

# Proteolysis Regulates Exposure of the IIICS-1 Adhesive Sequence in Plasma Fibronectin<sup>†</sup>

Tatiana P. Ugarova,<sup>\*,‡</sup> Alexander V. Ljubimov,<sup>§</sup> Lynn Deng,<sup>‡</sup> and Edward F. Plow<sup>‡</sup>

Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Department of Molecular Cardiology, Cleveland Clinic Foundation, Cleveland, Ohio 44195, and Ophthalmology Research Laboratories, Cedars-Sinai Medical Center, UCLA Medical School Affiliate, Los Angeles, California 90048

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**ABSTRACT:** The alternatively spliced type III connecting segment (IIICS) of fibronectin (Fn) contains an amino acid sequence, CS-1, which is recognized by the integrin receptor,  $\alpha_4\beta_1$ . Plasma Fn inhibits  $\alpha_4\beta_1$ -dependent binding of lymphocytes and monocytes to CS-1 containing Fn derivatives poorly, suggesting limited exposure of the CS-1 sequence in Fn. To test the availability of CS-1 in plasma Fn, an antibody was raised to the synthetic peptide CS-1. The CS-1 sequence was found to be minimally exposed in plasma Fn; and immobilization of Fn, a model of matrix deposition, caused only a modest increase in its exposure. Digestion of Fn with selected proteases, however, induced substantial expression of the CS-1 sequence. The acid protease cathepsin D generated fragments of 31–33.5 kDa from the COOH-terminal heparin-binding domain of Fn which possessed high immunoreactivity with anti-CS-1. Digestion of Fn with cathepsin B also resulted in the exposure of CS-1 sequence in a 140 kDa fragment. Although the digestion of Fn with neutral proteases (neutrophil elastase, cathepsin G, chymotrypsin, trypsin) generated fragments from the COOH-terminal heparin-binding domain of similar molecular weight as with cathepsin D, the exposure of CS-1 did not occur. Exposure of the CS-1 region by the cathepsins was supported by cell adhesion experiments; digestion of Fn with cathepsins D and B transformed inert plasma Fn to an effective inhibitor of adhesion of lymphoblastoid B and T cells (Ramos, Jurkat, Molt-4) to an immobilized CS-1 conjugate. These results suggest that exposure of the CS-1 sequence in plasma Fn by proteolysis with cathepsins D and B, enzymes implicated in several pathological processes, may serve a regulatory function in cell adhesion. The adhesive function of the CS-1 region in intact Fn appears to be suppressed by the native conformation of the molecule.

Fibronectin (Fn)<sup>1</sup> profoundly influences a wide range of cellular properties such as growth, differentiation, migration, and apoptosis (Hynes, 1990; Ruoslahti & Reed, 1994). These effects depend upon the interaction of Fn with cellular receptors, including several members of the integrin family. Two distinct regions of Fn are primarily responsible for the recognition of Fn by integrins. The central cell-binding domain contains the RGDS motif in the 10th type III repeat, a second adhesive motif in the 9th type III repeat, and flanking synergistic sites (Yamada, 1991; Aota et al., 1994; Bowditch et al., 1994). The RGD motif is recognized by several integrins including the broadly distributed  $\alpha_5\beta_1$  integrin (Ruoslahti, 1991). Recently, we have shown that exposure of the RGD-containing 10th type III repeat is highly dependent upon Fn conformation (Ugarova et al., 1995). A

number of effectors of Fn conformation modulate the availability of this region at the hydrated surface of Fn, thereby influencing the adhesive properties of Fn.

The second adhesive region is contained in the more carboxyl-terminal aspects of Fn and includes the COOH-terminal Hep II domain and the adjacent 120 amino acid stretch, the type III connecting segment (IIICS). The presence of IIICS in Fn is regulated by alternative mRNA splicing. Only one of two subunits of the plasma Fn dimer (subunit A) contains a IIICS, whereas Fn within extracellular matrices may contain one copy of this segment per subunit (Schwarzbauer et al., 1985; Paul et al., 1986; Hershberger & Culp, 1990). Moreover, several segments within the IIICS region can undergo independent alternative splicing (Kornblihtt et al., 1985). Two of these segments within the 120 amino acid IIICS region support cell adhesion: CS-1 (residues 1–25) and CS-5 (residues 90–109) (Humphries et al., 1986, 1987). The minimal active sites within CS-1 and CS-5 have been identified as LDV and REDV, respectively (Komoriya et al., 1991; Humphries et al., 1986).

Integrin  $\alpha_4\beta_1$  is a receptor for sequences within both the Hep II domain and the IIICS segment (Wayner et al., 1989; Guan & Hynes, 1990; Mould et al., 1991), including the CS-1 site. A variety of cells, including B and T lymphocytes and their transformed counterparts, monocytes, melanoma cells, natural killer cells, some fibroblasts, and neural crest cells, bear  $\alpha_4\beta_1$  and adhere the CS-1 peptide conjugates (Wayner et al., 1989; Ferreira et al., 1990; Humphries et al., 1986,

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<sup>\*</sup> To whom correspondence should be addressed at the Cleveland Clinic Foundation, 9500 Euclid Ave., FF20, Cleveland, OH 44195. Telephone: (216) 445-8213. FAX: (216) 445-8204. E-mail: ugarovt@cesmtp.ccf.org.

<sup>‡</sup> Cleveland Clinic Foundation.

<sup>§</sup> Cedars-Sinai Medical Center.

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<sup>1</sup> Abbreviations: Fn, human plasma fibronectin; IIICS, type III connecting segment; Hep II, heparin binding domain in the carboxyl-terminal portion of Fn; PBS, phosphate-buffered saline (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.3); BSA, bovine serum albumin; HSA, human serum albumin; OVA, ovalbumin; CS-1-OVA, CS-1.3 peptide conjugated to ovalbumin; HBSS, Hank's balanced salt solution; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay;  $\mu$ , ionic strength; t-PA, tissue plasminogen activator.

1988; Gismondi et al., 1991). While lymphocytes and monocytes adhere to immobilized Fn, these  $\alpha_4\beta_1$ -bearing cells interact poorly with soluble plasma Fn (Hosein & Bianco, 1985; Molnar et al., 1987). Furthermore, soluble Fn does not inhibit the attachment of T and B lymphocytes to Fn or CS-1-containing fragments (Garcia-Pardo & Ferreira, 1990; Garcia-Pardo et al., 1989, 1990; Ferreira et al., 1990). These observations suggest that, like the RGD motif in the central cell-binding domain, the availability of the CS-1 in the carboxyl-terminal cell-binding domain may also be regulated. To explore this possibility, an antibody to the CS 1–25 sequence has been used to probe the exposure of this region in Fn. Based upon our results, we propose that the adhesive functions of the CS-1 region are controlled not only by alternative splicing but also by the conformation of Fn.

