Atger, M., & Milgrom, E. (1976) *J. Biol. Chem. 251*, 4758–4762.
Bailly, A., LeFevre, B., Savouret, J.-F., & Milgrom, E. (1980) *J. Biol. Chem. 255*, 2729–2734.
Carlstedt-Duke, J., Wrange, O., Okret, S., Stevens, J., Stevens, Y.-W., & Gustafsson, J.-A. (1983) in *Gene Regulation and Steroid Hormones* (Roy, A. K., & Clark, J. H., Eds.) pp 151–180, Springer-Verlag, Heidelberg.
Dahmer, M. K., Housley, P. R., & Pratt, W. B. (1984) *Annu. Rev. Physiol. 46*, 67–81.
Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., & Evans, R. M. (1986) *Cell (Cambridge, Mass.) 46*, 645–652.
Godowski, P. J., Rusconi, S., Miesfeld, R., & Yamamoto, K. R. (1987) *Nature (London) 325*, 365–368.
Gorski, J., & Gannon, F. (1976) *Annu. Rev. Physiol. 38*, 425–450.
Green, S., & Chambon, P. (1986) *Nature (London) 324*, 615–617.
Green, S., & Chambon, P. (1987) *Nature (London) 325*, 75–78.
Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.-M., Argos, P., & Chambon, P. (1986) *Nature (London) 320*, 134–139.
Grody, W. W., Schroder, W. T., & O'Malley, B. W. (1982) *Endocr. Rev. 3*, 141–163.
Hansen, J. C., & Gorski, J. (1985) *Biochemistry 24*, 6078–6085.
Hansen, J. C., & Gorski, J. (1986) *J. Biol. Chem. 261*, 13990–13996.
Hollenberg, S. M., Giguere, V., Segui, P., & Evans, R. M. (1987) *Cell (Cambridge, Mass.) 49*, 39–46.
Hutchens, T. W., Dunaway, H. E., & Besch, P. K. (1985) *J. Chromatogr. 327*, 247–259.
Katzenellenbogen, J. A., Johnson, H. J., Jr., & Carlson, K. E. (1973) *Biochemistry 12*, 4092–4099.
Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.-M., & Chambon, P. (1986) *EMBO J. 5*, 891–897.
Kumar, V., Green, S., Staub, A., & Chambon, P. (1986) *EMBO J. 5*, 2231–2236.
Luttge, W. G., & Densmore, C. L. (1984) *J. Neurochem. 42*, 242–247.
Moudgil, V. K., & Hurd, C. (1987) *Biochemistry 26*, 4994–5001.
Moudgil, V. K., Eessalu, T. E., Buchou, T., Renoir, J.-M., Mester, J., & Baulieu, E.-E. (1985) *Endocrinology (Baltimore) 116*, 1267–1274.
Moudgil, V. K., Lombardo, G., Eessalu, T., & Eliezer, N. (1986) *Biochem. J. 99*, 1005–1016.
Moudgil, V. K., Vandenheede, L., Hurd, C., Eliezer, N., & Lombardo, G. (1987) *J. Biol. Chem. 262*, 5180–5187.
Muller, R. E., Traish, A. M., Hirota, T., Bercel, E., & Wotiz, H. H. (1985) *Endocrinology (Baltimore) 116*, 337–345.
Notides, A. C., Hamilton, D. E. & Auer, H. E. (1975) *J. Biol. Chem. 250*, 3945–3950.
Redeuilh, G., Secco, C., Mester, J., & Baulieu, E.-E. (1987) *J. Biol. Chem. 262*, 5530–5535.
Renoir, J.-M., Buchou, T., & Baulieu, E.-E. (1986) *Biochemistry 25*, 6405–6413.
Sakai, D., & Gorski, J. (1984) *Biochemistry 23*, 3541–3547.
Sherman, M. R., & Stevens, J. (1984) *Annu. Rev. Physiol. 46*, 83.
Skafar, D. F., & Notides, A. C. (1985) *J. Biol. Chem. 260*, 12208–12213.
Willmann, T., & Beato, M. (1986) *Nature (London) 324*, 688–691.

Complexation of Fibronectin with Tissue Transglutaminase†

P. M. Turner and L. Lorand*
Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208
Received March 30, 1988; Revised Manuscript Received August 30, 1988

ABSTRACT: Previous work [Lorand, L., Dailey, J. E., & Turner, P. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1057–1059] showed that fibronectin might serve as a specific carrier for transglutaminases accidentally discharged from erythrocytes or other cells into plasma. In the present study we examined the association of these proteins in purified systems. Complexation was readily demonstrable by nondenaturing electrophoresis, using dansylcadaverine-dependent activity staining as well as immunoblotting procedures, and also by HPLC gel filtration. The results indicate a stoichiometry of 2:1 for the binding of the human erythrocyte transglutaminase (80K) to human plasma fibronectin (440K). The attachment is noncovalent in nature and does not involve cross-linking of the proteins either to themselves or to each other. Binding occurs in the absence of Ca^{2+}, suggesting that a domain on the transglutaminase molecule other than the catalytic site is needed for complexation with fibronectin. Limited proteolysis with chymotrypsin for delineating the relevant region in fibronectin yielded two gelatin- (collagen) binding fragments (56K and 46K), each displaying affinity for transglutaminase. Moreover, these fragments—like intact fibronectin—bound erythrocyte transglutaminase and gelatin simultaneously in ternary complexes.

In terms of homeostatic regulation, the binding of erythrocyte transglutaminase to fibronectin in plasma (Lorand et al., 1988) may be analogous to the association of hemoglobin with haptoglobin [for a review, see Putnam (1975) and Lowe and Ashwell (1982)]. In intravascular hemolysis, for instance, both transglutaminase and hemoglobin would be released from red cells and would have to be cleared from the fluid phase of blood by processes in which the respective carrier proteins can be assumed to play important roles. Being widely distributed in tissues, transglutaminase could also be discharged as a result

† This work was aided by a USPHS Research Career Award (HL-03512) and by grants from the National Institutes of Health (HL-16346 and HL-02212).

