exert its effects from another location. A 117 amino acid segment inserted between type III-15 and type I-10 in a V0 DN (i.e., 10 kD COOH-terminal to the normal location) totally restored a high level of secreted dimers that in most cases exceeded the level of V120 dimers (Fig. 5, Table I). Clearly, the effects of V on FN dimer formation are to some extent independent of location within the sequence.

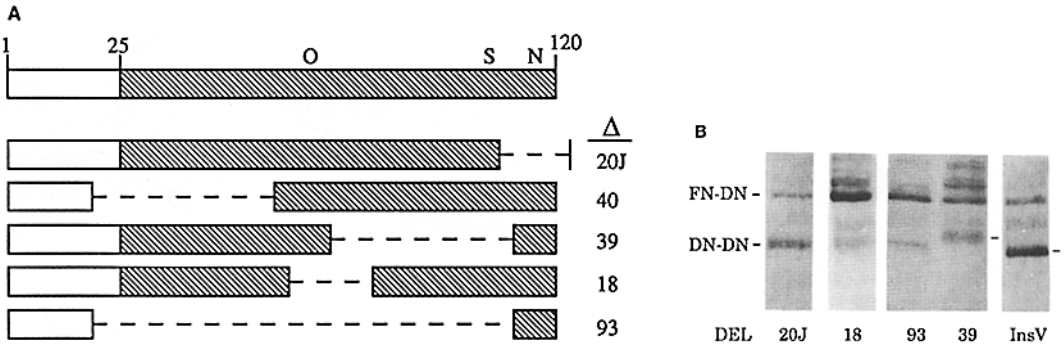

Figure 5. Deletion mapping of the V segment. *A*, This diagram illustrates V120 (*top*) and the locations of the different deletions (*dashed lines*) made within the V region. All are internal deletions with the exception of Δ20J that spans the junction between V and type III-15. V120 is at the top; cross-hatching defines the V95 section of V120. *O* and *N* mark the sites of O- and N-linked glycosylation identified in bovine pFN. *S* denotes tyrosine sulfation. *B*, Deletions were made in the V region cDNA, mutant DNs were expressed, and secreted dimers were separated by SDS-PAGE. Relative amounts of FN-DN heterodimers and DN-DN homodimers can be seen for Δ20J, Δ18, Δ93, Δ39, and Insert-V. Samples are from different experiments and are aligned via the heterodimer bands. Some samples also contain slight amounts of FN monomer.

Discussion

Multiple forms of FN arise by alternative splicing of the primary transcript at three sites. One of these sites, the complex V region, exists in three forms in rat, V0, V95, or V120. In this report, we have demonstrated that the presence of the V region in at least one subunit is required for secretion of FN dimers. V+ DNs are efficiently secreted as homodimers, while V0 DNs transit the secretory pathway as dimers only when coupled with a V+ DN or FN subunit. The V0 homodimers that form during FN biosynthesis are retained inside the cell. Monomeric V0 DNs are secreted and stable in the cell culture medium ruling out a requirement for the V region in intracellular transport or extracellular stability and suggesting that V0 homodimers themselves are defective.

FN monomers are disulfide-bonded into dimers in the lumen of the ER shortly after synthesis (Choi and Hynes, 1979). Assembly of dimers containing V+ subunits occurs by random assortment of monomers (Schwarzbauer et al., 1987*a*). Dimers are then transported through the Golgi apparatus and on to the cell surface. Posttranslational processing, transport, and secretion of V0–V120 and V120–V120 DN dimers occur at a rate similar to that of endogenous FN. However, analysis of the biosynthesis of V0 DNs by pulse labeling the cells and chasing for varying times demonstrates that V0 DN dimers are indeed formed inside the cell, but that these dimers never appear at the cell surface. In fact, the levels of intracellular V0 homodimers exceed that of heterodimers and would be readily observable in the cell conditioned medium if present. Longer V0 DNs display the same lack of secreted homodimers, excluding the amino terminal half of FN as playing a major role in dimer formation. In addition, effects of pre/pro sequence processing are ruled out as two different signal sequences, from parathyroid hormone (Hellerman et al., 1984) and FN (Patel et al., 1987), gave identical results.