## EXPERIMENTAL PROCEDURES

**Materials.** Cathepsin D (bovine and human) from spleen, cathepsin B from bovine spleen,  $\alpha$ -chymotrypsin, trypsin, protease type X (thermolysin), cathepsin G and elastase from neutrophils, pepsin, gelatin, BSA, HSA, pepstatin A, and iodoacetamide were from Sigma Chemical Co., St. Louis, MO. Gelatin–Sephacrose and heparin–Sephacrose were from Pharmacia LKB Biotechnology (Piscataway, NJ). T-PA was from Genentech, Inc. (South San Francisco, CA), and plasmin from ERL, Inc. (South Bend, IN). Reagents for immunochemical analyses and the 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) cross-linker were purchased from Pierce Chemical Co., Rockford, IL. Polystyrene microtiter plates for immunoassays were from Titertek (ICN Pharmaceuticals, Inc., Costa Mesa, CA), and 48-well plates for adhesion assays were from Costar (Cambridge, MA).

**Cell Cultures.** The Ramos B lymphoblastoid cell line and the Molt-4 and Jurkat T lymphoblastoid cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cells were cultured in RPMI-1640 medium supplemented with 7.5% heat-inactivated fetal bovine serum, 100  $\mu$ g/mL penicillin G, 0.1 mg/mL streptomycin, 2 mM L-glutamine, and 25 mM HEPES, pH 7.2.

**Isolation of Human Plasma Fn and Other Proteins.** Fn was isolated from fresh human blood, anticoagulated with acid/citrate/dextrose, by affinity chromatography on gelatin–Sephacrose using 1 M arginine for elution (Vuento & Vahery, 1979). After extensive dialysis against PBS, Fn was stored in aliquots at  $-70^\circ\text{C}$ . Upon thawing, Fn was kept at  $4^\circ\text{C}$  and used within 3 days. Fibrinogen (Fg) was purified from fresh human plasma by differential ethanol precipitation (Doolittle et al., 1967). von Willebrand Factor (vWF) was purified by cryoprecipitation and gel filtration as previously described (Agbanyo et al., 1993).

**Fragmentation of Fn.** Fn (0.3 mg/mL) was digested at an enzyme to substrate ratio of 1:100 (w/w) at  $22^\circ\text{C}$ . Digestion with chymotrypsin, trypsin, plasmin, t-PA, thrombin, neutrophil cathepsin G, and neutrophil elastase was performed in PBS, and proteolysis was terminated by addition of phenylmethanesulfonyl fluoride (final concentration, 1 mM). Thermolysin digestion of Fn was conducted in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 2.5 mM  $\text{CaCl}_2$ , and proteolysis was terminated by addition of EDTA (final concentration, 5 mM). Cathepsin D and pepsin digestion was performed in 0.1 M citrate buffer,

pH 3.6, and the reactions were stopped by addition of a 10-fold excess of pepstatin A. Cathepsin B digestion was performed in 0.1 M phosphate buffer with 10 mM cysteine, and 10 mM iodoacetamide was the inhibitor. The various digests were diluted to 3  $\mu$ g/mL with PBS, coated onto the wells of microtiter wells, and tested in ELISA, or diluted in sample buffer for analysis by SDS–PAGE and Western blotting.

To investigate the effect of pH on the expression of the CS-1 sequence, incubations of Fn with cathepsin D were performed in 0.2 M formate buffer, pH 3.3–5.1, at an enzyme to substrate ratio of 1:100. At selected times, aliquots of the digests were inhibited with pepstatin A and tested in ELISA with anti-CS-1.

**Purification of Fn Fragments.** Fn (1 mg/mL) was digested with cathepsin D at an enzyme to substrate ratio of 1:100 in 0.1 M citrate buffer, pH 3.6, for 20 h at  $22^\circ\text{C}$ . Digestion was terminated with pepstatin A, and the sample was adjusted to pH 7.4. The digest was applied to a heparin–agarose column (bed volume 7 mL), equilibrated with PBS. The bound fragments were eluted sequentially with PBS containing 0.1 M NaCl ( $\mu$  0.25) followed by PBS containing 0.35 M NaCl ( $\mu$  0.5). Fractions eluting with  $\mu$  0.5 were collected as pools I and II, with pool II containing the 33.5 and 31 kDa fragments.

**Peptides.** Peptide (KY)D<sup>1</sup>ELPQLVTLPHPNLHGPEILDVPS<sup>25</sup> (CS-1.3), spanning the entire alternatively spliced IIICS 1–25 region of Fn, was synthesized by solid phase technology using an Applied Biosystems Model 430 peptide synthesizer (Foster City, CA). Smaller CS-1 peptides from within the CS-1.3 segment also were synthesized including (KY)N(13–25), (KYGG)L(14–25), D(1–20), V(7–20), and E(18–25). (Amino acids in parentheses are not naturally occurring; the amino acid without parentheses is the naturally occurring N-terminus, and the numbers indicate the residues within the CS-1.3 sequence.) In addition, peptides CGGT-DVNGDGRHDL(B<sub>12</sub>) from the  $\alpha$  subunit of  $\alpha_{IIb}\beta_3$  and KYMEILRGDFSSC (Fg-1) and GWTVFQKRLDGSV (Fg-2) from the  $\alpha$  and  $\gamma$  chains of fibrinogen, respectively, were used as control peptides to test the specificity of anti-CS-1 antibody. Peptides were purified by HPLC on a preparative C18 $\mu$  Bondapak column with a linear gradient of 0–100% acetonitrile in 0.1% TFA. Before use, the CS-1.3 peptide was additionally purified by HPLC using a 5–90% gradient of acetonitrile. CS-1.3 was conjugated to ovalbumin using EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide) as a cross-linker (Iida et al., 1992). The CS-1-OVA preparations were tested in ELISA to verify the preservation of CS-1 epitopes. The conjugates were stored at  $-70^\circ\text{C}$  until use.

**Antibodies.** To produce anti-CS-1, CS-1.3 was coupled to keyhole limpet hemocyanin with glutaraldehyde (Kagan & Glick, 1978), and the conjugate was used as immunogen to raise antibodies in rabbits. Antisera titer was monitored after each round of immunization by ELISA using Fn- and CS-1.3-coated microtiter plates. Bleeds containing high-titer anti-peptide antibody were precipitated with 35% ammonium sulfate, and the pellet was dissolved and extensively dialyzed against PBS.