0006-2960/89/0428-0628$01.50/0

of damage to cells other than erythrocytes, and its presence in the free form in plasma would probably pose great danger. For example, the uncontrolled cross-linking of proteins in the circulation might give rise to thrombotic plugs even though neither thrombin nor fibrin generation occurred. These considerations prompted us to examine the fate of a tissue transglutaminase coming in contact with plasma. Simulating the hemolytic situation, the membrane-depleted lysate of human erythrocytes was added to human plasma, and the striking observation was made that, immediately upon mixing, there was a major change in the electrophoretic behavior of transglutaminase. The otherwise fast anodic mobility of transglutaminase was reduced to about half of its value in plasma under the nondenaturing conditions of the analytical procedure (pH 7.25, $\mu \sim 0.1$). Since fibronectin-depleted plasma had no effect, it was inferred that the shift in the mobility of transglutaminase was due to complexation with fibronectin (Lorand et al., 1988).

In order to corroborate the conclusion that the transglutaminase-binding property of plasma resided with the fibronectin molecule and in order to gain further insights into the complexation process between the two proteins, experiments with purified components were necessary. The present paper describes the interaction of the transglutaminase, isolated from human erythrocytes, with purified human fibronectin and also with gelatin-binding fragments obtained by the chymotryptic digestion of fibronectin. An abstract of this work has been published (Turner & Lorand, 1988).

Materials and Methods

Protein Preparations. Human red blood cell transglutaminase was kindly prepared by J. Colaluca using a modification of published methods (Brenner & Wold, 1978). The product was taken up in 50 mM Tris-HCl[1] and 1 mM EDTA and stored at −70 °C.

Human plasma fibronectin was obtained as a byproduct of factor XIII isolation (Molnar et al., 1979). The protein was further purified by affinity chromatography according to Vuento and Vaheri (1979) using an Affi-Gel 10–gelatin column. This material was prepared by coupling 25 mL of 2% gelatin (ICN Nutritional Biochemical Co., Cleveland, OH) in 0.1 M $NaHCO_3$ and 0.1 M NaCl to 20 mL of Affi-Gel 10 (Bio-Rad Labs, Richmond, CA) at room temperature for 1 h. Unreacted sites were blocked by treatment with 50 mM Tris-HCl, pH 7.5; the gel was washed with 500 mL of 2 M NaCl in 50 mM Tris-HCl and 1 mM EDTA at pH 7.5, and a column was prepared in the same buffer without NaCl. Approximately 58 mg of fibronectin was applied to 10 mL of Affi-Gel 10–gelatin (1.5 × 6 cm), and the bound protein was eluted with 1 M arginine. This solute was removed by passing the eluate through a Bio-Gel P-60 column (1.5 × 27.5 cm) with 50 mM Tris-HCl plus 0.5 mM EDTA at pH 7.5.

Chymotrypsin (3× recrystallized, Worthington Biochemicals, Freehold, NJ) at a concentration of 10 μg/mL was employed at 25 °C to digest fibronectin (2.2 mg/mL). At 6 min, aprotinin (50 μg/mL; Sigma Chemical Co., St. Louis, MO) was added, and the mixture was chilled on ice. The fibronectin digest (corresponding to about 15 mg of the parent protein) was applied to Affi-Gel 10–gelatin (1.2 × 2.7 cm) equilibrated with 50 mM Tris-HCl and 0.5 mM EDTA, pH 7.5. Further washing with 25 mL of the buffer was followed by a 20-mL wash at 2 M NaCl in buffer and a 10-mL wash again with the buffer alone. The chymotryptic fragments retained on the gelatin column were released by elution with 6 M urea in 12.5 mM Tris-HCl–0.13 mM EDTA, pH 7.5. Mixed-bed resin [AG501-X8(D); 1 × 17 cm, Bio-Rad] was used for deionization of the freshly prepared urea solution (at 8 M) prior to addition of the Tris–EDTA components. The urea was removed from the fibronectin fragments by passage through a 2 × 8 cm column of Bio-Gel P-10 (Bio-Rad), 50 mM Tris-HCl–0.5 mM EDTA, pH 7.5, being used for gel filtration. The protein was concentrated on a PM30 membrane (2.5-cm diameter, Amicon Corp., Lexington, MA).

The gelatin-binding fragments (0.7 mg) isolated from the first phase of chymotrypsin digestion of fibronectin were further treated with chymotrypsin (15 μg) in 1 mL of solution for 25 min at 25 °C. Digestion was again terminated by addition of aprotinin (75 μg) and by placing the mixture on ice for 30 min. The gelatin-binding fragments obtained from this digestion were purified on Affi-Gel 10–gelatin as described above. An approximately 46K product was obtained by gel filtration on Bio-Gel P-60 (1.5 × 27.5 cm), with 50 mM Tris-HCl and 0.5 mM EDTA as eluant.

Protein determinations were carried out by the Miller (1959) procedure, and mole quantities were calculated on the basis of apparent molecular weights of the proteins obtained by $NaDodSO_4$ electrophoresis: 440K for fibronectin, 80K for transglutaminase, 56K for the gelatin-binding early chymotryptic fragment of fibronectin, and 46K for the later digestion product with similar properties.

Rabbit antiserum to human plasma fibronectin was purchased from Cooper Biomedical Inc. (Malvern, PA). Antiserum to guinea pig liver transglutaminase, highly cross-reactive against the human red cell enzyme, was raised in our laboratory in rabbit.