The relative proportions of alternatively spliced FN subunits vary in a cell type–specific manner. For example, fibroblasts synthesizing cellular FN make very few if any V0 subunits, while up to 50% of pFN subunits are V0, depending on the species (Schwarzbauer et al., 1985; Paul et al., 1986). With 40% V0 and 60% V+ subunits in rat pFN, the calculated percentages of FN dimers are 16% V0–V0, 48% V0–V+, and 36% V+–V+. However, two experiments suggest that the V0 homodimer species is not present in purified pFN: separation of dimers by two dimensional nonreduced/reduced SDS-PAGE and immunoprecipitation of all detectable pFN dimers with an anti-V segment antibody. If plasma contains any V0 FN dimers, their levels are so low as to be undetectable by these assays. Therefore, pFN must consist entirely of dimers containing at least one V+ subunit in the proportions 80% V0–V+ and 20% V+–V+.

The data for pFN are consistent with our results with cells synthesizing both V0 and V120 DNs. Three species of DN dimers (V0–V0, V0–V120, and V120–V120) are assembled intracellularly. Their relative proportions show that no single form is favored and suggest random assortment of monomers into dimers (see Fig. 2 *B*). However, only V0–V120 mixed dimers and V120 homodimers are secreted. V0 homodimers are selectively retained by the cell and are not found in the cell medium.

We carried out deletion mapping of the V segment to localize any specific sequences involved in dimer formation/secretion. Although removal of a large part of V, 93 amino acids, had a major effect on the level of DN homodimers secreted, shorter deletions from within the V95 region also decreased the efficiency of secretion. In fact, the shortest deletion tested, Δ18, caused a significant reduction in secreted homodimers. Flanking deletions of 40 and 39 amino acids had lesser but reproducible effects. Together the deletion data suggest that the central 18–amino acid portion is required for efficient secretion of FN dimers but probably cooperates with other segments within the V95 to allow maximum secretion. Interestingly, while the 18-residue segment would be present in all V+ subunits of human FN, the Δ39 overlaps considerably with the COOH terminal 31 amino acids omitted from one alternatively spliced form of the human FN transcript (Kornblihtt et al., 1984). Using an antiserum directed against these 31 amino acids, Sekiguchi and Titani (1989) have recently shown that V+ subunits containing and lacking this segment are present in human pFN. Determination of the combinations of variants comprising human pFN dimers may shed some light on the role of this region.

One important result that proves a functional role for the V segment is its ability to reverse completely the V0 phenotype to that of V120 dimers when inserted 10 kD carboxy terminal to its normal location. The two locations are very different structurally in that the normal site is between the last two type III repeats (III-14 and III-15) each of ~90 amino acids, while the heterologous site lies between the last type III unit and type I-10, a finger structure of half the size folded via a pair of intrachain disulfides (Petersen et al., 1983). Apparently, local information from adjacent repeats is not essential to this activity of the V region. In fact, the V-insert DN dimer levels were generally higher than those of V120 dimers.

Aside from the additional amino acids, one major difference between V+ and V0 subunits is the presence of posttranslational modifications. Within the V segment itself, there are three different modifications: one asparagine-linked oligosaccharide at the COOH terminus (position 117), one or several threonine-linked sugars around residue 73 (Skorstengaard et al., 1986), and a sulfate group on a tyrosine located between residues 80 and 110, most probably on tyrosine 105 (Paul and Hynes, 1984; Huttner and Baeuerle, 1988) (Fig. 5 *A*). V0 DNs lacking the carboxy terminal cysteines and, therefore, unable to form disulfide-bonded dimers, were efficiently secreted and accumulated in the culture medium as monomers showing that modifications within the V segment, and the V segment itself, are not essential for synthesis and secretion. Although Δ18 contains the site(s) of O-linked glycosylation identified in bovine pFN, other sites may exist in cFN and the diffuse appearance of the fluorographic bands from these subunits suggests that they retain a large degree of modification. Taken together, there is no apparent correlation between the deletion data and the presence or absence of specific sites of posttranslational modification.