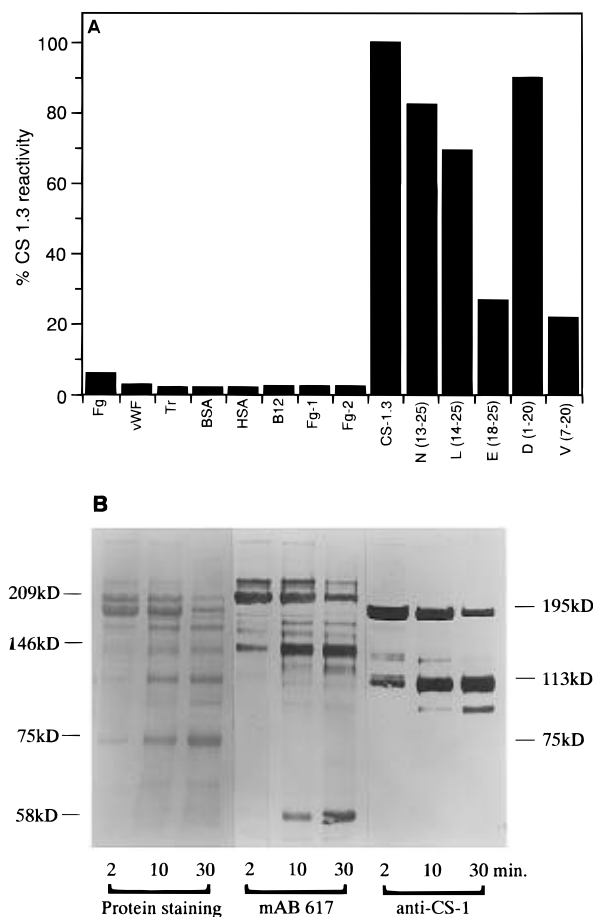
MAb 617 and mAb 618 were produced by immunizing mice with fibronectin secreted by human embryonic fibroblasts. MAb 617 recognizes the 31 kDa COOH-terminal fibrin-binding fragment (Ljubimov et al., 1995). MAb 618 recognizes the proteolytic fragment of Fn containing 2nd and

3rd type III repeats of Fn (unpublished data). MAb III-10 and mAb III-4, which recognize the 10th and 4th type III repeats in the central cell-binding domain of Fn, have been previously described (Ugarova et al., 1995). MAb 9D2, which recognizes an epitope in the 1st type III repeat (Chernousov et al., 1991), was kindly provided by Dr. Deane Mosher (University of Wisconsin). MABs IST-1 and IST-2 (Sekiguchi et al., 1985; Castellani et al., 1986) were purchased as culture media from Accurate Chemical Scientific Corp. (Westbury, NY). MAb IST-1 recognizes an epitope within the first 158 amino acids of the Hep II domain, and mAb IST-2 recognizes an epitope within the remaining 112 amino acids. MAb P4C2 to the integrin  $\alpha_4$  subunit was purchased from Gibco BRL (Gaithersburg, MD).

**Cell Adhesion Assays.** Tissue culture plates (48-well) were coated with 20  $\mu\text{g/mL}$  CS-1-OVA conjugate (0.2 mL/well) for 3 h at 37 °C. After being washed, the plates were postcoated with 10 mg/mL heat-inactivated BSA for 1 h at 22 °C. Cultured Jurkat, Molt-4, and Ramos cells were washed twice in HBSS and radiolabeled with  $\text{Na}_2^{51}\text{CrO}_4$  (0.5 mCi/mL) for 2 h and washed twice by centrifugation. The cells were resuspended at  $1 \times 10^6/\text{mL}$  in HBSS supplemented with 1 mg/mL BSA and 1 mM  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Mn}^{2+}$ , and mixed with selected concentrations of competitors, which had been dialyzed against HBSS prior to experiment. After 20 min at 22 °C, the cells were added to each well ( $10^5/0.1$  mL) for 1 h at 37 °C in a 5%  $\text{CO}_2$  humidified atmosphere. Nonadherent cells were removed by three washes with PBS. The adherent cells were solubilized with 2% SDS, and bound  $^{51}\text{Cr}$  was quantitated in a  $\beta$  counter. For inhibition experiments with anti-CS-1, coated wells were incubated for 1 h at 22 °C with 0.1 mL of selected dilutions of anti-CS-1 or normal rabbit serum.

**ELISA and Competitive Inhibition ELISA.** For solid-phase immunoassays, polystyrene microtiter plates were coated with Fn or its proteolytic digests (100  $\mu\text{L}/\text{well}$  at 3  $\mu\text{g/mL}$ ) for 16 h at 4 °C. The wells were postcoated with 3% gelatin in PBS for 1 h at 22 °C. The plates then were washed with PBS + 0.05% Tween 20 and incubated with anti-CS-1, diluted 1:100–1:200 in PBS + 0.05% Tween 20. After 90 min at 22 °C, the plates were washed, and goat anti-rabbit IgG conjugated with alkaline phosphatase was added. Antibody binding was detected by reaction with p-nitrophenyl phosphate. The signal was measured at 405 nm. For the competitive ELISA format, different amounts of the test competitor (Fn, CS-1.3 peptide) were mixed with anti-CS-1, and 100  $\mu\text{L}$  aliquots of the mixtures were added to the wells coated with CS-1.3 (1.67  $\mu\text{M}$ ) or the selected proteolytic digest of Fn (3  $\mu\text{g/mL}$ ).

**Analytical Procedures.** SDS-PAGE was performed under nonreducing conditions on 7% and 11% polyacrylamide gels in the Laemmli buffer system (Laemmli, 1970). For Western blots, the proteins were transferred onto Immobilon P membranes, which were then incubated with anti-CS-1 (1:100), mAb 617 cell culture fluid (1:10), mAb III-10, III-4 (5  $\mu\text{g/mL}$ ), mAb 618 (10  $\mu\text{g/mL}$ ), mAb IST-1, and IST-2 at dilution 1:6, or mAb 9D2 at a 1:1000 dilution of ascites. Bound antibodies were detected by reaction with a peroxidase-conjugated secondary antibody, followed by the addition of 4-chloro-1-naphthol.



**FIGURE 1:** Specificity of anti-CS-1. (A) ELISA of the immunoreactivity of anti-CS-1 with CS-1 peptides or unrelated peptides and proteins. Wells of microtiter plates were coated with 10 nM of the peptides [CS-1.3, N(13–25), L(14–25), D(1–20), E(18–25), B12, Fg-1, Fg-2] and 3  $\mu\text{g/mL}$  of each protein [Fg, vWF, transferrin (Tr), HSA, BSA]. Wells were postcoated with 3% gelatin and incubated with anti-CS-1 (1:150 dilution). The values are expressed as a percent of the absorbance obtained with CS-1.3 which usually was in the range of 3.0–3.5 at 405 nm. (B) Western blot of tryptic fragments of Fn using anti-CS-1 and mAb 617. Digests (2, 10, and 30 min) of Fn were electrophoresed on 7% SDS-PAGE under reducing conditions. Proteins were transferred to Immobilon-P membranes and stained for protein with Coomassie blue (left), probed with mAb 617 at 1:10 (middle) or anti-CS-1 at 1:100 (right). The molecular masses of Fn fragments originating from the A subunit are shown on the right and those from the B subunit on the left. (Fragments below 50 kDa were run off the gel.)

