Komoroski, R. A., & Allerhand, A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1804–1808.
Kowalewski, J., & Levy, G. C. (1977) *J. Magn. Reson. 26*, 533–536.
Levitt, M., & Warshel, A. (1978) *J. Am. Chem. Soc. 100*, 2607–2613.
Levy, G. C., Hilliard, P. R., Jr., Levy, L. F., Rill, R. L., & Inners, R. (1981) *J. Biol. Chem. 256*, 9986–9989.
Lipari, G., & Szabo, A. (1980) *Biophys. J. 30*, 489–506.
Lipari, G., & Szabo, A. (1981) *Biochemistry 20*, 6250–6256.
London, R. E. (1980) in *Magnetic Resonance in Biology* (Cohen, J. S., Ed.) pp 1–69, Wiley, New York.
London, R. E., & Avitable, J. (1978) *J. Am. Chem. Soc. 100*, 7159–7165.
Moore, A. C., & Browne, D. T. (1980) *Biochemistry 19*, 5768–5773.
Otvos, J. D., & Browne, D. T. (1980) *Biochemistry 19*, 4011–4021.
Patkowski, A., & Chu, B. (1979) *Biopolymers 18*, 2051–2072.
Schmidt, P. G., Tompson, J. G., & Agris, P. F. (1980) *Nucleic Acids Res. 8*, 643–656.
Schweizer, M. P., Hamill, W. D., Jr., Walkiw, I. J., Horton, W. J., & Grant, D. M. (1980) *Nucleic Acids Res. 8*, 2075–2083.
Serianni, A. S., Nunez, H. A., & Barker, R. (1980) *J. Org. Chem. 45*, 3329–3341.
Tompson, J. G., & Agris, P. F. (1979) *Nucleic Acids Res. 7*, 765–779.
Tompson, J. G., Hayashi, F., Paukstellis, J. V., Loeppky, R. N., & Agris, P. F. (1979) *Biochemistry 18*, 2079–2085.
Wittebort, R. J., & Szabo, A. (1978) *J. Chem. Phys. 69*, 1722–1725.
Woessner, D. E. (1962) *J. Chem. Phys. 36*, 1–4.
Yokoyama, S., Usuki, K. M. J., Yamaizumi, Z., Nishimura, S., & Miyazawa, T. (1980) *FEBS Lett. 119*, 77–80.

Topological Arrangement of Four Functionally Distinct Domains in Hamster Plasma Fibronectin: A Study with Combination of S-Cyanylation and Limited Proteolysis†

Kiyotoshi Sekiguchi and Sen-itiroh Hakomori*

ABSTRACT: Hamster plasma fibronectin was shown previously to consist of four functionally distinct domains which were isolated as M_r 150 000–140 000, 40 000, 24 000, and 21 000 fragments (150K–140K, 40K, 24K, and 21K fragments) after mild thermolysin digestion [Sekiguchi, K., Fukuda, M., & Hakomori S. (1981) *J. Biol. Chem. 256*, 6452–6462]. The alignment of these domains and the location of cysteine residues have been studied by S-cyanylation of cysteine residues with 2-nitro-5-thiocyanobenzoic acid. Hamster plasma fibronectin contained 1.1 mol of cysteine residue/mol of subunit chain. This cysteine residue was localized in the 150K–140K domain. Cleavage of intact fibronectin at the cysteine residue by S-cyanylation produced three major fragments, i.e., sc155K, sc145K, and sc125K fragments (the prefix "sc" specifies the S-cyanylation-cleaved fragment). Only the sc145K fragment was heavily radiolabeled by factor XIIIa/[^{3}H]putrescine, as well as galactose oxidase/NaB^3H_4 for carbohydrates, indicating that it contained 24K and 40K domains which were located at the NH_2-terminal side of the cysteine residue. The sc145K fragment was separated from other fragments by gelatin–Sepharose chromatography, followed by gel filtration on Sephacryl S-200. Thermolysin digestion of the sc145K fragment produced 24K, 40K, and 55K fragments but did not produce the 21K fragment. The first two fragments represent NH_2-terminal 24K domain and a next adjacent carbohydrate-rich 40K domain, respectively. The 55K fragment was considered to represent COOH-terminal region of the sc145K fragment and overlap with NH_2-terminal portion of the 150K–140K domain, because this portion was obtained as an sc58K fragment by S-cyanylation of the 150K–140K domain. In contrast, thermolysin digestion of the S-cyanylation fragments, which were nonadsorbed on gelatin columns and were considered to be derived from the COOH-terminal portion of intact fibronectin, produced 64K, 62K, and 21K fragments, but not 24K or 40K. These results, taken together, indicate that the alignment of the four domains in the α subunit is NH_2–24K–40K–150K(–140K)–21K–COOH and that in the β subunit is NH_2–24K–40K–(150K–)140K–COOH.

Fibronectin is a multifunctional glycoprotein abundantly present in the pericellular matrix and in body fluids (Vaheri & Mosher, 1978; Yamada & Olden, 1978; Mosesson & Amrani, 1980; Pearlstein et al., 1980; Mosher, 1980; Ruoslahti et al., 1981a). A decrease or deletion of this protein associated on oncogenic transformation aroused a great deal of initial interest for the study of this protein (Gahmberg & Hakomori, 1973a; Hynes, 1973). It is an adhesive protein which mediates in vitro cell attachment and spreading onto uncoated or collagen-coated plastic surfaces (Klebe, 1974; Pearlstein, 1976; Grinnell, 1978). In plasma, it modulates the phagocytic activity of the reticuloendothelial system as a nonspecific opsonin (Saba et al., 1978). It shows an affinity toward various substances, including collagen (or gelatin), fibrin, glycosaminoglycans, bacterial surface components, actin, and de-

† From the Program of Biochemical Oncology, Fred Hutchinson Cancer Research Center, and Departments of Pathobiology, Microbiology, and Immunology, School of Public Health and School of Medicine, University of Washington, Seattle, Washington 98104. *Received May 18, 1982; revised manuscript received November 5, 1982.* This investigation was supported by Research Grant CA23907 from the National Institutes of Health. A part of the present investigation was presented at the 21st Annual Meeting of the American Society for Cell Biology at Anaheim, CA (Sekiguchi & Hakomori, 1981).

* Address correspondence to this author at the Program of Biochemical Oncology, Fred Hutchinson Cancer Research Center.

