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Reactivity of Chemically Cross-Linked Fibrinogen and Its Fragments D toward the Staphylococcal Clumping Receptor†

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Received December 5, 1986; Revised Manuscript Received April 15, 1987

ABSTRACT: It has been established that the binding domain for the staphylococcal clumping receptor exists in fragment D of human fibrinogen [Hawiger J., Timmons, S., Strong, D. D., Cottrell, B. A., Riley, M., & Doolittle, R. F. (1982) *Biochemistry 21*, 1407; Strong, D. D., Laudano, A., Hawiger, J., & Doolittle, R. F. (1982) *Biochemistry 21*, 1414]. To examine the role of valency in the adhesive function of fibrinogen, its fragments were prepared by digestion with plasmin in the presence of calcium and purified by a two-step chromatographic procedure. Fragments D_1 and E did not induce the staphylococcal clumping reaction. After they were prepared in oligomeric form by chemical cross-linking with glutaraldehyde, fragment D_1 (M_r 94000) became functionally reactive toward the staphylococcal clumping receptor, and fragment D_3 (M_r 75000) and fragment E (M_r 50000) remained inactive. Fragment D dimer derived from enzymatic cross-linking was not reactive. Human fibrinogen cross-linked with glutaraldehyde usually reached a 250 times higher reactivity toward the staphylococcal clumping receptor, depending on the condition of the cross-linking reaction. It is concluded that the valency of fibrinogen in regard to its receptor binding domain and the availability of this domain are essential for the staphylococcal clumping reaction.

Fibrinogen, which has a dimeric structure, is able to cross-bridge receptors present on individual staphylococci or platelets, forming visible aggregates of these cells (Hawiger et al., 1982a,b; Strong et al., 1982; Kloczewiak et al., 1984). Although a plasmin degradation product of fibrinogen, fragment D_1,[1] still binds to staphylococci and inhibits the binding and the clumping reaction of intact fibrinogen, no staphylococcal clumping reaction could be elicited by fragment D_1 (Hawiger et al., 1982a). To test the hypothesis that the cell agglutinating property of fibrinogen toward staphylococci was dependent on the dimeric structure of fibrinogen, we isolated monovalent fibrinogen fragments D_1 and D_3 which possess or lack, respectively, the binding domain for the staphylococcal clumping receptor (Hawiger et al., 1982a; Strong et al., 1982). The 15-residue carboxy-terminal segment of the γ-chain that interacts with the staphylococcal clumping receptor also bears sites for enzymatic cross-linking by factor XIIIa (Chen & Doolittle, 1970; Strong et al., 1982). Therefore, we compared chemically cross-linked D_1 oligomers with enzymatically cross-linked fragment D (D dimer) to see whether factor XIIIa induced cross-links interfere with receptor recognition of the

† This work was supported by Research Grants HL-30647, HL-33014, and HL-35437 from the National Institutes of Health, U.S. Public Health Service.

[1] Abbreviations: CU, casein unit(s); TIU, trypsin inhibitor unit(s); Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; CM, carboxymethyl; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; PPACK, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone; $NaBH_4$, sodium borohydride. Fragments obtained by plasmic degradation of fibrinogen are denoted as follows: D_1 is a 94-kDa fragment from the carboxy-terminal domain encompassing chain remnants α105-206, β134-461, and γ63-411; D_3 is a 75-kDa fragment derived from D_1 by proteolytic removal of the carboxy terminal of the γ-chain resulting in the remnant γ63-302; D dimer is an enzymatically cross-linked fragment D via ϵ-(γ-glutamyl)lysyl isopeptide bonds between the carboxy-terminal regions of the two γ-chain remnants; E is a 50-kDa fragment derived from the central, amino-terminal domain of fibrinogen and encompassing chain remnants α24-78, β54-120, and γ1-53.

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carboxy-terminal domain of the human fibrinogen γ-chain.

Materials and Methods

Human fibrinogen (grade L, Kabi, Stockholm, Sweden) was used in most experiments without further purification. In some experiments human fibrinogen was purified further on Sepharose CL-4B according to the procedure described by Timmons and Hawiger (1986).