## RESULTS

**Specificity of Anti-CS-1.** CS-1.3, corresponding to first 25  $\text{NH}_2$ -terminal amino acids of the IIICS segment, was conjugated to keyhole limpet hemocyanin and used to elicit an antiserum in rabbits. Several lines of evidence demonstrate that the resultant anti-CS-1 recognized epitopes within the CS-1 sequence of IIICS. First, the antibody reacted with CS-1.3 and several shorter CS-1 peptides (13–25, 14–25, and 18–25) in ELISA (Figure 1A). Since these shorter peptides were truncated at the  $\text{NH}_2$ -terminus, at least a portion of the anti-CS-1 response was directed toward the IIICS region recognized by the  $\alpha_4$  integrin. The antibody also recognized the amino-terminal aspects of CS-1 as indicated by its reaction with D(1–20) and V(7–20) and with the 29K thermolysin fragment (see Table 1 below) which contains only the first five amino acids of CS-1. Boiling of CS-1.3 for 15 min prior to immobilization did

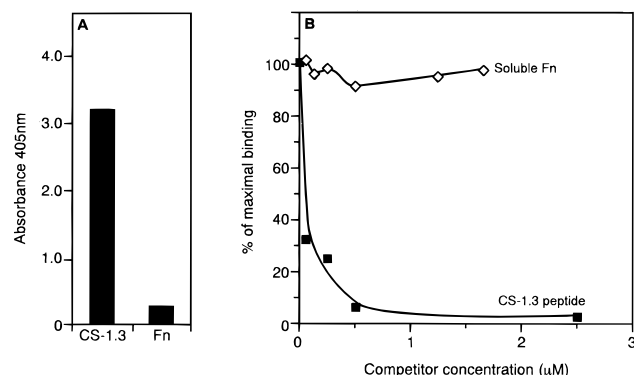


FIGURE 2: Lack of immunoreactivity of plasma Fn with anti-CS-1. (A) Immunoreactivity of CS-1.3 peptide and plasma Fn in ELISA. CS-1.3 and Fn were coated at 10 nM, and the reaction was developed as in Figure 1. (B) Competitive ELISA with CS-1.3 and Fn as inhibitors of anti-CS-1 binding to immobilized CS-1.3. The wells of a microtiter plate were coated with CS-1.3 (1.7  $\mu$ M) overnight at 4 °C. CS-1.3 or Fn was mixed with antibody (1:150), incubated for 15 min, and added to the wells. Data are expressed as the percent of maximal binding of antibody in the absence of inhibitor. The molecular mass of Fn was taken as 250 kDa assuming one CS-1 sequence per Fn heterodimer.

not influence its reaction with the antibody. Thus, anti-CS-1 reports on several linear epitopes within the CS-1 region. Second, anti-CS-1 did not react with several unrelated proteins and peptides (Figure 1A). The signal obtained with CS-1 peptides was at least 10 times greater than that with the unrelated peptides. Third, anti-CS-1 reacted specifically with the A but not the B subunit of Fn (Figure 1B). In the plasma Fn dimer, the A and B subunits differ in size due to the presence (A subunit) or absence (B subunit) of the IIICS region, and digestion with selected proteases results in subunit cleavage products differing in size (Hayashi & Yamada, 1983; Borsi et al., 1986). Brief digestion of Fn with trypsin produces B chain fragments of 209, 146, and 58 kDa, whereas the IIICS region in the A chain introduces an additional trypsin-sensitive site in the middle part of the IIICS segment such that fragments of 195, 113, and 38 kDa are generated (Hayashi & Yamada, 1983). In Western blotting analyses, anti-CS-1 interacted only with the latter set of fragments originating from the A chain (195, 113, and 38 kDa). As a control, mAb 617, which recognizes the COOH-terminal fibrin II binding domain in both Fn chains, exhibited the appropriate pattern of reactivity including interaction with the B chain-derived 209, 146, and 58 kDa fragments.

**Anti-CS-1 Reacts Poorly with Intact Fn.** The immunoreactivity of the CS-1 antibody with immobilized Fn was significantly lower than its reactivity with the CS-1.3 peptide (Figure 2A). The slight reactivity of the anti-CS-1 with immobilized Fn was specific as it was blocked by soluble CS-1.3 (not shown).

Consistent with the poor exposure of the CS-1 region in native Fn, soluble Fn failed to inhibit the binding of anti-CS-1 to immobilized CS-1.3 (Figure 2B). Soluble CS-1.3 inhibited the binding of anti-CS-1 to immobilized peptide in a dose-dependent manner; 50% inhibition of antibody binding was achieved at a CS-1.3 concentration of  $\sim 0.2 \mu$ M. On the other hand, soluble Fn was noninhibitory at a 10-fold higher concentration. Previously, we have shown that interaction of Fn with certain ligands (glycosaminoglycans, gangliosides, or gelatin) and exposure to high ionic strength

increase the expression of epitopes within the 10th type III repeat (Ugarova et al., 1995). Under the same conditions, these effectors of Fn conformation did not enhance exposure of the CS-1 region; i.e., soluble Fn still failed to compete in the ELISA assay in the presence of these effectors (not shown). Thus, the epitopes recognized by anti-CS-1 are not available on the Fn surface and are not readily exposed. Some exposure does occur when Fn is adsorbed onto the surface of a microtiter plate.

**Exposure of CS-1 Epitopes by Proteolysis.** Digestion of Fn with selected proteases resulted in exposure of the CS-1 sequence. In these experiments, proteolysis was conducted at an enzyme:substrate ratio of 1:100 at 22 °C, and reactions were terminated at selected time points with appropriate inhibitors for each enzyme. The digests were then coated onto microtiter plates and reacted with anti-CS-1. As shown in Figure 3A, digestion of Fn with cathepsin D resulted in a progressive increase in immunoreactivity; the signal reached maximum at 24 h and then declined slightly with additional incubation. The pH optimum of the reaction was 3.9 as judged by accumulation of fragments reactive with anti-CS-1, although digestion at pH 5.1 also generated fragments with exposed CS-1 sequence. Digestion of Fn with cathepsin B, a thiol-dependent protease, was also accompanied by a progressive increase in the immunoreactivity of the digest (Figure 3A). In contrast, digestion of Fn with other proteases failed to expose the CS-1 sequence. Falling into this category were neutrophil elastase and trypsin (Figure 3A). Chymotrypsin caused a rapid but only very modest exposure of the CS-1 region.

The selective exposure of CS-1 in the catheptic digests was confirmed in competitive ELISA analyses (Figure 3B). The 20 h cathepsin D and cathepsin B (not shown) digests effectively inhibited the binding of anti-CS-1 antibody to the wells coated with 20 h catheptic D digest, whereas the 4 h trypsin and 2 h chymotrypsin digests produced limited inhibition. Moreover, CS-1.3 peptide efficiently inhibited the binding of antibody to the immobilized 20 h cathepsin D digest.