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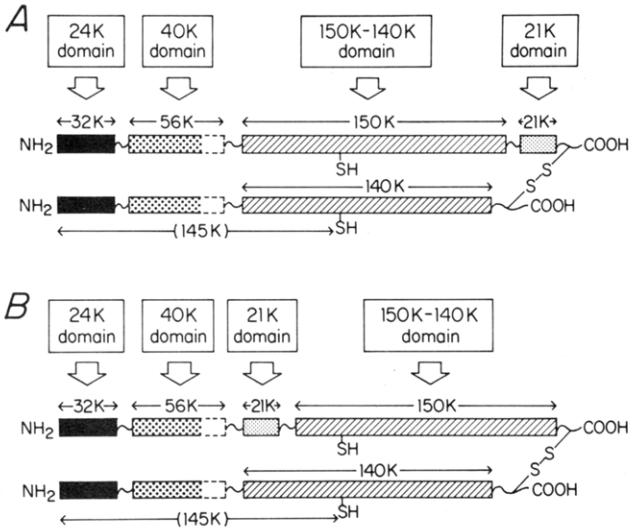

FIGURE 1: Two possible models for the topological arrangement of four structural domains of hamster plasma fibronectin. The positions of sulfhydryl groups are placed on the basis of the results obtained in the present investigation. For details, see the text.

oxyribonucleic acid (Mosher, 1980; Ruoslahti et al., 1981a, and references therein).

Fibronectin is composed of two identical or nonidentical subunit polypeptides with M_r ~230 000, which are held together by disulfide bonding. Several lines of evidence indicate that it is composed of several structural domains which are linked by flexible, protease-sensitive peptide segments (Alexander et al., 1978; Fukuda & Hakomori, 1979; Wagner & Hynes, 1979; Hahn & Yamada, 1979; Sekiguchi & Hakomori, 1980; McDonald & Kelley, 1980). Recently, we reported that sequential digestion with trypsin and thermolysin or thermolysin digestion alone could separate these domains almost quantitatively (Sekiguchi & Hakomori, 1980; Sekiguchi et al., 1981). Thus, fibronectin was found to be composed of four distinctive domains which were recovered as 150K–140K,[1] 40K, 24K, and 21K thermolysin-released fragments. The first three domains (i.e., 150K–140K, 40K, and 24K domains) are equally present in both the larger (α) and smaller (β) subunits, while the 21K domain seems to be present only in the α subunit (Sekiguchi et al., 1981). These domains were also functionally distinct from each other (Sekiguchi & Hakomori, 1980; Sekiguchi et al., 1981).

The alignment of these domains, however, has not been fully determined. There is some evidence (Furie & Rifkin, 1980; Balian et al., 1980; Wagner & Hynes, 1980) indicating that the 24K domain is located at the NH_2 terminus and the 40K gelatin-binding domain is adjacent to the 24K domain. Thus, we proposed two alternative models for the topological arrangement of the domains, depending on the location of the 21K domain (Sekiguchi et al., 1981; Figure 1). In the present investigation, we attempted to determine the alignment of the domains by employing the chemical cleavage at cysteine residues by S-cyanylation, which was further combined with limited proteolysis by thermolysin. The results obtained support the alignment of the domains shown in model A of Figure 1.

Materials and Methods

Materials. 2-Nitro-5-thiocyanobenzoic acid (NTCB)[2] was obtained from Eastman Kodak Co. (Rochester, NY) and recrystallized from ethanol. Ultrapure urea and guanidine hydrochloride (Gdn·HCl) were purchased from Schwarz/Mann (Orangeburg, NY) and used without further purification. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), iodoacetamide, gelatin (from swine skin, type I), and thermolysin (protease, type X) were obtained from Sigma; [^{3}H]putrescine, K^{14}CN, and NaB^3H_4 were from Amersham (Arlington Heights, IL); iodo[^{14}C]acetamide was from New England Nuclear; Sephacryl S-200, Sepharose 4B, and Sephadex G-25 were from Pharmacia (Piscataway, NJ); DEAE-cellulose was from Whatman (Kent, England); galactose oxidase was from Kabi (Stockholm, Sweden); sialidase of *Vibrio cholerae* was from Behring Diagnostics (Somerville, NJ). Factor XIII was a generous gift from Dr. S. I. Chung (Laboratory of Biochemistry, National Institute of Dental Research). Highly purified bovine thrombin was provided by Dr. W. Kisiel (Department of Biochemistry, University of Washington). Gelatin–Sepharose was prepared according to Cuatrecasas & Anfinsen (1971).

Purification of Fibronectin. Fibronectin was purified from freshly drawn citrated hamster plasma by affinity chromatography on gelatin–Sepharose, as described previously (Sekiguchi et al., 1981). Carboxamidated fibronectin was prepared as follows: Fibronectin dissolved in buffer A (0.2 M Tris–acetate–1 mM EDTA, pH 8.0) containing 7.5 M Gdn·HCl was incubated with 5 mM iodoacetamide at 22 °C for 30 min in the dark. Modified protein was dialyzed against buffer B (10 mM Tris-HCl–0.5 mM EDTA–50 mM NaCl, pH 7.6) in the dark at 4 °C for 3 days and stored at −80 °C.

[^{3}H]Putrescine Labeling of Fibronectin by Factor XIIIa. Factor XIIIa dependent putrescine labeling was performed essentially according to Mosher et al. (1980), with a slight modification (Sekiguchi et al., 1981). Fibronectin (0.82 mg/mL) was dissolved in 10 mM Tris-HCl buffer (pH 7.6) containing 0.5 mM EDTA, 100 mM NaCl, 10 mM $CaCl_2$, 0.42 mM [^{3}H]putrescine (0.29 Ci/mmol), and factor XIII (50 μg/mL). The reaction was started by adding thrombin (1 unit/mL). After incubation at 22 °C for 1.5 h, EDTA (20 mM) was added to terminate the reaction. The labeled protein was dialyzed against buffer B at 4 °C for 3 days. It contained 1.8 mol of putrescine/mol of intact fibronectin.

Labeling of Carbohydrate Units by Galactose Oxidase. Carbohydrate chains of intact fibronectin were labeled with galactose oxidase according to Gahmberg & Hakomori (1973b), as described previously (Sekiguchi et al., 1981). Briefly, fibronectin (1 mg/mL) was incubated with galactose oxidase (10 units/mL) and sialidase from *V. cholerae* (5 units/mL) at 22 °C for 3 h. It was reduced with NaB^3H_4 (1 mCi/mL) at 22 °C for 30 min, followed by incubation with $NaBH_4$ (1 mM) for 30 min. The labeled fibronectin was absorbed on gelatin–Sepharose to remove the enzymes. The labeled protein was eluted from the column with 6 M urea, dialyzed against buffer B at 4 °C. The specific activity of the labeled protein was 4.5×10^6 cpm/mg of protein.

Purification of 150K–140K Thermolysin-Released Fragments. Intact fibronectin (1–2 mg/mL) was digested with thermolysin (2.5 μg/mL) at 22 °C for 4 h in buffer B containing 2.5 mM $CaCl_2$. The 150K–140K fragments were purified as described previously (Sekiguchi et al., 1981).