Other Reagents. Heparin, single-stranded DNA, Coomassie Brilliant Blue, EDTA, NaCl, dansylcadaverine hydrochloride (converted to the fumarate salt by K. N. Parameswaran), 2-mercaptoethanol, dithiothreitol, buffers, and other reagents were obtained from Sigma Chemical Co. The ArgGlyAspSer tetrapeptide was a product of Peninsula Labs (Belmont, CA). Electrophoretic supplies were from Bio-Rad, agarose, and Gel Bond plastic support medium was from FMC Corp. (Rockland, ME). Nitrocellulose sheets (BA83) were purchased from Schleicher & Schuell (Keene, NH). Porous polyethylene was obtained from Fischer Scientific (Chicago, IL), nonfat dry milk from Carnation Co. (Los Angeles, CA), Vectastain Kit from Vector Labs (Burlingame, CA), and Hammersten casein from Mann Research (Malvern, PA).

Nondenaturing electrophoresis was performed (3 V/cm, 4 °C) essentially as previously described (Lorand et al., 1988) by applying 10-μL samples to 1% or 2% agarose gels (10 × 15 cm), with 75 mM imidazole hydrochloride and 0.5 mM EDTA, pH 7.25. Hammersten casein (0.3%, w/v) was included in those gels which were to be used for locating transglutaminase activity by staining with dansylcadaverine for about 3 h at 37 °C. The stain comprised 0.5 mM of the fluorescent amine, 6 mM $CaCl_2$, and 6 mM dithriothreitol in 50 mM Tris-HCl, pH 7.5. Unreacted dansylcadaverine was removed by washing in 20% methanol–10% acetic acid. Enzyme activity bands were viewed and photographed under UV illumination.

Immunoblots. Following nondenaturing electrophoresis, proteins were transferred by placing the agarose gel onto moist nitrocellulose above a wetted porous polyethylene sheet and

[1] Abbreviations: dansylcadaverine, *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

applying uniform pressure (ca. 30 g/cm^2) for 3–6 h. Unbound sites on the nitrocellulose were blocked by immersion for 30 min in 1% (w/v) nonfat dry milk dispersed in a solution of 200 mM NaCl and 25 mM Tris-HCl, pH 7.5. Incubation with the primary antisera against transglutaminase (1:2000 dilution) or against fibronectin (1:10 000 dilution) was carried out overnight in the above mixture of buffer and milk. Treatment with biotinylated goat antibody against rabbit IgG and with avidin–peroxidase, as well as color development, was carried out as described previously (Lorand et al., 1988).

Polyacrylamide electrophoresis in NaDodSO$_4$ was performed by the Laemmli procedure (1970). If necessary, samples were reduced prior to electrophoresis with 3% (v/v) 2-mercaptoethanol in 1.5% (w/v) NaDodSO$_4$, 15% glycerol, and 0.12 M Tris-HCl (pH 7) for 15 min at 75 °C. Gels were stained overnight at room temperature with 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid and were then destained by washing in 20% methanol–10% acetic acid. The same procedure was used to stain agarose gels for proteins after nondenaturing electrophoresis, as required.

The combining ratio between transglutaminase and fibronectin was assessed by HPLC analysis of a mixture (25 μL) of the two proteins using an Altex (0.75 × 30 cm) TSK-3000 SW column (with a 0.75 × 7.5 cm TSK GSWP guard; Beckman Instruments, Berkeley, CA) with 75 mM imidazole hydrochloride–0.1 mM EDTA, pH 6.8, at a flow rate of 0.5 mL/min (300 psi). The elution profile was monitored with a Waters Associates detector (Model 450; Milford, MA) at 280 nm, and 1.2-mL fractions were collected. They were taken to dryness (Savant Instruments, Speed-Vac, Hicksville, NY) were dissolved by heating in NaDodSO$_4$, 2-mercaptoethanol, and glycerol prior to NaDodSO$_4$–polyacrylamide electrophoresis. After staining with Coomassie Blue, the relative intensities of protein bands were evaluated with the use of a LKB Ultrascan XL laser densitometer (Bromma, Sweden). In calculating molar binding ratios, the assumption was made that the uptakes of stain by unit weights of the two proteins were identical.

Results

Charge-Shift Analysis of Binary Mixtures of Fibronectin and Tissue Transglutaminase. Our first aim was to demonstrate that the shift in the electrophoretic mobility of transglutaminase, originally observed with plasma, could be reproduced in pure mixtures of transglutaminase and fibronectin. Constant amounts (ca. 30 pmol) of the red cell enzyme were added to different amounts of fibronectin (ca. 4.3–30 pmol), and the mixtures were electrophoresed on a casein–agarose gel. Enzyme activity was localized by fluorescence staining with dansylcadaverine in the presence of Ca^{2+}, followed by removal of the unreacted amine by acidic methanol (Lorand et al., 1988). The photograph of the gel (Figure 1), taken under UV light, clearly shows that the shift in the mobility of transglutaminase could be readily observed also in simple binary mixtures with purified fibronectin. By increasing the proportion of fibronectin over transglutaminase, a point was reached above which the fast-moving enzymatic species was no longer detectable and only the slower form of activity was present.

Whereas the interaction between transglutaminase and fibronectin caused a significant reduction in the mobility of transglutaminase, the other partner, fibronectin, experienced only a slight anodic shift. This is illustrated in Figure 2, with the use of Coomassie Blue stain after electrophoresis in agarose.