Table 1 lists the proteases tested for their effects on CS-1 exposure. In each case, a time course of digestion was performed over 72 h, and the digests were tested by ELISA as in Figure 3A and by Western blotting to identify fragments containing the CS-1 region. Beside cathepsins D and B, thermolysin and pepsin digests contained fragments which expressed CS-1, i.e., were reactive with anti-CS-1 when immobilized onto microtiter plates. All other tested proteases failed to expose CS-1 sequence. Among these, plasmin, thrombin, and t-PA generated high molecular mass fragments (150–200 kDa) reactive with anti-CS-1 in immunoblots. Thus, these CS-1-containing fragments were similar in size to those within the cathepsin B digest, but their CS-1 epitopes were not exposed. Digestion with trypsin, chymotrypsin, neutrophil elastase, and cathepsin G produced smaller CS-1-containing fragments (28–38 kDa), similar to those present in the cathepsin D digest, as determined by Western blotting. Nevertheless, in the ELISA format, anti-CS-1 only reacted with the cathepsin D, thermolysin, and pepsin fragments (Table 1). Three of the four proteases which induced CS-1 exposure have acidic pH optima (cathepsins D and B, and pepsin); however, incubation of Fn in the pH range of 3.5–5.1 for 72 h caused no exposure of the CS-1 sequence.

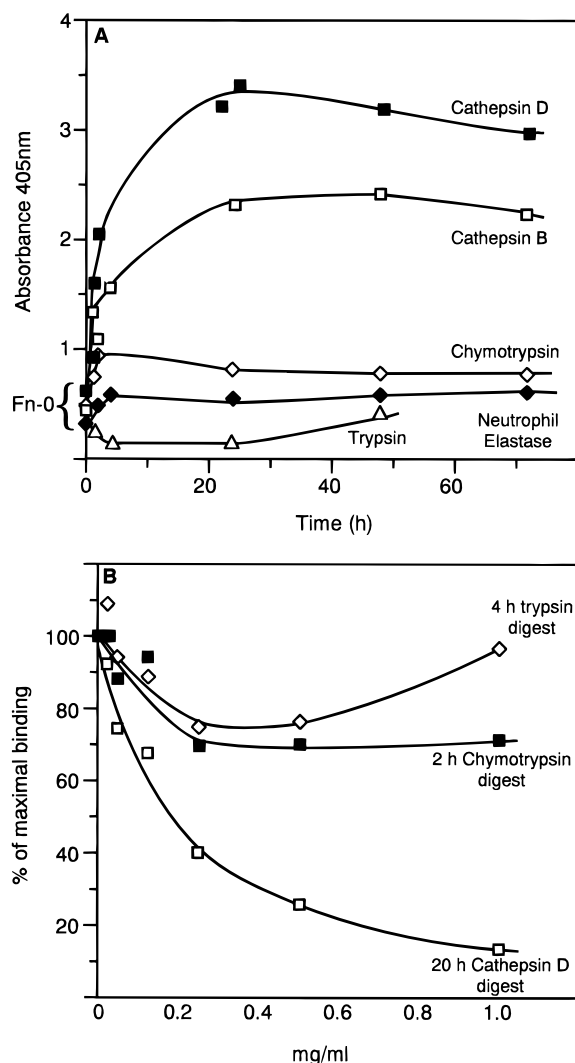


FIGURE 3: Exposure of CS-1 by proteolysis. (A) Fn, 0.3 mg/mL, was digested with cathepsin D (in 0.1 M citrate buffer, pH 3.6) or cathepsin B (in 0.1 M phosphate buffer at pH 6.4 in the presence of 10 mM cysteine) at an enzyme:substrate ratio of 1:100, at 22 °C. Digestions with trypsin, chymotrypsin, and neutrophil elastase were performed as described under Experimental Procedures. At selected times, the digestions were terminated with appropriate inhibitors. Intact Fn or the digests were coated onto the wells of microtiter wells at 3  $\mu$ g/mL and tested in the ELISA format. Fn-0 indicates the range of signal obtained with intact Fn. (B) Competitive ELISA with cathepsin D (20 h), chymotrypsin (2 h), and trypsin (4 h) digests. The digests were mixed with anti-CS-1, incubated for 50 min at 22 °C, and then added to the wells of microtiter plates coated with the 20 h cathepsin D digest as ligand (3  $\mu$ g/mL used to coat the wells).

**Nature of the Fn Fragments with Exposed CS-1 Epitopes.** Experiments were performed to identify the immunoreactive fragments in the cathepsin D and B digests. SDS-PAGE and Western blots of cathepsin D digests are shown in Figure 4A and Figure 4B, respectively. The appearance of immunoreactivity in ELISA (Figure 3A) coincided with the generation of a 36 kDa fragment. Maximal immunoreactivity occurred with the transformation of the 36 kDa fragment to a 33.5 kDa fragment, and the partial decrease in the CS-1 expression was associated with conversion of the 33.5 kDa fragment to a 31 kDa derivative. A similar pattern of degradation and exposure of the CS-1 epitope(s) was obtained with both bovine and human cathepsin D. The group of CS-1-containing fragments in the 31–36 kDa range originated from the Hep II domain of plasma Fn as they also

Table 1: Exposure of CS-1 Sequence by Different Proteases

enzyme	pH <sup>a</sup>	exposure of IIICS-1 <sup>b</sup>	CS-1-containing fragment <sup>c</sup>
cathepsin D (bovine, human)	3.5–5.1	6.0	36→33.5→31 kDa
cathepsin B	5.4–6.4, 10 mM cysteine	5.4	140 kDa
pepsin	3.6, 10 mM cysteine	3.3	35 kDa
thermolysin	7.5 (Ca <sup>2+</sup> )	5.3	155→95→60→29 kDa
chymotrypsin	7.2	1.6	150→32→29 kDa
trypsin	7.2	1.0	195→113→38 kDa
plasmin	7.2	1.0	~180K–190 kDa
thrombin	7.2	1.0	~150 kDa
t-PA	7.2	1.0	~190–200 kDa
cathepsin G (from neutrophils)	7.2	1.0	135→32, 29 kDa
neutrophil elastase	7.2	1.0	150→31, 28 kDa

<sup>a</sup> Conditions of digestion. <sup>b</sup> Immunoreactivity of digests tested by ELISA with anti-CS-1 antibody. Numbers represent the ratio of maximal immunoreactivity of the tested digest versus the immunoreactivity of intact Fn. <sup>c</sup> Fragments stained with anti-CS-1 antibody in Western blot analysis. Arrows show sequential transformation of fragments with time.

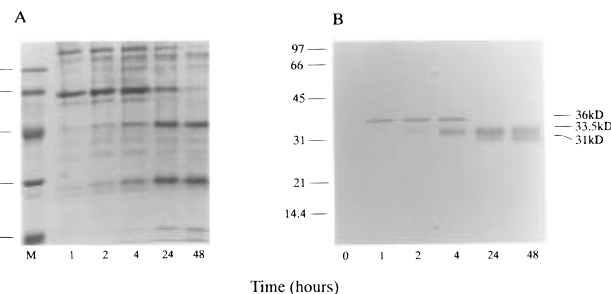


FIGURE 4: SDS-PAGE (A) and Western blot analysis (B) of cathepsin D digests. The samples were separated on an 11% gel under nonreducing conditions. Proteins were transferred to Immobilon-P membranes which were probed with anti-CS-1 at a 1:100 dilution. The positions of molecular mass markers (M) are shown on the left, and the estimated molecular masses of the fragments reactive with anti-CS-1 are shown on the right.

reacted in Western blots with mAbs IST-1 and IST-2, specific for this domain (not shown).