[1] Proteolytic fragments with M_r 150 000–140 000 are designated as 150K–140K fragments in the text. Other fragments are designated in the same manner.

[2] Abbreviations: NTCB, 2-nitro-5-thiocyanobenzoic acid; Gdn·HCl, guanidine hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); buffer A, 0.2 M Tris–acetate–1 mM EDTA, pH 8.0; buffer B, 10 mM Tris-HCl–0.5 mM EDTA–50 mM NaCl, pH 7.6; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Briefly, the digest was passed through a gelatin–Sepharose column (2.5 × 10 cm) to remove a gelatin-binding 40K fragment. The unbound fragments were chromatographed on a DEAE-cellulose column (2.5 × 10 cm). The column was washed with buffer B containing 80 mM NaCl, followed by elution of the 150K–140K fragments with the same buffer containing 200 mM NaCl.

Determination of Cysteine Residue. Cysteine residues were quantitated by either DTNB (Ellman, 1959) or iodo[^{14}C]-acetamide in the presence or absence of 7.5 M Gdn·HCl. In some experiments, 7.5 M Gdn·HCl was replaced by 8 M urea. Both procedures gave almost the same results.

Cleavage by S-Cyanylation with NTCB. Cleavage of polypeptides at cysteine residues by one-step S-cyanylation with NTCB was carried out according to Jacobson et al. (1973), with a slight modification as follows. Two protocols (procedure 1 and procedure 2) were employed for analytical and preparative purposes, respectively. Both procedures gave essentially the same results.

Procedure 1. Intact fibronectin or its proteolytic fragments (1–2 mg/mL) were dissolved in 0.19 mL of buffer A containing 0.1% $NaDodSO_4$. The protein solution was boiled for 2–3 min to complete denaturation. To the denatured protein was added 0.1 mL of 10 mM NTCB dissolved in the same buffer. After 30-min incubation at 37 °C, the modified protein was dialyzed against the same buffer containing 0.1% $NaDodSO_4$, pH 9, at 37 °C for 20 h. The cleaved protein was lyophilized and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Procedure 2. Fibronectin (2–4 mg/mL) dissolved in buffer A containing 8 M urea was reacted with 0.5 mM NTCB at 37 °C for 30 min. The reaction was quenched by adjusting the pH to 4–5 with acetic acid. The modified protein was desalted by passage through Sephadex G-25 equilibrated with 0.1% acetic acid and then lyophilized. For cleavage, it was dissolved in 0.2 M Tris–acetate buffer containing 8 M urea, pH 9, and incubated at 37 °C for 20 h. The cleavage products were dialyzed against buffer B at 4 °C for 3 days.

Cleavage by Two-Step S-Cyanylation with K^{14}CN. Polypeptides were cleaved by two-step S-cyanylation with K^{14}CN to label the newly formed NH_2 termini. The 150K–140K thermolysin fragments were dissolved in 0.95 mL of buffer A containing 7.5 M Gdn·HCl, pH 8. To the sample was added 0.025 mL of 40 mM DTNB dissolved in buffer A. After 10-min incubation at 22 °C, 0.025 mL of 183 mM K^{14}CN (27.3 mCi/mmol) was added and further incubated 15 min at 22 °C. The reaction was quenched by adding 0.04 mL of acetic acid. The sample was desalted by passage through a Sephadex G-25 column (1 × 25 cm) equilibrated with 0.1% acetic acid and lyophilized. For cleavage, the cyanylated fragments were dissolved in 0.2 M Tris–acetate buffer containing 7.5 M Gdn·HCl, pH 9, and incubated for 20 h at 37 °C.

Isolation of S-Cyanylation-Cleaved 145K (sc145K) Fragment. Fibronectin (40 mg) was cleaved by one-step cyanylation with procedure 2. After extensive dialysis against buffer B at 4 °C, the cleavage products were fractionated with a gelatin–Sepharose column (2.5 × 10 cm). After the column was washed with buffer B, the bound fragments were eluted with buffer B containing 4 M urea, dialyzed against buffer B at 4 °C for 3 days, and then concentrated by vacuum dialysis with Micro-ProDiCon (BioMolecular Dynamics, Beaverton, OR). The sc145K[3] fragment was further purified on Sephacryl S-200 (2.5 × 110 cm) equilibrated with buffer B.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) with 9.5% polyacrylamide gels. Samples were reduced with 2% (v/v) 2-mercaptoethanol. The apparent molecular weight was estimated by using the following proteins as standards: skeletal muscle myosin, M_r 200 000; β-galactosidase, M_r 116 000; phosphorylase B, M_r 94 000; bovine serum albumin, M_r 68 000; ovalbumin, M_r 43 000; soybean trypsin inhibitor, M_r 21 000; hemoglobin, M_r 16 000. Fluorography of slab gels was performed according to Bonner & Laskey (1974).

Results

Quantitation and Localization of Cysteine Residues. Fibronectin has been shown to contain one, or at most two, cysteine residues per subunit chain, which were barely detectable under nondenaturing conditions (Chen et al., 1977; Fukuda & Hakomori, 1979; McDonald & Kelley, 1980; Wagner & Hynes, 1980). We also quantitated the cysteine residues of hamster plasma fibronectin under denaturing and nondenaturing conditions. In the presence of 7.5 M Gdn·HCl or 8 M urea, 1.1 mol of cysteine residue was detected per mol of subunit chain.[4] However, only 0.25 mol was detectable per mol of subunit chain under nondenaturing condition. This indicates that each subunit chain contains mostly one cysteine residue readily available by SH reagents under the denaturing condition, and the cysteine residue is mostly cryptic under nondenaturing condition. Since slightly more than one cysteine residue was detectable per subunit chain, the second cysteine residue could be present in a minor population of hamster plasma fibronectin, or the second cysteine residue could be deeply cryptic (see also Discussion).

Localization of the cysteine residue was investigated by labeling the fibronectin with iodo[^{14}C]acetamide under the denaturing condition either before or after the thermolysin digestion (Figure 2). Thermolysin cleaved iodo[^{14}C]acetamide-labeled fibronectin into four fragments (i.e., 140K, 40K, 24K, and 21K fragments), among which the iodo[^{14}C]acetamide was only localized in the 140K fragment, indicating that the cysteine residue is localized in the 150K–140K domain. This domain was normally recovered as 150K–140K fragments upon the thermolysin digestion of intact fibronectin (Figure 2A, lane c). However, only the 140K fragment was generated from the SH-modified fibronectin. This could be due to an irreversible alteration of the protein conformation caused by the modification of the cysteine residue or by the exposure to a strong denaturant, i.e., 8 M urea, during the modification.