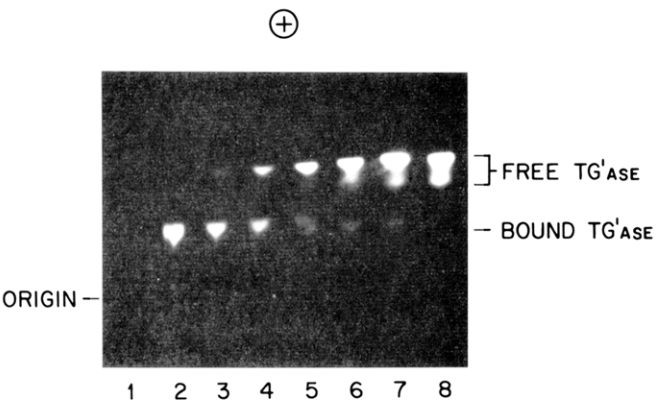

FIGURE 1: Change in the electrophoretic mobility of transglutaminase upon mixing with fibronectin. Human red cell transglutaminase (30 pmol) was mixed with varying amounts of human plasma fibronectin (approximately 30, 14, 10, 7, 5.7, and 4.3 pmol for lanes 2–7, respectively), electrophoresed on 1% agarose with casein, and stained for enzyme activity with dansylcadaverine as substrate. Following removal of unreacted dansylcadaverine, fluorescent bands were photographed under UV illumination. Lane 1 represents the control with 30 pmol of fibronectin alone, and lane 8 corresponds to 30 pmol of transglutaminase without any fibronectin added.

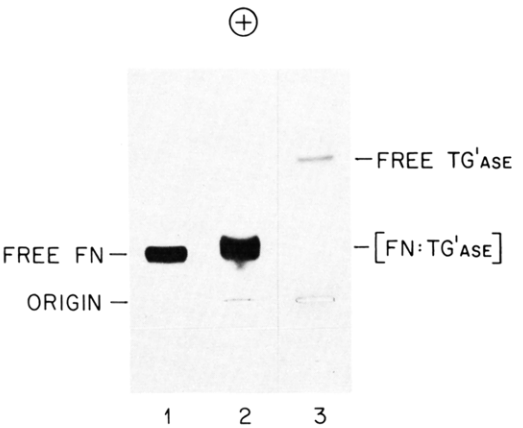

FIGURE 2: Slight anodic shift in the migration of fibronectin upon binding to transglutaminase. Fibronectin (FN; approximately 60 pmol) was electrophoresed on 1% agarose either alone (lane 1) or in combination with 50 pmol of erythrocyte transglutaminase (lane 2), and the gels were stained with Coomassie Blue. Lane 3 represents a control, with 50 pmol of transglutaminase alone.

It was presented in Figure 1 that, upon the addition of fibronectin, the enzymatic activity of transglutaminase shifted to a less anodic position. Nevertheless, it was also essential to prove that there was a transfer of the protein itself to the slower migrating form. Using immunoblotting with a monospecific antiserum against transglutaminase, it could be shown that, as the relative amount of fibronectin was increased in the mixtures, the transglutaminase shifted to the slow form (Figure 3, panel A). Probing the nitrocellulose transblots with an antiserum to fibronectin (panel B) showed a slight increase in the anodic mobility of fibronectin, in accordance with the findings in Figure 2.

Complexation between Fibronectin and Transglutaminase Does Not Involve Cross-Linking by Covalent Bonds. By exclusion of Ca^{2+} from the mixtures, precaution was taken that the enzymatic activity of transglutaminase would not play a role in the complexation process. Nevertheless, it had to be shown by more direct means that no covalent cross-linking of the proteins occurred. When fibronectin and transglutaminase were mixed at mole ratios of about 1.1:2 and the mixture was analyzed after treatment with NaDodSO$_4$ alone or with NaDodSO$_4$ plus 2-mercaptoethanol by polyacrylamide gel elec-

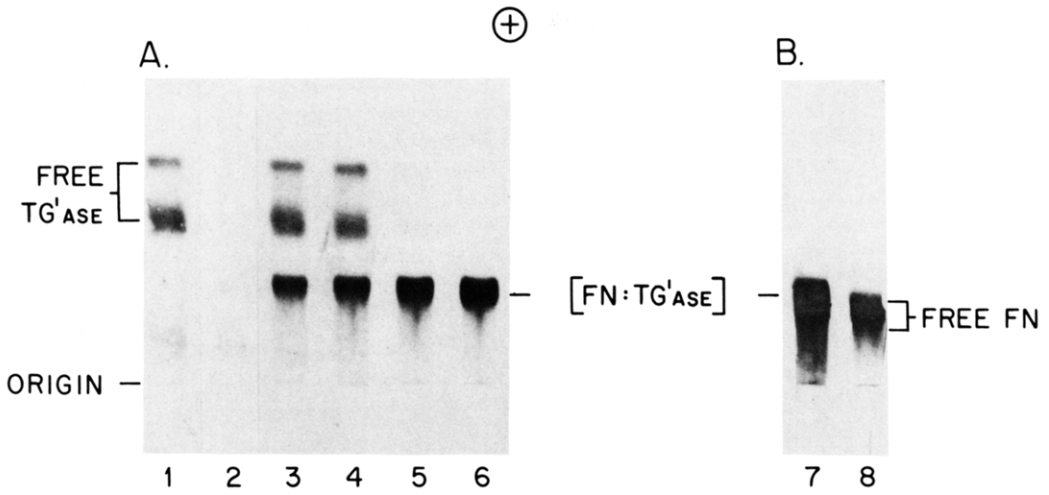

FIGURE 3: Demonstration of complex formation between transglutaminase and fibronectin by immunoblotting with antibodies to the two proteins. Various amounts of fibronectin (7 pmol in lane 3; 10 pmol in lane 4; 16 pmol in lane 5; 30 pmol in lane 6) were mixed with 30 pmol of erythrocyte transglutaminase. The samples were analyzed by nondenaturing electrophoresis in 1% agarose and were transblotted to nitrocellulose for immunostaining with antiserum to transglutaminase (panel A). Lane 7 (panel B) corresponds to the sample in lane 6, immunostained with antiserum to fibronectin. Lane 8 is 30 pmol of fibronectin alone, using anti-fibronectin immunostain. Lanes 1 and 2 in panel A show transblots for 30 pmol of transglutaminase and 30 pmol of fibronectin, respectively, immunostained with anti-transglutaminase antiserum.