Proteolytic degradation of fibrinogen with plasmin was carried out in the presence of 1 mM calcium with 4 CU of human plasmin (Kabi, Stockholm, Sweden) for 1 g of fibrinogen. The reaction was conducted for 4 h at 37 °C and stopped with 16 TIU of aprotinin.

Preparation of Fragments of D_1, D_3, and E. The mixture of fibrinogen degradation products was dialyzed overnight against 0.01 M Tris-HCl buffer, pH 7.4, with 4 mM $CaCl_2$ at 4 °C. The dialyzed solution was filtered through Whatman No. 1 filter paper and applied on a DEAE-Sepharose CL-6B (Pharmacia, Sweden) column (2 × 25 cm) equilibrated with the above buffer. Protein was eluted with a linear gradient (1500 mL) of NaCl (0–0.5 M) in the same buffer. Protein fragments were identified by $NaDodSO_4$–polyacrylamide (5–15% gradient) microslab gel electrophoresis (Fling & Gregerson, 1986). Peaks containing fragments D_1, D dimer, and E were pooled, freeze-dried, and dissolved in water. The pool containing fragment D_1 and D dimer was chromatographed on a Sepharcyl S-200 superfine (Pharmacia, Sweden) column (2.5 × 100 cm) equilibrated in 0.01 M Tris-HCl buffer, pH 7.4, with 0.1 M NaCl and 2 mM $CaCl_2$. Fractions containing fragment D_1 and D dimer were collected, freeze-dried, dissolved in water, and dialzyed against the buffer used for the cross-linking reaction. The occurrence of fragment D dimer in the plasmin digest of commercial fibrinogen was prevented when the thrombin inhibitor (PPACK) was added to the incubation mixture.[2]

Fractions containing fragment E were dialyzed against 0.05 M sodium acetate buffer, pH 5.0, and applied on a CM-Sepharose CL-6B (Pharmacia, Sweden) column (2 × 25 cm) equilibrated with the same buffer. Protein was eluted with a NaCl gradient (0.0–0.3 M) in the same buffer (800-mL total volume). If not pure, fragment E was rechromatographed on a Sephadex G-100 column (2 × 100 cm) equilibrated with 10% acetic acid. Fragment E prepared in this way was soluble in the buffers used for cross-linking.

Fragment D_3 was prepared by further degradation of fragment D_1 by addition of 0.2 CU of plasmin to 50 mg of fragment D_1 in 0.02 M Tris-HCl, buffer, pH 7.6, with 0.1 M NaCl and 2 mM EDTA. Proteolysis was carried out for 16 h at 37 °C in the presence of 0.01% NaN_3. Fragment D_3 was isolated from the reaction mixture by gel filtration on a Sephadex G-100 column (2 × 100 cm) equilibrated with 0.01 M Tris-HCl buffer, pH 7.4, with 0.1 M NaCl, 2 mM EDTA, and 0.01% NaN_3. The purity of prepared fragments was checked as above by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Determination of sedimentation velocity of purified fragments D_1 and D_3 was carried out in a Beckman Spinco E analytical ultracentrifuge equipped with a schlieren optical system. All measurements were carried out at 20 °C. No correction for salt viscosity (0.1 M NaCl) was introduced. Values of the sedimentation coefficient, $s^0_{20,w}$, were obtained by linear extrapolation of the experimental data to zero protein concentration.

[2] M. Kloczewiak, unpublished experiments.