Localization of the cysteine residue in the 150K–140K domain was also confirmed by labeling the thermolysin digest of intact fibronectin with iodo[^{14}C]acetamide (Figure 2B). As expected, the major label was found in the 150K and 140K fragments but not in the 40K, 24K, or 21K. A minor label was found in an unexpected fragment which migrated slightly above the 24K band. On the Coomassie blue staining of the gel, the presence of a very minor band was detected in the same region. This fragment may contain the second cysteine residue which could be present only in the minor population of the

[3] S-Cyanylation-cleaved fragments are distinguished from proteolytic fragments by adding a prefix "sc", as in "sc145K fragment".

[4] Average of five separate determinations. The number of cysteine residues varied 0.8–1.7 mol/mol of subunit.

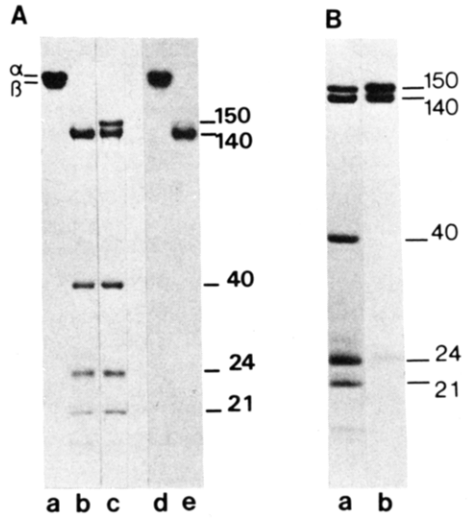

FIGURE 2: Localization of cysteine residues. (A) Cysteine residues of intact fibronectin were labeled by reacting with 1 mM iodo-[^{14}C]acetamide at 22 °C for 20 min in the presence of 8 M urea, as described under Materials and Methods. After extensive dialysis against buffer B, the labeled protein (1.2×10^5 cpm/mg of protein) was digested by thermolysin (2.5 μg/mL) at 22 °C for 4 h. (Lanes a and d) Iodo[^{14}C]acetamide-labeled fibronectin; (lanes b and e) thermolysin digest of the labeled fibronectin; (lane c) thermolysin digest of unlabeled intact fibronectin. (Lanes a–c) Protein staining with Coomassie blue; (lanes d and e) fluorogram. (B) Cysteine residues of fibronectin were labeled by iodo[^{14}C]acetamide after thermolysin digestion. Intact fibronectin was digested by thermolysin (2.5 μg/mL) for 2 h at 22 °C. The digest was labeled by iodo[^{14}C]acetamide as described above. (Lane a) Thermolysin digest stained by Coomassie blue; (lane b) fluorogram of lane a.

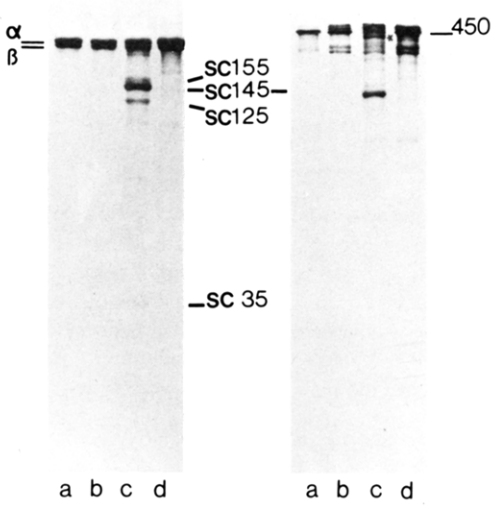

FIGURE 3: Cleavage of intact fibronectin by one-step S-cyanylation. Intact fibronectin was cleaved at cysteine residues by one-step S-cyanylation with NTCB according to procedure 1 (for details, see Materials and Methods). Cleavage products were analyzed by Na-DodSO$_4$–polyacrylamide gel electrophoresis under reducing (left) and nonreducing (right) conditions. (Lane a) Intact fibronectin; (lane b) carboxamidated fibronectin; (lane c) cyanylation-cleaved intact fibronectin; (lane d) cyanylation-cleaved carboxamidated fibronectin. The asterisk in the right panel points to the band which may represent a hetero dimer consisting of an uncleaved and a cleaved subunit (for details, see text). A blockade of free sulfhydryl residues by iodoacetamide reproducibly caused partial cleavage of interchain disulfide bonds (see lane b of right panel).

fibronectin or could be highly cryptic even in the presence of a strong denaturant.

Cleavage of Intact Fibronectin by One-Step S-Cyanylation. Polypeptide can be cleaved at cysteine residues by S-cyanylation (Jacobson et al., 1973; Degani & Patchornik, 1974). Fibronectin, denatured in buffer A containing 0.1% NaDodSO$_4$, was cyanylated by one-step reaction with 2-nitro-5-thiocyanobenzoic acid (NTCB), and the modified protein was cleaved by incubation in the same buffer of pH 9 (for details, see Materials and Methods). The cleavage products contained sc155K, sc145K, and sc125K fragments, as well as uncleaved α and β subunit polypeptides (Figure 3). The sc145K fragment gave a much denser band in a polyacrylamide gel than sc155K and sc125K. Another fragment with M_r 35000 was also faintly detected in the cleavage products. The presence of intact α and β subunit chains among the cleavage products indicates that the cleavage was not complete under the present conditions. Since increase of NTCB concentration up to 5 mM did not affect the cleavage profile (K. Sekiguchi and S. Hakomori, unpublished observation), it could be due to β-elimination of β-thiocyanoalanine residues or incomplete modification of cysteine residues (Degani & Patchornik, 1974; Stark, 1977).

The sc145K fragment appears to be devoid of interchain disulfide bonding, because it showed the same mobility on the acrylamide gels both in the presence and in the absence of a reducing agent (Figure 3, lane c of right panel). In contrast, sc155K, sc125K, and sc35K fragments were significantly reduced under nonreducing conditions, suggesting that these fragments are connected to other fragments or intact subunit chains through disulfide linkage. In addition, a fragment with M_r ~320000 was detected on the acrylamide gel under nonreducing conditions (as marked by an asterisk in Figure 3, right panel). This may represent a "hetero dimer" which consists of a fragment of a cleaved, sc145K-depleted subunit disulfide bonded to an uncleaved subunit.

The cleavage by one-step S-cyanylation with NTCB appears to be specific for cysteine residues, because none of these fragments was generated when the sulfhydryl groups of fibronectin were blocked by iodoacetamide before cyanylation (Figure 3, lane d). On the other hand, two-step cyanylation with DTNB and KCN employed in the previous studies (Fukuda & Hakomori, 1979; Wagner & Hynes, 1980) was found to cause significant cleavage at cystine residues (Wagner & Hynes, 1980; K. Sekiguchi and S. Hakomori, unpublished observation).