The regulation of CS-1 exposure by cathepsin D was further explored. A 4 h tryptic digest contained a 38 kDa fragment which reacted well with anti-CS-1 in Western blots (Figure 5A) but not in ELISA (Figure 5B). When this tryptic digest was exposed to cathepsin D, the 38 kDa fragment was converted to a 33 kDa fragment, similar to the 33.5 kDa fragment generated by direct degradation of Fn with cathepsin D (Figure 5A). Concomitantly, the immunoreactivity of the digest in ELISA was substantially augmented (Figure 5B), suggesting that the CS-1 sequence was still cryptic in the 38 kDa fragment but was exposed by further digestion with cathepsin D.

To verify that the increased exposure of the CS-1 region in cathepsin D digests of Fn was associated with the formation of 33.5 and 31 kDa fragments, these fragments were purified as the mixture from a 20 h cathepsin D digest by affinity chromatography on heparin–agarose. When an ELISA was performed with Fn or a 33.5 + 31 kDa mixture coated onto a microtiter plate at equal molar concentrations, Fn was substantially less reactive (Figure 6). This difference in reactivity was not due to differences in coating efficiency as these coated wells were equally reactive with mAbs IST-1 and IST-2. The specificity of the reaction between anti-CS-1

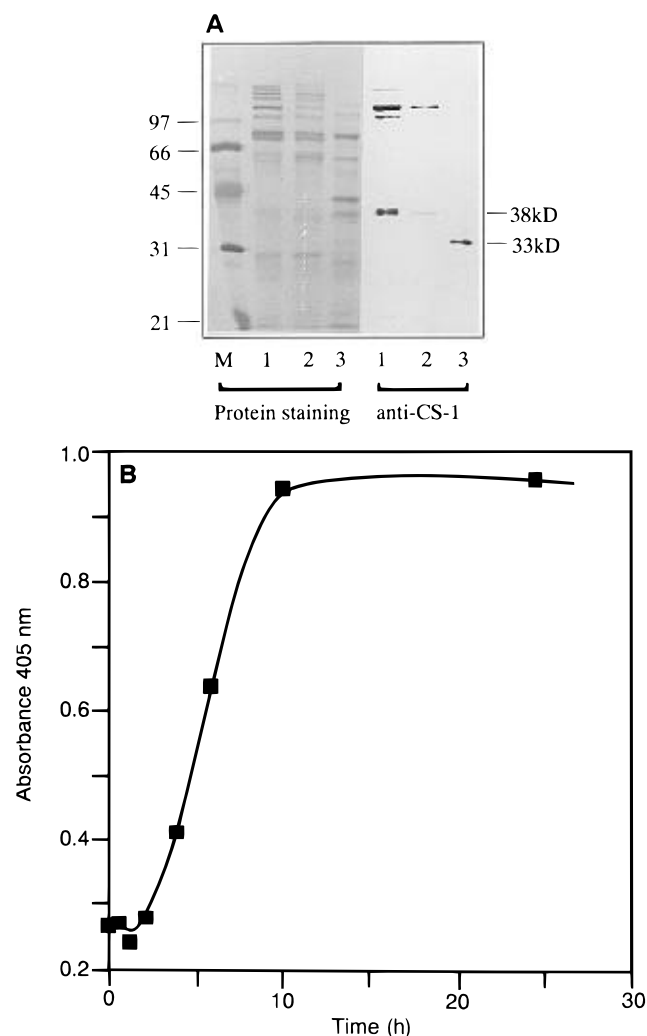


FIGURE 5: Augmentation of CS-1 immunoreactivity during the transformation of the 38 kDa tryptic fragment to the 33 kDa cathepsin D fragment. A 4 h tryptic digest of Fn (0.3 mg/mL) was acidified to pH 3.5–4.0 and digested with cathepsin D at an enzyme:substrate ratio of 1:100 at 22 °C. Digestion was stopped at selected time intervals with pepstatin A. (A) SDS-PAGE and Western blot analyses with anti-CS-1 of the initial 4 h tryptic digest of Fn (lane 1), the same digest acidified to pH 4.0 and incubated for 20 h (lane 2), and the same digest after 20 h of incubation with cathepsin D (lane 3). (B) The initial 4 h tryptic digest (zero time) and its catheptic D digests were diluted to 3  $\mu$ g/mL with PBS, coated onto microtiter wells, and tested in an ELISA.

and 31 + 33.5 kDa fragments was confirmed as the CS-1 peptide completely inhibited the binding of anti-CS-1 to the immobilized fragments (not shown).

Incubation of Fn with cathepsin B resulted in the limited degradation of the molecule and the generation of fragments of 140, 60, 27, and 25 kDa. Only the 140 kDa fragment reacted with anti-CS-1 in Western blotting analysis (not shown). In Western blots, this 140 kDa fragment reacted with mAb III-10, mAb III-4, and mAb 618 directed against 10th, 4th, 2nd, and 3rd type III repeats, respectively. The fragment also reacted with mAb IST-1 and IST-2, recognizing the epitopes in the Hep II domain, but failed to react with mAbs 617 and 9D2, which recognize the COOH-terminal portion of Fn and the 1st type III repeat, respectively. Therefore, based on this immunoreactivity pattern and its estimated molecular mass, the 140 kDa catheptic B fragment must span the 2nd to the 14th type III repeats plus the adjacent IIICS domain.

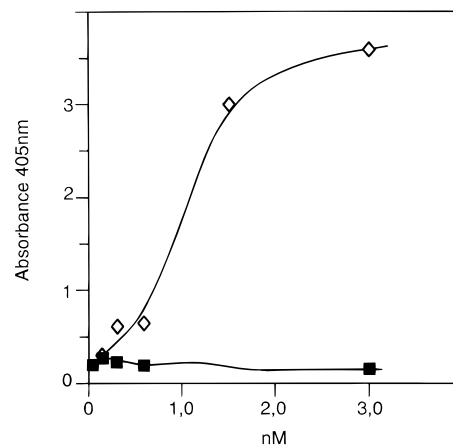


FIGURE 6: Immunoreactivity of the 33.5 + 31 kDa fragments of Fn produced by cathepsin D. Intact Fn (■) and 31 + 33.5 kDa catheptic D fragments (◇) were immobilized onto microtiter plates at the same molar concentration, assuming that the absorbance at 280 nm of a 1 mg/mL solution of the 31 + 33.5 kDa fragments is 1.0 (Homandberg & Erickson, 1986).