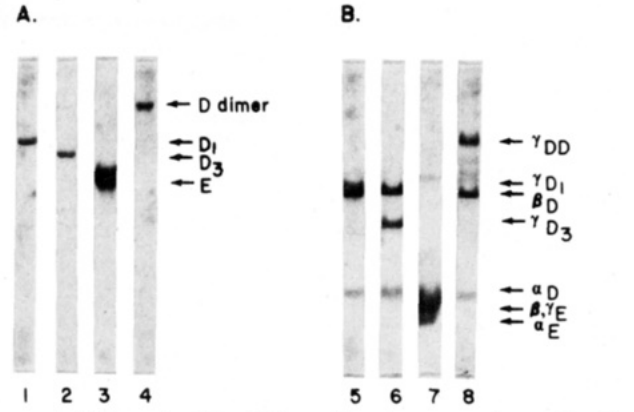

FIGURE 1: Polyacrylamide gel electrophoresis pattern of nonreduced (panel A) and reduced (panel B) fragments of fibrinogen (D_1, D_3, and E) and fibrin (D dimer). Electrophoresis was developed in the presence of $NaDodSO_4$ in a 5–15% linear gradient gel. Fragments: (lanes 1 and 5) D_1; (lanes 2 and 6) D_3; (lanes 3 and 7) E; (lanes 4 and 8) D dimer.

Staphylococcal clumping reaction was carried out in the system described by Hawiger et al. (1982a) with *Staphylococcus aureus* Newman D_2C possessing the staphylococcal clumping receptor and *Staphylococcus epidermidis* Zak strain lacking the staphylococcal clumping receptor.

Chemical Cross-Linking of Fibrinogen and Its Fragments. Purified fragments D_1, D dimer, D_3, and E and human fibrinogen (2 mg/mL each) were cross-linked with 10 mM glutaraldehyde following the conditions used by Furlan and Beck (1975), employing a buffer composed of 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.5, and containing 4 mM EDTA or 4 mM $CaCl_2$. The time of the cross-linking reaction usually varied from 0.5 to 192 h. The reaction was stopped by addition of lysine to a final concentration of 20 mM. As a comparative control, human serum albumin was also cross-linked under the same conditions. Reduction of Schiff bonds was carried out in the lysine-blocked samples by the addition of 0.5 M $NaBH_4$ in water to a final concentration of 35 mM. After 3 h of reduction, the excess of reagents was removed by dialysis overnight at 4 °C against the buffer used for the cross-linking reaction.

Results

Characteristics of Fibrinogen Fragments Used for Chemical Cross-Linking. Under nonreducing (Figure 1A) and reducing (Figure 1B) conditions, the fragments D_1 and D dimer were homogeneous and were composed of the remnants of chains very similar to those previously described by Cierniewski et al. (1986). Fragment D_3 has an apparent M_r of 75 000 containing a γ-chain remnant of apparent M_r 25 000. Fragment E (apparent M_r 50 000) was composed of remnants of α-, and β-, and γ-chains of apparent M_r 7000–10 000 (Figure 1B).

Sedimentation velocity analysis was carried out to show that fragments D_1 and D_3 appear in the monomeric form in the buffers used for the clumping reaction. Fragment D_1 sedimented as a single symmetrical peak with $s^0_{20,w}$ = 5.0 S in 0.05 M Tris-HCl–0.1 M NaCl buffer, pH 7.4, containing 2 mM $CaCl_2$ or 2 mM EDTA. Fragment D_3 had $s^0_{20,w}$ = 4.3 S in EDTA-containing buffer and $s^0_{20,w}$ = 3.8 S in calcium-containing buffer. These sedimentation coefficient values correspond to fragments D_1 and D_3 of M_r 9400 and 75 000, respectively, calculated from the approximate formula (Halsall, 1967). The value of the M_r of 94 000 calculated from the sedimentation coefficient determined by us for fragment D_1 is very close to the value obtained for fragment D_{CATE} by van der Drift and Poppema (1982).

Table I: Effect of Chemical Cross-Linking on Reactivity of Fibrinogen and Its Fragments toward Staphylococcal Clumping Receptor

	staphylococcal clumping reaction[a]						
		1 hr		8 h		192 h	
tested material	0 h[b]	EDTA	$CaCl_2$	EDTA	$CaCl_2$	EDTA	$CaCl_2$
fibrinogen	3.9	0.03	0.03	0.06	0.004	0.24	0.97
fragment D_1 (94 kDa)	none	7.8	7.8	3.9	1.9	7.8	7.8
fragment D_3 (75 kDa)	none	none	none	none	none	none	none
fragment E (50 kDa)	none	none	none	none	none	none	none

[a] Reactivity is expressed as the minimal concentration (μg/mL) of fibrinogen and its fragments causing the staphylococcal clumping reaction. [b] Duration of the cross-linking reaction with 0.01 M glutaraldehyde buffered with 0.05 M Tris buffer, pH 7.5, containing 0.1 M NaCl and 4 mM EDTA or 4 mM $CaCl_2$.