For correlation of these cyanylation-cleaved fragments with the four distinct structural domains, fibronectin was radiolabeled either by factor XIIIa and [^{3}H]putrescine or by galactose oxidase and NaB^{3}H$_4$ and then subjected to S-cyanylation. The NH$_2$-terminal 24K domain was specifically labeled by the former, and the 40K carbohydrate rich domain was labeled by the latter (Sekiguchi et al., 1981). The sc145K fragment was heavily radiolabeled by [^{3}H]putrescine, while other fragments were only faintly labeled (Figure 4A). Similarly, galactose oxidase–NaB^{3}H$_4$ also heavily labeled the sc145K among the cleavage products, except uncleaved subunits (Figure 4B). These results clearly indicate that sc145K fragment contains both 24K and 40K domains and, therefore, it is derived from the NH$_2$-terminal side of the cysteine residue which is located at the 150K–140K domain. The presence of the 40K domain in the sc145K fragment was further supported by the fact that this fragment could specifically bind to gelatin (see below).

Isolation and Thermolysin Digestion of the sc145K Fragment. The sc145K fragment was isolated from whole cyanylation cleavage products by gelatin–Sepharose chromatography, followed by gel filtration on Sephacryl S-200 (Figures 5 and 6). Since 0.1% NaDodSO$_4$ caused irreversible denaturation of fibronectin, S-cyanylation was performed in the presence of 8 M urea to this end (procedure 2; for details see

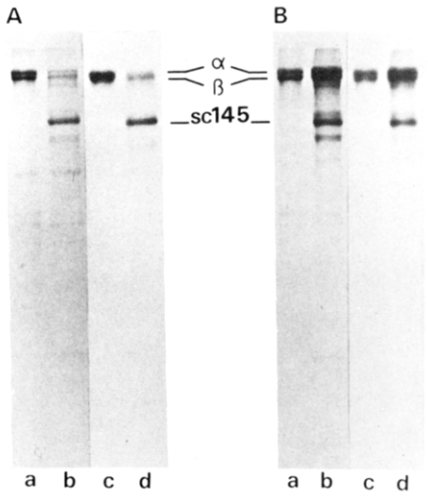

FIGURE 4: S-Cyanylation cleavage of [^{3}H]putrescine- and carbohydrate-labeled fibronectin. (A) Fibronectin was labeled with [^{3}H]-putrescine by factor XIIIa, as described under Materials and Methods. Labeled fibronectin was cleaved by one-step S-cyanylation (procedure 1) and analyzed on NaDodSO$_4$–polyacrylamide gel electrophoresis. The gel was first stained by Coomassie blue (lanes a and b) and then subjected to fluorography (lanes c and d). (Lanes a and c) [^{3}H]-Putrescine-labeled fibronectin; (lanes b and d) cleavage products. (B) Carbohydrate units were labeled by a galactose oxidase–NaB^{3}H$_4$ method described under Materials and Methods. The labeled protein was cleaved by one-step S-cyanylation and analyzed in the same way. (Lanes a and b) Protein staining with Coomassie blue; (lanes c and d) fluorogram. (Lanes a and c) Labeled fibronectin; (lanes b and d) cleavage products.

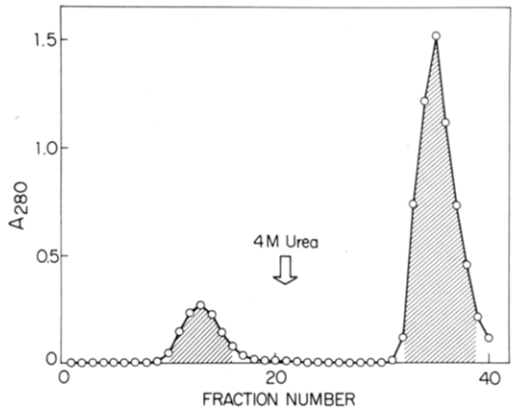

FIGURE 5: Gelatin–Sepharose chromatography of S-cyanylation-cleaved fragments of intact fibronectin. Intact fibronectin (40 mg) was cleaved by one-step S-cyanylation (procedure 2), as described under Materials and Methods. Cleavage products were fractionated on gelatin–Sepharose. Gelatin-bound fragments were eluted with buffer B containing 4 M urea. Unbound and bound fractions were pooled and dialyzed against buffer B (only bound fractions). For details, see Materials and Methods. Fractions of 3 mL were collected.

Materials and Methods). The cleavage profile was almost the same as that of procedure 1 (Figure 7, lane b). Among the cleavage products, sc145K fragment was capable of binding to gelatin, while sc155K, sc125K, sc35K, and other minor fragments failed to bind to gelatin (Figure 7, lanes c and d). Although a small amount of sc155K, sc125K, and sc35K fragments were still present in the gelatin-bound fraction (Figure 7, lane d), they are considered to be connected to uncleaved subunit chains through which they were absorbed on gelatin–Sepharose. The gelatin-bound fragments were further separated into two fractions on Sephacryl S-200 (Figure 6). The first peak mostly consisted of uncleaved and partially cleaved subunits held together by disulfide bonding (Figure 7, lane e), whereas the second peak consisted of

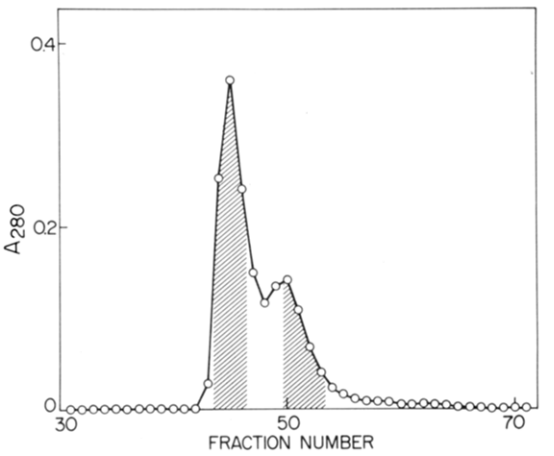

FIGURE 6: Sephacryl S-200 column chromatography. Gelatin-bound S-cyanylation-cleaved fragments were further fractionated on a Sephacryl S-200 column (2.5 × 100 cm). The first peak and a small second peak were separately pooled, concentrated, and analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. Fractions of 4.5 mL were collected.