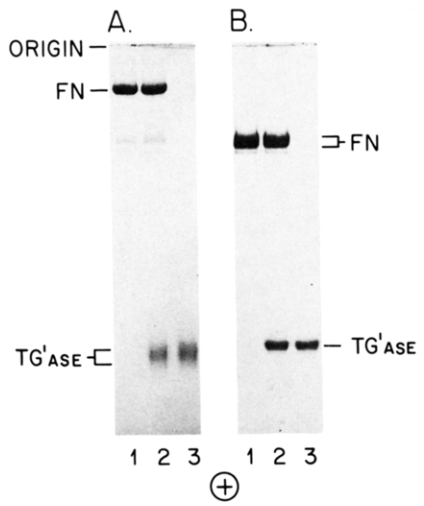

FIGURE 4: The fibronectin–transglutaminase complex can be dissociated in sodium dodecyl sulfate. Mixtures of fibronectin (11 pmol) with red cell transglutaminase (20 pmol) were electrophoresed on a polyacrylamide gel with $NaDodSO_4$, according to Laemmli (1970) and stained with Coomassie Blue. Samples which were treated with $NaDodSO_4$ alone are presented in panel A, whereas those in panel B were reduced prior to electrophoresis with 2-mercaptoethanol in the detergent. Lane 1 is fibronectin alone; lane 2 is the mixture of fibronectin and transglutaminase; lane 3 is transglutaminase alone.

trophoresis, the two proteins could be readily separated from each other, and no polymeric form of either protein was found (Figure 4). In the absence of the reducing agent (panel A) the apparent molecular weight of fibronectin was approximately 440K and that of transglutaminase 80K, whereas following reduction (panel B) fibronectin appeared as a doublet of about 225K and 220K and transglutaminase showed as a more compact band at about 80K of relative mass.

Attempts To Interfere with the Complexing of Transglutaminase and Fibronectin by Agents Which Are Known either To Bind to Fibronectin or To Mimic a Specific Site of This Protein. When heparin, gelatin, DNA, or the tetrapeptide ArgGlyAspSer was added to transglutaminase (Figure 5, lanes 4, 6, 8, and 10), the mobility of the enzyme remained the same as that of the control (lane 2) with no additive. However, upon creation of ternary mixtures by inclusion of fibronectin, the free anodic form of the enzyme disappeared in every instance (lanes 5, 7, 9, and 11). In the presence of heparin (lane 5) or ArgGlyAspSer (lane 11) the activity, monitored by dansylcadaverine staining, shifted to a position close to that seen in the binary mixture of transglutaminase with fibronectin (lane 3). With gelatin (lane 7) or DNA (lane 9), fluorescence staining was found near the origin, and also a faint trail was visible from the position of bound transglutaminase toward the site of application.

Collagen-Binding Domain of Fibronectin Is the Locus for Transglutaminase Binding. Following digestion with chymotrypsin, the collagen-binding domain of fibronectin can be purified by an Affi-Gel–gelatin chromatographic procedure. We have isolated a predominantly 56K (called FN/56) fragment first which, on further digestion with chymotrypsin, yielded a 46K polypeptide (called FN/46). Both of these polypeptides bound tissue transglutaminase in a saturable manner. This is illustrated for the 56K fragment in Figure 6. When the mole ratio of transglutaminase to FN/56 was approximately 1:1, as in lane 2, the anodic free form of the enzyme antigen could no longer be detected by immunoblotting with anti-transglutaminase antiserum. However, as seen also in Figure 7, the shift in the mobility of the enzyme with FN/56 was less pronounced than that observed with intact fibronectin.

Upon formation of a ternary mixture of transglutaminase, FN/56, and gelatin (marked as TG′ase:FN/56:GEL in Figure 7), the enzyme antigen actually moved as a cathodically migrating band. This same phenomenon was evident also with the FN/46 gelatin-binding chymotryptic fragment of fibronectin, as presented in Figure 8. In order to emphasize the less anodic character of the complex of transglutaminase with the relatively small FN/46 fragment, it was necessary to use 2% agarose rather than the 1% concentration previously employed for nondenaturing electrophoresis.

Complexation between Transglutaminase and Fibronectin, Examined by Gel Filtration on HPLC. Analysis of fibronectin–transglutaminase mixtures by gel filtration chromatography provided independent confirmation for complexation between the two proteins. Figure 9 shows the elution profiles from a TSK-3000 column for fibronectin (ca. 27 pmol), erythrocyte transglutaminase (ca. 100 pmol), and for the mixture of these two. Though the experiment presented in this figure was performed at a salt concentration of less than physiological (75 mM imidazole hydrochloride, 0.1 mM