Reactivity of Monovalent and Polyvalent Fibrinogen Fragments toward Staphylococcal Clumping Receptor. Whereas native fibrinogen induced the staphylococcal clumping reaction at a concentration range of 1.9–3.9 μg/mL, neither of the isolated "monomeric" fragments D_1 or D_3 induced the clumping reaction up to a 1000-fold higher concentration (4 mg/mL). A control protein, human serum albumin, did not cause the clumping reaction at concentrations up to 10 mg/mL. However, after chemical cross-linking some of the fragments of fibrinogen became reactive with the staphylococcal clumping receptor and induced the clumping reaction (Table I). It is evident that fragment D_1, possessing an intact carboxy-terminal part of fibrinogen's γ-chain, after chemical cross-linking reached a reactivity comparable to that of native fibrinogen. On the other hand, the more degraded product, fragment D_3, which lacks the binding domain for the staphylococcal clumping receptor (Hawiger et al., 1982a), exhibited no clumping reactivity after chemical cross-linking. No reactivity of cross-linked fragment E or human serum albumin (data not shown) was observed for any time of the cross-linking reaction. Fragment D dimer resulting from enzymatic cross-linking of fibrin by factor XIIIa was unable to induce the staphylococcal clumping reaction up to 8 mg/mL. However, after subsequent chemical cross-linking with glutaraldehyde, it became reactive toward staphylococci at 50 μg/mL. When fragment D dimer was treated with plasmin (0.25 CU/mg of protein) in the presence of 5 mM $CaCl_2$ for 3 h and then chemically cross-linked, it caused the staphylococcal clumping reaction at 200 μg/mL. This was a 33 times lower reactivity than chemically cross-linked fragment D_1 (6 μg/mL). The electrophoretic pattern of nonreduced fragment D dimer remained unchanged after digestion by plasmin, indicating that only a very small portion, most likely the carboxy-terminal end of the γ-chain, was cleaved.

The reactivity of fibrinogen after 8 h of the cross-linking reaction in the presence of $CaCl_2$ increased by 2–3 orders of magnitude as compared to that of fibrinogen not treated with glutaraldehyde (0 h). Prolongation of the reaction time beyond 8 h (up to 192 h) decreased the reactivity of all preparations presumably because the carboxy-terminal segment of their γ-chains was also chemically cross-linked and less available for interaction with the clumping receptor on staphylococci. The results obtained with chemically cross-linked human fibrinogen (Kabi) were verified with preparations of Kabi fibrinogen that was further purified. The minimal concentration of fibrinogen causing staphylococcal clumping reaction was 1.5 μg/mL before cross-linking with glutaraldehyde and 0.006 μg/mL after cross-linking, a 250-fold increase in clumping reactivity. None of the chemically cross-linked fragments or fibrinogen gave positive clumping reaction with the Zak strain of *Staphylococcus epidermidis*, which does not possess the clumping factor receptor (Hawiger et al., 1982a).

Chemical modification of proteins with glutaraldehyde results in the formation of a labile Schiff base forming bonds between side-chain amino groups of modified fragments of fibrinogen. To prevent the possible exchange of amino groups of the treated fragment with amino groups of the staphylococcal clumping receptor, which could cause "nonspecific" clumping, we used $NaBH_4$ to reduce the Schiff bases to stable amino–alkyl bonds (Means & Feeney, 1968). Chemically cross-linked (0.5 h) fragment D_1, which was treated with $NaBH_4$, had similar reactivity toward staphylococci (22 μg/mL) as a preparation not treated with $NaBH_4$.