*Effect of Fn and Its Proteolytic Digests on Adhesion of Lymphoblastoid Cells to CS-1 Peptide.* We sought to determine if exposure of the CS-1 sequence within Fn and its proteolytic fragments, as reported by anti-CS-1 reactivity, correlated with changes in cell adhesive functions. Toward this end, adhesion assays were performed using Jurkat and Molt-4 T lymphoblastoid cell lines and the Ramos B lymphoblastoid cell line. Jurkat and Molt-4 cells express both  $\alpha_5\beta_1$  and  $\alpha_4\beta_1$  as Fn receptors, whereas Ramos cells express  $\alpha_4\beta_1$  as its only Fn receptor (Garcia-Pardo & Ferreira, 1990; Hemler, 1990; Sánchez-Aparicio et al., 1994). The adhesion of  $^{51}\text{Cr}$ -labeled cells to wells coated with CS-1.3 peptide conjugated to ovalbumin was measured. The  $\alpha_4$ -specific mAb P4C2 (Wayner et al., 1989) completely inhibited the adhesion of all three cell types to CS-1-OVA. Soluble CS-1.3 also inhibited adhesion; the  $\text{IC}_{50}$  was 15  $\mu\text{M}$  for Jurkat and 10  $\mu\text{M}$  for Ramos cells. Anti-CS-1 efficiently inhibited cell adhesion to CS-1-OVA (80% inhibition at dilution 1:50), while nonimmune rabbit serum did not affect cell adhesion. The effect of intact Fn and the 20 h cathepsin D and cathepsin B digests on the adhesion of the lymphoid cells was compared. The data shown in Figure 7A were obtained with Ramos cells, but similar results were observed with Jurkat and Molt-4 cells. Cathepsin D and cathepsin B digests were potent inhibitors of the adhesion of the lymphoblastoid cell lines to the CS-1.3 conjugate. The  $\text{IC}_{50}$  values for the digests were 0.5 mg/mL for the cathepsin D digest and 0.365 mg/mL for the cathepsin B digest. In contrast, the maximal testable concentration of soluble Fn (2.0 mg/mL) inhibited adhesion by only 20%. The effects of other proteolytic digests of Fn on cell adhesion to the CS-1.3 conjugate are shown in Figure 7B. The digests generated by the neutrophil proteases, elastase and cathepsin G, were similar to intact Fn and failed to significantly inhibit cell adhesion. On the other hand, a tryptic and chymotryptic digest (not shown) was inhibitory, with a potency similar to that of the catheptic digests. Thus, the capacity of proteolytic digests to inhibit cell adhesion paralleled the reactivity with anti-CS-1 in certain cases (cathepsins B and D) but not in others (trypsin and chymotrypsin).

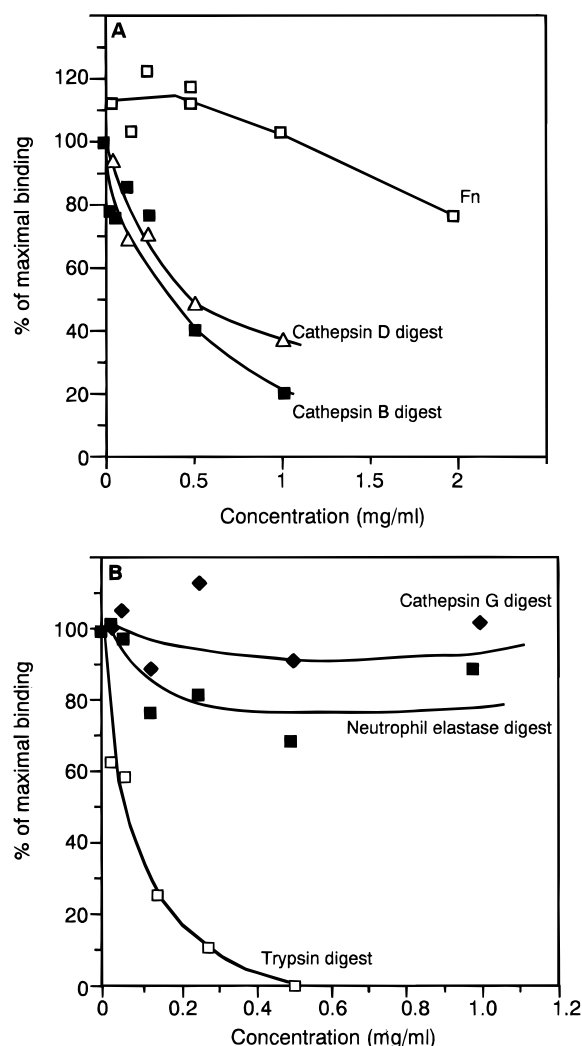


FIGURE 7: Effect of Fn and its proteolytic digests on the adhesion of Ramos cells to the CS-1.3-ovalbumin conjugates.  $^{51}\text{Cr}$ -labeled cells were preincubated with increasing concentrations of (A) intact Fn or its 20 h cathepsin D and 20 h cathepsin B digests or (B) its 4 h trypsin, 24 h cathepsin G, and 24 h neutrophil elastase digests for 20 min at 22 °C, and then added to the CS-1-ovalbumin-coated wells. Plates were incubated for 1 h at 37 °C. At the end of this time, the nonadherent cells were removed by washing, and the adherent cells were solubilized in SDS and bound  $^{51}\text{Cr}$  was quantitated. Values are expressed as the percentage of adherent cells in the absence of Fn or its digests.

## DISCUSSION

In this study, we have demonstrated that the exposure of the CS-1 sequence (residues 1–25) of IIICS is tightly regulated within the conformation of Fn and its proteolytic fragments. The sequence is not available to antibody in soluble plasma Fn, and conditions and/or effector molecules (high ionic strength, glycosaminoglycans, gangliosides), which induce a molecular expansion of Fn (Lai et al., 1993; Ankel et al., 1986) and increase exposure of the 9th and 10th type III repeats (Ugarova et al., 1995), fail to expose the CS-1 region. This region does become slightly exposed by the unfolding of Fn associated with surface immobilization. In contrast, degradation by selected proteases results in extensive epitope exposure in the immobilized or soluble fragments.

Several observations are consistent with the lack of exposure of the CS-1 region in soluble Fn. Hydropathy plots indicate that the CS 1–25 amino acid stretch is extremely

hydrophobic (Humphries et al., 1986) and, therefore, should be unavailable at the hydrated surface. Experimentally, soluble Fn interacts poorly with  $\alpha_4\beta_1$ -bearing hematopoietic cells and does not inhibit the adhesion of these cells to immobilized Fn (Garcia-Pardo et al., 1989, 1990; Garcia-Pardo & Ferreira, 1990; Ferreira et al., 1990). No interaction of Ramos cells with soluble Fn was observed, even in the presence of mAb 8A2 (Faull et al., 1994), which activates  $\alpha_4\beta_1$  on cells to bind to the minimal LDV sequence in CS-1 (Wayner & Kovach, 1992).

Immobilization of Fn onto a plastic surface was associated with only a slight exposure of the CS-1 sequence as judged by anti-CS-1 reactivity. Other studies support the limited expression of the CS-1 region in immobilized Fn. Wayner et al. (1989) found that YT cells, which express  $\alpha_4\beta_1$  but not  $\alpha_5\beta_1$ , adhere poorly to plasma Fn, and Hemler et al. (1990) observed that, although transfected K562 cells expressed similar levels of  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$ , their adhesion to intact plasma Fn was not inhibited by anti- $\alpha_4\beta_1$ . Other immunological studies also suggest that the CS-1 sequence is not exposed in immobilized Fn. Garcia-Pardo et al. (1992) and Paul et al. (1986) reported that antibodies to CS 1–25 did not react with immobilized plasma Fn. Furthermore, since antiserum to 29–36 kDa catheptic D fragments reacted weakly with intact Fn, Gold et al. (1983) suggested that this region may not be exposed.