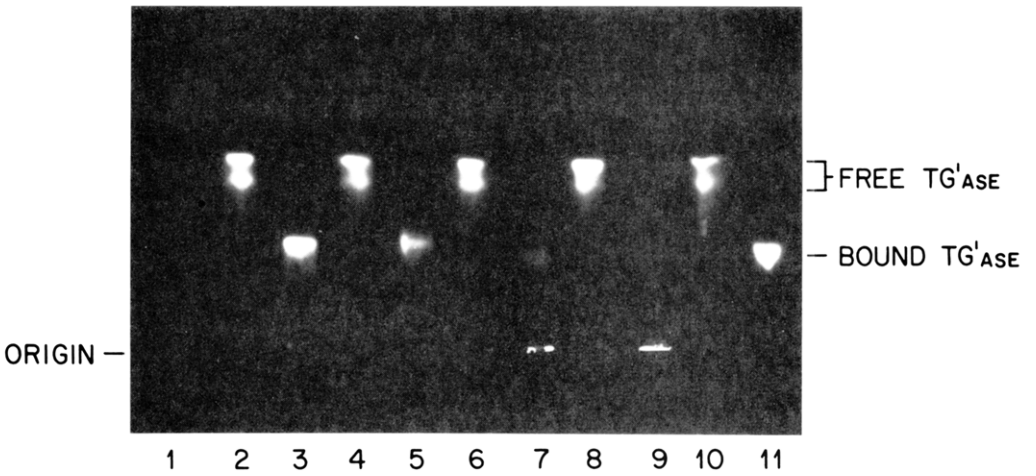

FIGURE 5: Binding of transglutaminase is not prevented by heparin, gelatin, DNA, or the tetrapeptide ArgGlyAspSer. Approximately 9 pmol of red cell transglutaminase (even-numbered lanes) was mixed with compounds known to bind to fibronectin or to represent a specific site in this protein: lane 4, heparin (7.4 μg); lane 6, gelatin (7.4 μg); lane 8, DNA (3.6 μg); lane 10, ArgGlyAspSer (7.4 μg). Lane 2 is the enzyme control without additives. Samples in the odd-numbered lanes contained approximately 8 pmol of fibronectin, starting with fibronectin alone (in lane 1) and fibronectin plus transglutaminase (in lane 3). The remaining lanes represent mixtures of fibronectin and transglutaminase in the presence of heparin (lane 5), gelatin (lane 7), DNA (lane 9), and ArgGlyAspSer (lane 11) at the concentrations given for the corresponding even-numbered lanes. Following electrophoresis in 1% agarose with casein, fluorescence staining for transglutaminase activity was developed with dansylcadaverine.

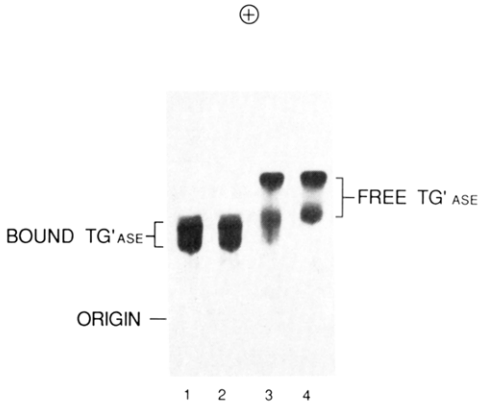

FIGURE 6: Saturable complexation between a chymotryptic fragment of fibronectin and transglutaminase. Mixtures corresponding to approximately 10 pmol of red cell transglutaminase and varying amounts of the 56K fragment from fibronectin (20, 10, and 5 pmol for lanes 1–3) were electrophoresed in 1% agarose. Lane 4 is transglutaminase alone (free TG′ase). Nitrocellulose transblots were immunostained, with anti-transglutaminase serum as the primary antibody.

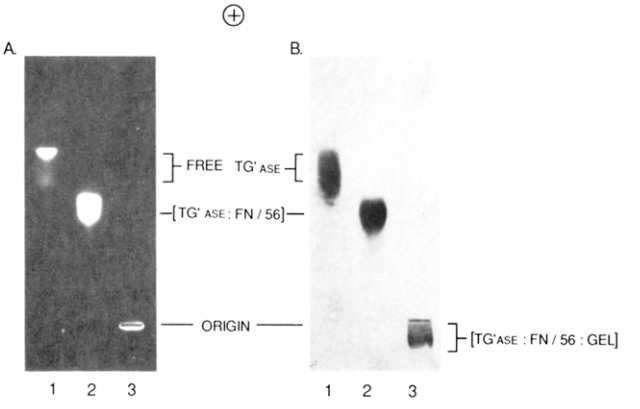

FIGURE 7: The 56K chymotryptic fragment of fibronectin binds transglutaminase and gelatin simultaneously. (Panel A) Transglutaminase activity staining with dansylcadaverine after electrophoresis on 1% agarose and casein. (Panel B) Immunoblots from 1% agarose gels, with anti-transglutaminase antiserum as the primary antibody. All lanes represent about 9 pmol of red cell transglutaminase (TG′ase); mixtures for lanes 2 comprised 40 pmol of the chymotryptic fragment (FN/56) and those for lanes 3 contained also 40 μg of gelatin (GEL).

EDTA, pH 6.8), very similar data were obtained when the solute composition was 25 mM Tris-HCl, 140 mM NaCl, and 0.5 mM EDTA at pH 7.4. Transglutaminase eluted at 17.1 min (panel A), whereas fibronectin was essentially excluded from this column, emerging as an early peak at about 13.3 min (panel B). The elution profile of the mixture (panel C) shows an increased peak at the excluded range and a much diminished peak in the transglutaminase region, with increase in the former matching the decrease in the latter. It was confirmed with $NaDodSO_4$ electrophoresis that transglutaminase was, indeed, present in the excluded peak in panel C.

Since the gel filtration profile in panel C still showed a significant amount of free transglutaminase, it was assumed that the fibronectin in the excluded peak approached saturation with the enzyme. The Coomassie Blue stained gel derived from the material emerging at 13.3 min in panel C was scanned by a densitometer, and an approximate ratio of 1 mol of fibronectin to 1.95 mol of transglutaminase was calculated.

Discussion

Fibronectin is a large plasma protein (ca. 440K), comprising two nearly identical constituent chains which are disulfide bonded near the C-termini. A somewhat different, insoluble form of the protein is an integral part of the connective tissue matrix, serving as an adhesive structure between cells, collagen, and sulfated polysaccharides. Through limited proteolytic approaches, discreet domains have been identified along the two polypeptide chains with well-defined, biologically interesting affinities [see Yamada (1983) and Hynes (1985)]; these include attachment regions for bacteria, fibrin, collagen, acute-phase C-reactive protein (Salonen et al., 1984), heparin, and an ArgGlyAspSer sequence which can recognize specific receptors on a variety of cell surfaces. By virtue of such

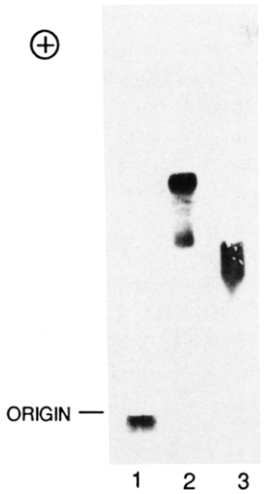

FIGURE 8: Simultaneous binding of transglutaminase and gelatin to the 46K chymotryptic fragment of fibronectin. Following electrophoresis in 2% agarose, immunostaining was carried out on a nitrocellulose transblot as in Figure 7B. Lane 1 represents a mixture of approximately 34 pmol of red cell transglutaminase, 68 pmol of the 46K fragment of fibronectin, and 23 μg of gelatin; lane 2 is transglutaminase alone; and lane 3 is transglutaminase plus the 46K fragment without gelatin.

multiple binding sites, plasma fibronectin is thought to act as an opsonin in the circulation, promoting the phagocytosis of certain particles (Saba et al., 1978; Molnar et al., 1979).