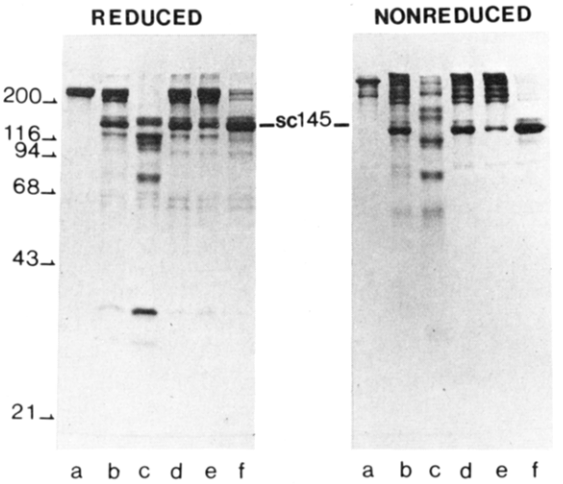

FIGURE 7: Gel electrophoretic pattern of gelatin-bound and unbound fraction of S-cyanylation cleavage products and purified sc145K fragment. The sc145K fragment was purified by gelatin–Sepharose chromatography and subsequent Sephacryl S-200 column chromatography, as described under Materials and Methods. Fragments obtained by each purification step were analyzed on NaDodSO$_4$–polyacrylamide gels with (left) or without (right) prior reduction. (Lane a) Intact fibronectin; (lane b) S-cyanylation-cleaved fibronectin; (lane c) gelatin-unbound fragments; (lane d) gelatin-bound fragments; (lane e) fragments eluted at the first peak on Sephacryl S-200 chromatography; (lane f) fragments eluted at the second peak on Sephacryl S-200 chromatography.

sc145K and a trace of other fragments (Figure 7, lane f).

Both purified sc145K fragment, which was derived from the NH$_2$-terminal side, and gelatin-unbound fragments, which were derived from the COOH-terminal side of the cysteine residue, were separately digested by thermolysin to examine their domain composition. The sc145K fragment was cleaved into three fragments, i.e., 24K, 40K, and 55K (Figure 8, lane c), among which the first two fragments were considered to be derived from NH$_2$-terminal 24K domain and the adjacent 40K domain as expected from the labeling experiments with factor XIIIa and galactose oxidase (Figure 4). No 21K fragment was detected. In contrast, thermolysin digestion of gelatin-unbound fragments produced 64K, 62K, and 21K fragments (Figure 8, lane b). These results clearly indicate that the 21K domain, which was previously identified as the fibrin-binding domain characteristic for α subunit, is present in the COOH-terminal side of the cysteine residue. The 55K

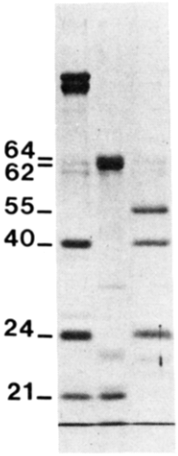

FIGURE 8: Thermolysin digestion of the purified sc145K fragment. Intact fibronectin or its S-cyanylation-cleaved fragments were digested by thermolysin (2.5 μg/mL) at 22 °C for 4 h. The digest was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions. (Lane a) Thermolysin digest of intact fibronectin; (lane b) thermolysin digest of gelatin-unbound S-cyanylation-cleaved fragments; (lane c) thermolysin digest of purified sc145K fragment.

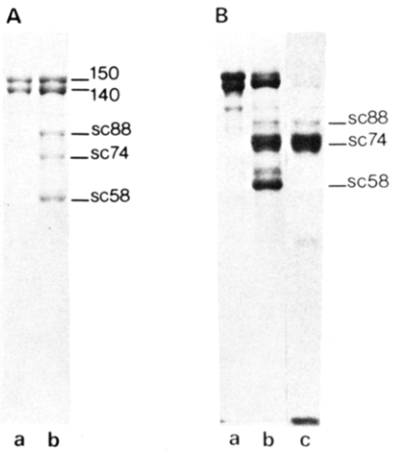

FIGURE 9: Cleavage of 150K–140K thermolysin released fragments by S-cyanylation. (A) Purified 150K–140K thermolysin fragments were cleaved by one-step S-cyanylation with procedure 1. (Lane a) Purified 150K–140K thermolysin fragments; (lane b) cyanylation cleavage products. (B) 150K–140K thermolysin fragments were cleaved by two-step S-cyanylation with $K^{14}CN$, as described under Materials and Methods. Cleavage products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and subsequent fluorography. (Lane a) 150K–140K thermolysin fragments (protein staining); (lane b) cleavage products (protein staining); (lane c) cleavage products (fluorogram).

fragment, derived from sc145K fragment, may represent the NH_2-terminal portion of the 150K–140K domain, as well as the COOH-terminal region of the sc145K fragment. The 64K–62K fragments, derived from gelatin-unbound fragments, are considered to be COOH-terminal portion of the 150K–140K domain.

S-Cyanylation of the 150K–140K Domain. For confirmation of the above possibility, the 150K–140K domain was isolated after thermolysin digestion of intact fibronectin, as described under Materials and Methods. S-Cyanylation of the 150K–140K thermolysin fragments produced three fragments with M_r 88 000, 74 000, and 58 000 (Figure 9A). The sc58K fragment always gave a much denser band than that of sc88K and sc74K fragments, suggesting that the sc58K is derived from both 150K and 140K fragments, while sc88K and sc74K were derived from either 150K or 140K. Since the sc58K fragment had almost the same size as the 55K fragment, which was produced from sc145K fragment by thermolysin digestion, it appears to be derived from NH_2-terminal portion of the 150K–140K domain.

NH_2-Terminal location of the sc58K fragment in the 150K–140K domain was confirmed by two-step S-cyanylation of 150K–140K fragments with DTNB and $K^{14}CN$ (Figure 9B). Cleavage of polypeptides at cysteine residues produces new NH_2 termini which are blocked with an iminothiazolidine carboxylyl group (Jacobsen et al., 1973; Degani & Patchornik, 1974). Cyanylation with DTNB and $K^{14}CN$ results in the incorporation of the ^{14}CN group into an iminothiazolidine carboxylyl group. Thus, most of the cleavage products can be radiolabeled, except a fragment which contains an original NH_2 terminus. Two-step cyanylation of 150K–140K fragments with DTNB and $K^{14}CN$ produced sc88K, sc74K, and sc58K fragments, as was the case for one-step cyanylation (Figure 9B). However, the sc88K fragment was relatively decreased, while the sc74K was increased and apparently became heterogeneous (Figure 9B, lane b). Since it has been shown that two-step cyanylation always accompanies cleavage at cystine residues to some extent (Wagner & Hynes, 1980), this seems to be due to the additional cleavage at the cystine residue(s). Among these three fragments, only sc58K fragment was not labeled by $K^{14}CN$, indicating that this fragment contained the original NH_2 terminus of the 150K–140K fragments. These results are in good agreement with model A of Figure 1.