The acidic proteases—cathepsin D, cathepsin B, and pepsin—degraded Fn and generated fragments with exposed CS-1-containing sequences. In cathepsin D digests, the heparin-binding 33.5 kDa fragment contained the CS-1 sequence and interacted well with anti-CS-1 antibody. Although several tested neutral proteases—trypsin, chymotrypsin, neutrophil elastase, and cathepsin G—produced CS-1-containing fragments of similar molecular mass, these derivatives did not express CS-1 epitope(s) when presented to the anti-CS-1 as either soluble or immobilized fragments. Analogously, another acidic protease, cathepsin B, exposed CS-1 epitope in a 140 kDa fragment, whereas neutral proteases, which produced CS-1-containing fragments of similar size, did not expose CS-1 epitopes. Acidic conditions alone did not expose the CS-1 epitopes. Thus, the combination of proteolysis of specific peptide bonds and acidic conditions is required for exposure of the CS-1 sequence as a possible mechanism. The interactions that occur between domains within the same subunit and between domains within the two constituent chains of Fn (Homandberg & Erickson, 1986; Robinson & Hermans, 1984; Hörmann & Richter, 1986; Litvinovich et al., 1991; Matsuka et al., 1994) may shield the CS-1 region. Such interactions may be preserved between proteolytic fragments produced at neutral pH, but may be destabilized at low pH.

The lack of exposure of the CS-1 sequence in the 38 kDa tryptic fragment as contrasted to the 33.5 kDa catheptic D fragment merits specific consideration. Previous characterization of a 35 kDa catheptic D fragment (Homandberg & Erickson, 1986), presumably the same as our 33.5 kDa fragment, showed that its amino terminus was Lys-1641 of Fn, which resides in the mid-portion of 12th type III repeat [numbering based on Kornblihtt et al. (1985)], and that it contained a free SH, which is present in the 15th type III repeat. The 38 kDa tryptic fragment has Thr-1558 in the 11th type III repeat as it amino terminus and terminates at residue 67 in the IIICS region (Garcia-Pardo et al., 1987).

Thus, these two fragments, which are structurally very similar, must differ substantially in conformation to account for their differential exposure of the CS-1 region. Moreover, it is difficult to reconcile with common usage of the 38 kDa tryptic fragment as the CS-1-containing ligand in adhesion assays if this derivative does not present the region in an exposed conformation. Our attempts to isolate this fragment from a 4 h tryptic digest of Fn by one-step affinity chromatography on heparin-agarose resulted in copurification of 38 kDa fragment and 58 kDa fragment originating from the B subunit of Fn (data not shown). As suggested above, a complex between fragments in different subunits could prevent antibody access to the CS-1 region. The described procedure (Garcia-Pardo et al., 1987) for purification of the 38 kDa fragment for adhesion assays involves several chromatographic steps performed in 4 M urea. Thus, dissociation and/or denaturation may be necessary to expose the CS-1 sequence in the 38 kDa fragment.

Soluble Fn and the proteolytic fragments produced by cathepsin G and neutrophil elastase were poor inhibitors of  $\alpha_4\beta_1$ -mediated cell adhesion to CS-1.3. These results are consistent with the poor exposure of the CS-1 region in these Fn derivatives. On the other hand, tryptic and chymotryptic digests, which express the CS-1 region poorly, were as potent in inhibiting cell adhesion as the cathepsin B and D digests, which express this region well. Two possibilities may explain this disparity. First, the  $\alpha_4\beta_1$  receptor may be able to interact with determinants within the CS-1 sequence which are cryptic to the antibody. While this possibility cannot be excluded, it seems remote since the antibody was capable of blocking cell adhesion. Second, the inhibitory digests may present other sequences which interact with  $\alpha_4\beta_1$  and compete with CS-1 for receptor recognition. In support of this possibility Garcia-Pardo et al. (1990) showed that soluble CS-1 peptide, even at concentrations of 500  $\mu\text{g/mL}$ , inhibited the adhesion of HUT-78 cells to the 38 kDa fragment by only 37%, consistent with a weak cross-reaction rather than a direct competition. In addition, CS-1, CS-5, and H1 (contained in the 14th type III repeat of Hep II domain) peptides have been shown to cross-compete with one another for  $\alpha_4\beta_1$  recognition (Mould & Humphries, 1991). Indeed, several sequences flanking the CS-1 region are recognized by  $\alpha_4\beta_1$ -bearing cells (Mould & Humphries, 1991; Iida et al., 1992; Humphries et al., 1986), suggesting that the CS-1 region functions as one of several alternative cell adhesive sequences within the carboxyl-terminal cell binding domain. This explanation implies that these alternative cell adhesive sequences are also poorly exposed in soluble plasma Fn.

The exposure of the CS-1 sequence by cathepsin D and cathepsin B may be of pathophysiological significance. While both enzymes are found predominantly in lysosomes under physiological circumstances, pathological conditions result in their extracellular release. Cathepsin D is released as a consequence of myocardial cell damage in vivo and in vitro (Katagiri et al., 1983) and is found at increased levels in the serum of patients with chronic liver disease (Murawaki & Hirayama, 1980). The plasma and synovial fluid of patients with rheumatoid arthritis (Poole et al., 1976) also contain high levels of this enzyme. Both cathepsins have been implicated in tumor invasion and metastasis due to their ability to degrade extracellular matrices (Nicolson, 1989; Recklies & Poole, 1982). In this regard, cathepsin B levels have been correlated with tumor malignancy (Sloane, 1990).

Phagocytic cells secrete cathepsin D (Rossman et al., 1990; Young et al., 1991), and inflammatory mediators, such as  $\gamma$ -interferon, increase the secretion of cathepsin D from blood monocytes and alveolar macrophages by several fold (Rossman et al., 1990). It has been suggested that the low pH environment necessary for optimal proteolysis by the cathepsins can be maintained by cells at their adhesive contacts when they undergo a metabolic switch to aerobic glycolysis and secrete lactic acid (Young & Zygas, 1987; von Ardenne & Kröger, 1979; Etherington et al., 1981). Thus, appropriate circumstances may arise in vivo for the cathepsins to degrade Fn and expose the cryptic adhesive properties of the CS-1 region. It remains to be determined whether the detection of the CS-1 immunoreactivity in inflamed tissues by immunohistochemical analyses (Elices et al., 1994) may reflect the presence of Fn fragments produced by the cathepsins or related proteases. However, events other than proteolysis also might influence exposure of the CS-1 region. One such mechanism is insertion of other alternatively spliced domains into Fn (French-Constant, 1995) which could alter the organization of the molecule and expose the CS-1 region. Systematic evaluation of this possibility is currently underway.

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