We have recently described yet another binding function for plasma fibronectin which is complexation with tissue transglutaminase (Lorand et al., 1988). Normally, there is no transglutaminase in plasma, and it can be surmised that the transfer of such enzyme from tissues (e.g., red cells or vascular endothelium) would pose a serious threat because endoglutaminyl residues in many proteins could be modified through hydrolysis or aminolysis, including cross-linking to other proteins by N^{ϵ}-(γ-glutamyl)lysine peptide bridges [for a review of these reactions, see Lorand and Conrad (1984)]. In fact, it would be difficult to predict the consequences of the unwarranted posttranslational remodeling of native proteins in the circulation. Such considerations prompted us to initiate a search for the carrier of transglutaminase, and we developed appropriate methodologies to detect the complex. The evidence pointed to fibronectin as the specific transglutaminase-binding protein in plasma. The present paper is an extension of this work and deals with complexation between the purified human erythrocytic transglutaminase and the fibronectin molecule.

Though clearly noncovalent in nature (see Figure 4), the attachment between the two proteins is sufficiently tight to survive the analytical procedures employed, i.e., nondenaturing electrophoresis on an agarose matrix and gel filtration by HPLC using a TSK column. Moreover, as also observed earlier, complex formation seems to occur rapidly upon mixing of the two proteins.

It is important to bear in mind that the cross-linking enzymatic activity of the transglutaminase is not involved in the phenomenon observed by us. Thus, a region of the protein other than its catalytic center must be responsible for binding fibronectin. Apart from the fact that all experiments described in this paper were carried out with the exclusion of Ca^{2+}, an essential activator of the red cell transglutaminase, it could be directly shown (Figure 4) that neither homologous nor heterologous cross-linking by N^{ϵ}-(γ-glutamyl)lysine or by disulfides occurred in the mixture of transglutaminase with fibronectin.

Nondenaturing electrophoresis proved to be a very useful

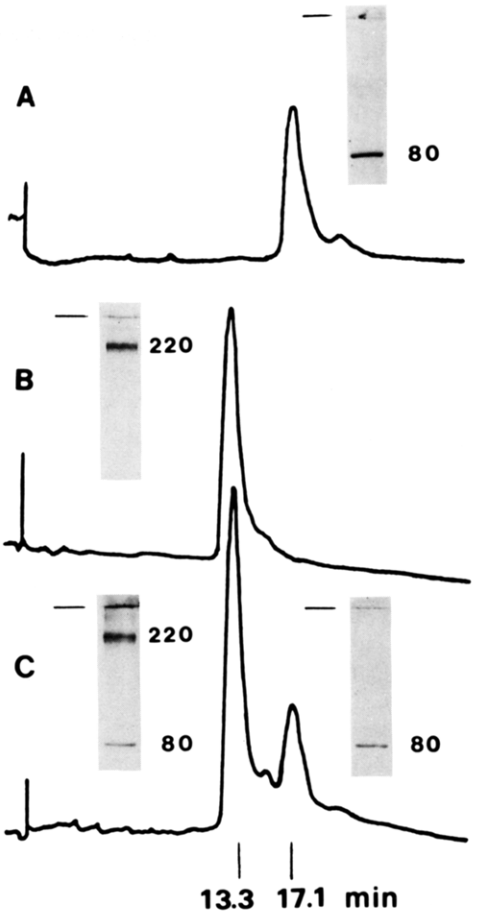

FIGURE 9: Complexation between transglutaminase and fibronectin, analyzed by HPLC gel filtration. Approximately 100 pmol of human red cell transglutaminase (panel A) and about 27 pmol of human plasma fibronectin (panel B) were applied to the TSK-3000 column. Appropriate peak fractions (revealed by absorbancy at 280 nm and emerging at 17.1 min for transglutaminase and at 13.3 min for fibronectin) were reduced with 2-mercaptoethanol and electrophoresed with $NaDodSO_4$. The Coomassie Blue stained patterns are shown in the inserts for the two proteins: one band at a relative mass position of 80K (marked 80) for transglutaminase and a doublet near 220K (marked 220) for fibronectin. Tops of the separating gels are marked by horizontal lines, with proteins moving toward the anode at the bottom. Panel C shows the gel filtration profile for the mixture of transglutaminase and fibronectin and the $NaDodSO_4$ electrophoretic patterns for peaks emerging at 13.3 min (left-hand gel) and at 17.1 min (right-hand gel). The left-hand gel, representative of the complex between fibronectin and transglutaminase, was examined by scanning densitometry, which indicated the presence of about 1 mol of the 80K species per 1 mol of 220K unit.

method for demonstrating complexation between fibronectin and transglutaminase. A variety of analytical modalities were employed for detecting the migration of the two proteins: activity staining with fluorescent dansylcadaverine (Figures 1 and 5), staining with Coomassie Blue (Figure 2), and immunoblotting with antibodies to transglutaminase or fibronectin (Figure 3). In the electrophoretic system employed, the mobility of the complex was about half of that of free transglutaminase and it was slightly higher than that of free fibronectin, with all species moving in an anodic direction.