Many studies of protein biosynthesis, oligomerization, processing, and transport to the cell surface have been carried out with viral membrane glycoproteins, namely, VSV-G protein and influenza hemagglutinin. While normal forms of these proteins are modified, processed and transported quite efficiently, mutant proteins exist that cannot exit the rough

ER (Rose and Bergmann, 1983; Kreis and Lodish, 1986; Gething et al., 1986; Copeland et al., 1986, 1988). A similar phenomenon has been observed for the cellular proteins, immunoglobulins (Morrison and Scharff, 1975; Bole et al., 1986) and silk fibroin (Takei et al., 1984). Immunoglobulin heavy chains, when synthesized in the absence of light chains, are not secreted and persist in the ER in association with BiP (Bole et al., 1986). Some elegant studies using antibodies that distinguish native and nonnative conformations of hemagglutinin have shown that these mutants are deficient in their ability to oligomerize properly and consequently maintain an unfolded conformation which inhibits their movement along the pathway and results in intracellular degradation (Copeland et al., 1986; Gething et al., 1986). Although FN is "free" in the lumen of the ER as opposed to membrane-bound, it seems likely that these conclusions could apply to our results as well. Possibly V0 dimers are in some way misfolded and therefore cannot proceed along the normal secretory pathway. However, V0 DNs can traverse the pathway either as monomers or as members of heterodimers suggesting that something inherent in the V0 homodimers makes them conformationally defective.

The monomer orientation in bovine pFN dimers is reported to be antiparallel (Skorstengaard et al., 1986). That is, although the subunit sequences in the COOH terminus are identical, opposite cysteine residues disulfide bond with each other. From this, one possible explanation for misfolding of V0 dimers arises: the antiparallel structure is native while the parallel form is not and the V region is involved in forming antiparallel FN dimers. The ER localized enzyme, protein disulfide isomerase (PDI), plays a key role in rearranging disulfide bonds to generate conformationally native structures (Creighton et al., 1980; Freedman, 1984; Bulleid and Freedman, 1988). The V region may be a binding site for such an enzyme and would thus allow rearrangement of parallel bonds to form the preferred antiparallel type. The complete lack of a V region in either subunit would preclude the action of this enzyme and leave the dimers in an unacceptable form. Interestingly, PDI has recently been shown to be highly homologous, if not identical, to another luminal ER protein, glycosylation site binding protein (GSBP), a protein that recognizes and binds to Asn-X-Thr/Ser sites (Geetha-Habib et al., 1988). In this report, GSBP was postulated to facilitate the interaction between these sites and oligosaccharyl transferase. On the other hand, PDI apparently functions as a homodimer (Freedman, 1984). So, although these two activities are very different, they seem to have one component in common. With the presence of a glycosylation site in the V region, GSBP, and by analogy PDI, can presumably bind to sequences in or around this segment. We are currently testing this hypothesis by coimmunoprecipitation experiments.

An alternative hypothesis to explain our results is that the V region affects the association of FN monomers with each other. This segment may provide a FN binding site for antiparallel alignment of monomers (Ehrismann et al., 1982) or a specific conformation required for subunit interactions independent of disulfide bonding (Homandberg et al., 1985; Robinson and Hermans, 1984). Evidence exists for interaction between the COOH-terminal regions of FN monomers and between the COOH-terminal heparin domain and the NH_2-terminal fibrin domain (Homandberg and Erickson, 1986). Experiments to test the potential role of the V segment in FN self-association within the extracellular matrix are currently underway.

Why do cells control the types of FN dimers that they secrete in part by retaining V0 homodimers? Melanoma cells have been shown to use two sites within the V segment for cell attachment (Humphries et al., 1986, 1987; McCarthy et al., 1986). Furthermore, its proximity to the heparin and fibrin binding domains may suggest a role in modulating the interactions between FN and these molecules. Possibly construction of a functional extracellular matrix relies in part on the proportions of FN variants, and cells vary these at two levels, splicing and secretion. Different subunit combinations could then result in variable degrees of fibrillar branching. The putative association of the V region with a counterpart on other FN subunits in the ER may mimic interactions that occur within the extracellular matrix. Alternatively, the solubility of FN may be changed by inclusion of the V region, thus altering its properties once outside the cell. Only further experiments using this and other systems to test the activities of the V region, and of the other sites of alternative splicing, will determine the exact functions of variant FN subunits.

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