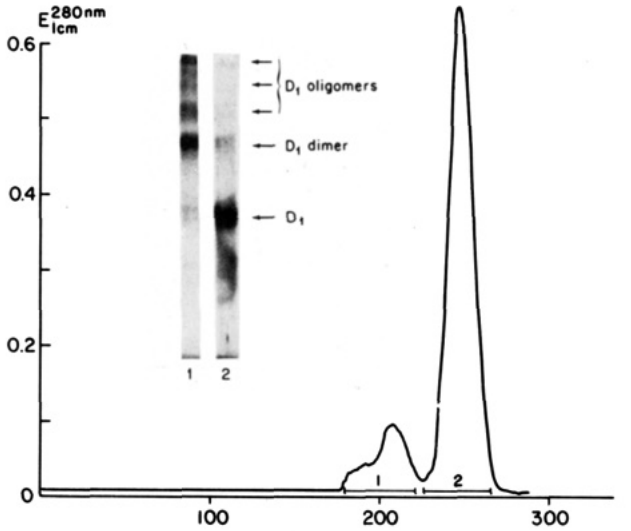

FIGURE 2: Sephacryl S-200 gel filtration of fragment D_1 preparation after cross-linking with glutaraldehyde. The conditions of gel filtration were the same as in the preparation of fragment D_1 (see Materials and Methods for details). The conditions for chemical cross-linking were as described under Materials and Methods except that the fragment D_1 concentration was 4 mg/mL. (Inset) $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5%) of nonreduced pooled fractions from peak 1 (lane 1) and peak 2 (lane 2).

To verify whether the treatment with glutaraldehyde results in formation of oligomers of fragment D_1 rather than modification and intrafragment cross-linking of monomeric fragment D_1, a mixture of fragment D_1 treated with glutaraldehyde in the presence of 4 mM $CaCl_2$ for 1 h was applied on a Sephacryl S-200 column (Figure 2). Two peaks were eluted; the first contained predominantly the dimer of fragment D_1 (apparent M_r 200000) and a small amount of higher molecular weight oligomers, as shown in $NaDodSO_4$–polyacrylamide gel electrophoresis (inset in Figure 2). The second peak contained predominantly monomeric fragment D_1 (M_r 94000). Only material present in the first peak was reactive with staphylococci at the concentration of 13 μg/mL.

Discussion

The interaction of fibrinogen with staphylococci is one of several examples of the cell agglutinating properties of this adhesive plasma protein. Fibrinogen is also known to interact with cell receptors on streptococci (Timmons et al., 1983),

platelets (Hawiger et al., 1982b), macrophages (Gonda & Shainoff, 1982), and endothelial cells (Timmons & Hawiger, 1984; Dejana et al., 1985). Positioning of the binding domain for the staphylococcal clumping receptor on the carboxy-terminal segment of the human fibrinogen's two γ-chains (residues 397–411) fulfills the criteria for divalency that is required for the agglutinating function of fibrinogen in regard to staphylococci. This was supported further by constructing an artificial clumping molecule by coupling the synthetic peptide analogue of this domain to bovine plasma albumin (Strong et al., 1982). Although this domain is present in fragment D_1 and not in fragment D_3 (Hawiger et al., 1982a), there is a possibility that an additional site in fragment D_1 may contribute to the interaction with staphylococci. However, the inability of monomeric fragment D_1 to induce staphylococcal clumping militates against such a possibility and indicates that the induction of the clumping reaction requires a higher-than-one level of valency of the reactive γ carboxy terminal. Chemical cross-linking with glutaraldehyde produced functionally reactive oligomers of fragment D_1 only when the specific binding domain was preserved and available to interact with the receptor on staphylococci. No other fragment of fibrinogen studied by us (fragment D_3 and fragment E) non-cross-linked or chemically cross-linked could support the clumping reaction. The enhancing effect of calcium on the functional properties of cross-linked fibrinogen and fragment D_1 is compatible with the localization of the calcium binding site in the human fibrinogen γ-chain in proximity to the binding domain for staphylococci (Strong et al., 1982; Dang et al., 1985).