Discussion

Fibronectin has been shown to consist of several structural domains which are connected through flexible, protease-sensitive peptide segments (Alexander et al., 1978; Hahn & Yamada, 1979; Sekiguchi & Hakomori, 1980; McDonald & Kelley, 1980; Engel et al., 1981). Much effort has been made in many laboratories to isolate such domains after limited proteolysis (Balian et al., 1979; Gold et al., 1979; Hahn & Yamada, 1979; Wagner & Hynes, 1979; Hörmann & Seidl, 1980; McDonald & Kelley, 1980; Sekiguchi & Hakomori, 1980; Hayashi & Yamada, 1981; Isemura et al., 1981; Keski-Oja & Yamada, 1981; Ruoslahti et al., 1981a,b; Sekiguchi et al., 1981). Recently, we found that hamster plasma fibronectin was almost quantitatively cleaved into four distinct domains by mild thermolysin treatment (Sekiguchi et al., 1981). These domains, represented by 150K–140K, 40K, 24K, and 21K thermolysin fragments, were also functionally distinct from each other (Sekiguchi et al., 1981). Similar set of fragments was also obtained after digestion by chymotrypsin (Hahn & Yamada, 1979), leukocyte elastase (McDonald & Kelley, 1980), and cathepsin B (Isemura et al., 1981). Several lines of evidence suggest that the 24K domain, which can be obtained as a 29–32K fragment after mild trypsin, thrombin, or plasmin digestion, is located at the NH_2-terminal region of intact subunit chains (Balian et al., 1980; Furie & Rifkin, 1980; Wagner & Hynes, 1980). The 40K gelatin binding domain appears to be located adjacent to the 24K domain, because 24K and 40K domains were recovered in a single fragment with M_r 70 000 after cathepsin D digestion (Balian et al., 1980; Furie et al., 1980). However, the location of the 150K–140K and 21K domains has not yet been determined, although 21K, representing one of the fibrin-binding domains, is present characteristically in the α subunit (Sekiguchi et al., 1981). Thus, two possible alignments of these domains, which differ in the location of the 21K domain (Figure 1), remained to be studied. In model A of Figure 1, the 21K domain is located at the COOH-terminal end of the α subunit, whereas

it is located between 40K and 150K–140K domains in the model B.

In the present investigation, we attempted to determine the alignment of these domains. The strategy we employed was as follows: Fibronectin was found to contain predominantly one cysteine residue per subunit chain, which was located in the 150K–140K domain. The same result was also obtained by McDonald & Kelley (1980) with human plasma fibronectin, although a slightly higher number (e.g., 1.3–1.6) was obtained by others (Fukuda & Hakomori, 1979; Smith et al., 1982). Therefore, cleavage at the cysteine residue by S-cyanylation releases a fragment which is derived from the NH_2-terminal side of the cysteine from both α and β subunits, while the COOH-terminal portions of these subunits are held together by disulfide bonding. Subsequent thermolysin digestion of either NH_2-terminal fragment or COOH-terminal fragments should clarify the domain composition of these fragments, through which we could determine the alignment of four functional domains.

For cleavage at cysteine residue, we employed one-step S-cyanylation with NTCB rather than the two-step reaction with DTNB and KCN, because the latter always accompanied the partial cleavage at cystine residues (Wagner & Hynes, 1980; also our unpublished observation). One-step cyanylation of fibronectin predominantly produced sc155K, sc145K, and sc125K fragments, among which the sc145K gave a much denser band than others in acrylamide gels. Besides these major fragments, some other fragments, including the sc35K fragment, were detectable.

The number of free SH groups per subunit chain was estimated to be slightly more than one, and it fluctuated between 0.8 and 1.7 among five separate determinations. The basis for such a partial expression of the second cysteine residue is not clearly understood. The following possibilities can be considered: (i) the second cysteine residue exists as a cryptic form and is partially exposed on extensive denaturation; (ii) hamster plasma fibronectin is heterogeneous, and the majority has one cysteine residue while the minority has two cysteine residues; (iii) the second cysteine is generated by a partial cleavage of cystine residue, i.e., the detection of the second cysteine is essentially an artifact. The domain bearing the second cysteine was recovered as a fragment which migrated slightly above the 24K fragment with very low yield by treatment of thermolysin digest of fibronectin with iodo-[^{14}C]acetamide. The same fragment was not detectable when fibronectin was labeled with iodo[^{14}C]acetamide followed by thermolysin digestion. This suggests that the second cysteine residue is present in highly cryptic locus which is exposed and partially digested by thermolysin. Thus, the first possibility is most likely to be supported although other possibilities cannot be denied.

Recently, Smith et al. (1982) identified two SH-containing fragments, 31K and 80K, in the tryptic digest of human plasma fibronectin. Two monoclonal antibodies with different specificities showed that these fragments were derived from distinct regions of the intact protein, suggesting that human fibronectin contains two free SH groups per subunit chain. However, only 1.3–1.6 sulfhydryl groups were detectable per subunit chain by titrating with 2,2′-dipyridyl disulfide and DTNB even under the denaturing condition. Thus, the second cysteine residue present in the 31K fragment appears to be only partially reactive while the other in the 80K fragment is fully reactive. Since the cleavage at the possible second SH group of hamster fibronectin by one-step S-cyanylation was, even if it occurred, far less pronounced than the cleavage at the major SH group, such minor cleavage would not conflict with the proposed topological arrangement of the four domains.

Several lines of evidence indicate that the sc145K fragment is derived from the NH_2-terminal side of the cysteine residue. First, labeling and binding property of this fragment indicates that it contains the domains located at the NH_2-terminal portion: (1) it contains 24K, for it was labeled by factor XIIIa–[^{3}H]putrescine; (2) it contains 40K, for it bound to gelatin and labeled by galactose oxidase–NaB^3H_4; (3) it gave on thermolysin digestion 24K and 40K fragments. Second, the sc145K fragment is devoid of an interchain disulfide bond, which was shown to be located at the COOH-terminal region (Furie & Rifkin, 1980; Wagner & Hynes, 1980). In addition to 24K and 40K fragments, the thermolysin digestion of the sc145K fragment produced also the 55K fragment. The 55K fragment may represent the NH_2-terminal half of the 150K–140K domain, as well as the COOH-terminal half of the sc145K fragment, because the NH_2-terminal portion of the 150K–140K domain was obtained as the sc58K fragment after S-cyanylation of this domain. Significantly, thermolysin digestion of the sc145K fragment did not produce the 21K fragment. Instead, it was generated from gelatin-unbound fragments, which are considered to be derived from the COOH-terminal portion of fibronectin. All these findings are in good agreement with model A of Figure 1, but do not support model B.