The interpretation of our results is illustrated in Figure 3. Isolated fragment D_1 with only one carboxy-terminal segment of the γ-chain is monovalent in regard to the staphylococcal clumping receptor and cannot induce clumping. Chemically cross-linked fragment D_1 has a valency of 2 or possibly higher. Since the carboxy-terminal segment of the γ-chain is not chemically cross-linked, it can react with the staphylococcal clumping receptor and mediate the clumping reaction at the concentration range of native fibrinogen. Interestingly, enzymatically cross-linked fragment D dimer, although it has two carboxy-terminal segments of the γ-chain thus fulfilling the requirement for divalency, does not mediate the clumping of staphylococci. Enzymatic cross-linking with factor XIII involves glutamine-398 and lysine-406 in the carboxy-terminal segment of the γ-chain, the same segment that is involved in the interaction with the staphylococcal clumping receptor (Chen & Doolittle, 1971; Strong et al., 1982). Apparently, enzymatically induced double cross-links form a nonpermissive arrangement of two adjoining carboxy-terminal segments of the γ-chain. In contrast, chemically induced cross-links with glutaraldehyde seem to spare this segment of the γ-chain when cross-linking is performed for 10 h or less, thus preserving its reactivity toward the staphylococcal clumping receptor. It should be noted that when enzymatically cross-linked fragment D dimer was further chemically cross-linked, it gained reactivity toward staphylococci, causing their clumping, although it required 4–8 times higher concentration as compared to chemically cross-linked fragment D_1. Moreover, 20% of the cross-linking sites available in fragment D_1 were still "free" in fragment D dimer on the basis of incorporation of dansylcadaverine.[2] These results indicate the presence of some partially cross-linked carboxy-terminal segments of the γ-chains that were able to cause clumping because fragment D dimer was chemically polymerized to a higher level of valency in regard to the staphylococcal clumping receptor. These

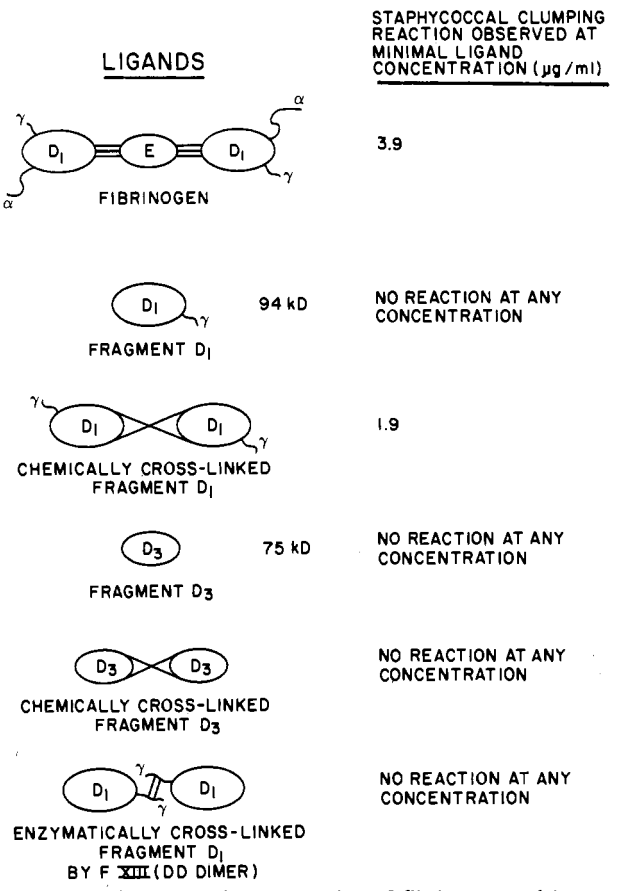

FIGURE 3: Diagrammatic presentation of fibrinogen and its non-cross-linked and cross-linked fragments. Please note the difference in reactivity toward the staphylococcal clumping receptor depending on the availability and valency of the carboxy-terminal segment of the γ-chain. Fragments D_1 that are chemically or enzymatically cross-linked differ in their reactivity although both possess carboxy-terminal segments of the γ-chain.

partially cross-linked carboxy-terminal segments were removed by plasmin, resulting in further reduction of clumping reactivity of chemically cross-linked fragment D dimer (3% reactivity as compared to that of chemically cross-linked fragment D_1).