Although model A of Figure 1 is consistent with most of the experimental data so far obtained, exact domain structure of the COOH-terminal region remained to be elucidated. S-Cyanylation of intact fibronectin predominantly produced three fragments, i.e., sc155K, sc145K, and sc125K, and there is much evidence indicating that the sc145K fragment is derived from NH_2-terminal portion of both α and β subunits, as described above. It is reasonable to assume that sc155K and sc125K fragments represent the COOH-terminal half of the subunit chains. Indeed, this is supported by the following observations: (1) These fragments do not contain 24K or 40K domain, which is located in the NH_2-terminal portion. (2) These fragments must be connected to each other or to uncleaved subunit chains by disulfide bonding(s), which has been shown to be located at the COOH-terminal end. Further studies are in progress to settle these domain structures by using specific antibodies directed to these regions and is the subject of the subsequent paper.

Previously, cleavage of cellular fibronectin of hamster fibroblasts by two-step cyanylation was reported from this laboratory (Fukuda & Hakomori, 1979). This paper tentatively proposed a model in which two cysteine residues were located at 95000 and 180000 daltons from one of the terminals of the subunit chain. Later, however, we found that the two-step cleavage by DTNB and KCN accompanied significant cleavage at cystine. Therefore, the previous model has to be revised with data obtained by one-step cleavage of the same material. Similarly, Wagner & Hynes (1980) also applied the two-step procedure to locate the cysteine residues of hamster cellular fibronectin. They observed that the two-step method cleaved both cysteine and cystine residues, but the former was more readily cleaved. Thus, they found that fragments with M_r 170000 and M_r 30000 were selectively produced in the early stage of the cleavage, although the 30K fragment gave a much fainter band on an acrylamide gel. These fragments seem to correspond to the sc145K and sc35K in the present report. They also detected several fragments with M_r 150000, 130000, and 110000, two of which may correspond to the sc155K and sc125K fragments of the present

investigation, although they assumed that these fragments were derived by gradual cleavage of the 170K fragment at cystine residues. The size difference between these fragments obtained by Wagner and Hynes and by us may reflect the structural difference between plasma and cellular fibronectin.

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References

Alexander, S. S., Jr., Colonna, G., Yamada, K. M., Pastan, I., & Edelhoch, H. (1978) *J. Biol. Chem. 253*, 5820–5824.

Balian, G., Click, E. M., Crouch, E., Davidson, J. M., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 1429–1432.

Balian, G., Click, E. M., & Bornstein, P. (1980) *J. Biol. Chem. 255*, 3234–3236.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Chen, A. B., Amrani, D. L., & Mosesson, M. W. (1977) *Biochim. Biophys. Acta 493*, 310–322.

Cuatrecasas, P., & Anfinsen, C. B. (1971) *Methods Enzymol. 22*, 345–378.

Degani, Y., & Patchornik, A. (1974) *Biochemistry 13*, 1–11.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Engel, J., Odermatt, E., Engel, A., Madri, J. A., Furthmayr, H., Rohde, H., & Timpl, R. (1981) *J. Mol. Biol. 150*, 97–120.

Fukuda, M., & Hakomori, S. (1979) *J. Biol. Chem. 254*, 5442–5450.

Furie, M. B., & Rifkin, D. B. (1980) *J. Biol. Chem. 255*, 3134–3140.

Furie, M. B., Frey, A. B., & Rifkin, D. B. (1980) *J. Biol. Chem. 255*, 4391–4394.

Gahmberg, C. G., & Hakomori, S. (1973a) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3329–3333.

Gahmberg, C. G., & Hakomori, S. (1973b) *J. Biol. Chem. 248*, 4311–4317.

Gold, L. I., Garcia-Pardo, A., Frangione, B., Franklin, E. C., & Pearlstein, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4803–4807.

Grinnell, F. (1978) *Int. Rev. Cytol. 53*, 65–144.

Hahn, L. E., & Yamada, K. M. (1979) *Cell (Cambridge, Mass.) 18*, 1043–1051.

Hayashi, M., & Yamada, K. M. (1981) *J. Biol. Chem. 256*, 11292–11300.

Hörmann, H., & Seidl, M. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1449–1452.

Hynes, R. O. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3170–3174.

Isemura, M., Yoshizawa, Z., Takahashi, K., Kosaka, H., Kojima, N., & Ono, T. (1981) *J. Biochem. (Tokyo) 90*, 1–9.

Jacobson, G. R., Schaffer, M. H., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248*, 6583–6591.

Keski-Oja, J., & Yamada, K. M. (1981) *Biochem. J. 193*, 615–620.

Klebe, R. J. (1974) *Nature (London) 250*, 248–251.

Laemmli, V. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McDonald, J. A., & Kelley, D. G. (1980) *J. Biol. Chem. 255*, 8848–8858.

Mosesson, M. W., & Amrani, D. L. (1980) *Blood 56*, 145–158.

Mosher, D. F. (1980) *Prog. Hemostasis Thromb. 5*, 145–158.

Mosher, D. F., Schad, P. E., & Vann, J. M. (1980) *J. Biol. Chem. 255*, 1181–1188.

Pearlstein, E. (1976) *Nature (London) 262*, 497–500.

Pearlstein, E., Gold, L. I., & Garcia-Pardo, A. (1980) *Mol. Cell. Biochem. 29*, 103–128.

Ruoslahti, E., Engvall, E., & Hayman, E. G. (1981a) *Collagen Res. 1*, 95–128.

Ruoslahti, E., Hayman, E. G., & Engvall, E. (1981b) *J. Biol. Chem. 256*, 7277–7281.

Saba, T. M., Blumenstock, F. A., Weber, P., & Kaplan, J. E. (1978) *Ann. N.Y. Acad. Sci. 312*, 43–55.

Sekiguchi, K., & Hakomori, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2661–2665.

Sekiguchi, K., & Hakomori, S. (1981) *J. Cell Biol. 91*, 156a.

Sekiguchi, K., Fukuda, M., & Hakomori, S. (1981) *J. Biol. Chem. 256*, 6452–6462.

Smith, D. E., Mosher, D. F., Johnson, R. B., & Furcht, L. T. (1982) *J. Biol. Chem. 257*, 5831–5838.

Stark, G. R. (1977) *Methods Enzymol. 47*, 129–132.

Vaheri, A., & Mosher, D. F. (1978) *Biochim. Biophys. Acta 516*, 1–25.

Wagner, D. D., & Hynes, R. O. (1979) *J. Biol. Chem. 254*, 6746–6754.

Wagner, D. D., & Hynes, R. O. (1980) *J. Biol. Chem. 255*, 4304–4312.

Yamada, K. M., & Olden, K. (1978) *Nature (London) 275*, 179–186.