An interesting finding in our experiments was a significant increase in the reactivity of human fibrinogen after chemical cross-linking with glutaraldehyde. Formation of fibrinogen oligomers reduced the concentration required for the clumping reaction by 1–3 orders of magnitude. In other words, instead of approximately 2000 molecules of fibrinogen required to saturate available binding sites on the staphylococcus and producing visible clumping reaction (Hawiger et al., 1982a), only 2 to 8 molecules of chemically cross-linked fibrinogen are needed. Such an increase may have a bearing on the interaction of staphylococci with "polymerized" but not enzymatically cross-linked fibrinogen deposited on damaged vascular surfaces, e.g., heart valves (Durack, 1975), thus preventing removal of firmly seeded staphylococci by the high shear rate of flowing blood. Our observations with chemically cross-linked fibrinogen and staphylococcal clumping are paralleled by recent evidence showing that soluble polymers of fibrinogen produced by its interaction with $F(ab)_2$ fragments of antibodies against fibrinogen's E (central) domain have a stronger propensity toward human platelets than native plasma fibrinogen (McManama et al., 1986).

In summary, our experiments with monomeric and dimeric fragment D obtained after chemical or enzymatic cross-linking show that the valency of fibrinogen in regard to its receptor

recognition domain and the availability of this domain are essential for the staphylococcal clumping reaction.

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A Gene for Rabbit Synovial Cell Collagenase: Member of a Family of Metalloproteinases That Degrade the Connective Tissue Matrix†

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Received March 9, 1987; Revised Manuscript Received May 15, 1987

ABSTRACT: We have determined the nucleotide sequence of a collagenase mRNA from rabbit synovial cells from which the primary structure of the encoded protein was deduced. This proteinase is 51% homologous to the enzyme that activates it from the zymogen form, rabbit synovial cell activator/stromelysin. Rabbit collagenase and activator/stromelysin thus share comembership in a gene family that includes human skin collagenase; the human and rabbit metalloproteinase, activator/stomelysin; and an oncogene-induced proteinase from rat named transin. The mRNA sequence of collagenase enabled us to completely map the structure of its gene, which is 9.1 kilobases and is composed of 10 exons and 9 introns. This is the first report of the structure of a collagenase gene. We show that it has striking similarity to additional members of this metalloproteinase gene family, transin genes I and II of rat. We have further sequenced genomic DNA flanking the collagenase gene and have identified nucleic acid elements of possible importance in gene regulation.

Collagen is the most prevalent protein in the body, and its metabolism plays a central role in many normal biological processes (Gross, 1982; Hay, 1984). The capacity to control collagen degradation also appears to be an essential characteristic of malignant tumors, allowing their successful invasion of surrounding tissues (Liotta et al., 1984; Mignatti et al., 1986). Furthermore, a number of other pathological conditions are marked by inappropriate or excessive collagenolysis (Wooley & Evanson, 1980). The metalloproteinase collagenase is the rate-limiting enzyme in this process. It is secreted from cells as an inactive proenzyme and can be activated by other proteinases found in the extracellular matrix (Harris et al., 1984). In rabbits, conversion of procollagenase to the active enzyme appears to require a specific metalloproteinase called activator, which is itself secreted as a proenzyme (Vater et al., 1983). Thus the control of collagenolysis in rabbits appears to be regulated via a minicascade of proteinases. Recently, rabbit activator has been shown to be identical with stromelysin, a metalloproteinase originally isolated from rabbit fibroblasts that has the ability to degrade noncollagenous matrix (Chin et al., 1985; Whitham et al., 1986; Fini et al., 1987).

This laboratory has focused on collagen turnover in rheumatoid arthritis, a disease in which excessive collagenase secretion by synovial fibroblasts lining the joints results in ex-

† Supported in part by grants from the USPHS (NIH-AR26599), from the New Hampshire and National Chapters of the Arthritis Foundation, from the RGK Foundation (Austin, TX), and from the Hitchcock Foundation.

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0006-2960/87/0426-6156$01.50/0