The nondenaturing electrophoretic procedure was also used to study complexation in ternary mixtures containing transglutaminase, fibronectin, and some other component such as heparin, DNA, or gelatin, macromolecules of undefined size and composition, known to bind to fibronectin. The effect of ArgGlyAspSer, a tetrapeptide equivalent to the sequence of the cell attachment domain on fibronectin, was also examined. As seen from the results in Figure 5, none of these compounds

interfered with the mobility of transglutaminase by themselves; nor did they impede the disappearance of the rapidly moving free form of the enzyme when fibronectin was present. The findings indicate that transglutaminase is not bound to fibronectin at its cell attachment region and that complex formation with transglutaminase depends on sites on the fibronectin molecule other than those involved in binding heparin, DNA, and gelatin. As discussed later, we focused in some details on the triple interactions of transglutaminase, fibronectin (or its fragments), and gelatin.

The disappearance of the rapidly migrating, free form of transglutaminase when the approximate mole ratio of the enzyme to fibronectin fell below 2:1 (Figures 1 and 3A) prompted us to design experiments for measuring the stoichiometry of association between the two proteins. As presented in Figure 9, panel C, the complex was isolated by gel filtration under conditions of excess transglutaminase, and the presence of both components was validated by $NaDodSO_4$ electrophoresis. Scanning densitometry was performed to evaluate the relative amounts of the 80K enzyme and the 220K half-fibronectin protein chains. When corrected for the difference in molecular weight, a combining ratio of 1.95 mol of transglutaminase to 1 mol of fibronectin was calculated. Other attempts to obtain the stoichiometry of combination between the two proteins yielded similar values. A mixture of fibronectin and transglutaminase (the latter added in 4-fold molar excess) was applied to an affinity column of gelatin (Affi-Gel–gelatin), and components of the retained complex were eluted with denaturing solvent (1 M urea, 3.3% $NaDodSO_4$, 5.5% 2-mercaptoethanol in 33% glycerol, and 62 mM Tris-HCl, pH 6.8). Scanning densitometry again gave a ratio of about 2 mol of transglutaminase to 1 mol of fibronectin.

Limited proteolysis of fibronectin is known to yield fragments with characteristic affinities for various ligands [see Hahn and Yamada (1979)]. In order to identify the transglutaminase-binding domain of the parent protein, digestion by chymotrypsin was employed. This method of fragmentation yielded an approximately 56K fragment which, upon further digestion, was degraded to a size of about 46K. With the electrophoretic binding assay, only these two fragments showed transglutaminase-binding properties in the 60-min chymotryptic digest of fibronectin (data, obtained with guinea pig liver transglutaminase, not shown). Both fragments bound to a gelatin column which, in fact, was used to purify the two products. More significantly, however, both the 56K and the 45K fragments also bound transglutaminase. The data in Figure 6 suggest an approximately 1:1 stoichiometry for complexation between transglutaminase and the chymotryptic fragment because, upon mixing of 10 pmol of the fragment with 10 pmol of transglutaminase (lane 2), the free form of the enzyme disappeared. It should also be noted that FN/56 can compete effectively against the intact fibronectin molecule in binding transglutaminase (data, obtained with guinea pig liver transglutaminase, not shown). The electrophoretic shift of the enzyme was somewhat less with the 56K fragment (Figures 6 and 7) than that seen with intact fibronectin; nevertheless, the mobility of the bound form of transglutaminase was still only about two-thirds that of the free enzyme. The mobility difference between the free and bound forms of the enzyme was smaller when the 46K fragment was tested for complexation, and in order to accentuate this difference, the concentration of the agarose support had to be increased from 1% to 2% (Figure 8).

The utility of the nondenaturing electrophoretic procedure for demonstrating complexes of transglutaminase is nowhere more obvious than in the examples given in Figures 7 and 8 for the associations of the enzyme with the 56K and 46K fragments of fibronectin in ternary mixtures with gelatin. In the presence of gelatin, the complexes moved to an essentially cathodically migrating position. These experiments buttress the conclusion (which, incidentally, was also evident from the retention of complexes of transglutaminase and the fibronectin fragments by gelatin columns; data not shown) that the fibronectin fragments must carry independent sites for accommodating transglutaminase and gelatin simultaneously.

Inasmuch as fibronectin of the extracellular matrix possesses a gelatin/collagen-binding domain identical with that of plasma fibronectin, our findings provide an explanation for the histological observations regarding the colocalization of fibronectin and transglutaminase in connective tissue preparations (Upchurch et al., 1987).

In the introduction, an analogy was drawn between the fibronectin–transglutaminase and haptoglobin–hemoglobin complexes. When defenses against dangerous enzyme activities are discussed, the complexes between α_2-macroglobulin and proteases also serve as appropriate examples [for relevant articles, Feinman (1983)]. Through binding to specific cell surface receptors, α_2-macroglobulin is thought to promote the elimination of proteases from the circulation even though the activity of the enzyme itself is only partially impeded during transit through plasma. As reflected in the staining intensities with casein and dansylcadaverine, qualitatively at least, the activity of the red cell transglutaminase was preserved during binary complexation with fibronectin (Figure 1), but activity was harder to detect in the ternary complexes with gelatin (Figure 5). Detailed kinetic analysis with native plasma and cell surface proteins (including fibronectin itself) would be required to answer the question whether the binding of transglutaminase to fibronectin would cause the downregulation of its hydrolytic and aminolytic activities, including the cross-linking of protein substrates. Regarding the analogy with the α_2-macroglobulin–protease complexes, we have initiated research to answer the question whether fibronectin would promote the endocytosis of tissue transglutaminase in vitro and the clearance of the enzyme from the circulation in vivo. In its complexes with transglutaminase, fibronectin could be stabilized in a configuration more favorable for attachment to and internalization by cells of the reticuloendothelial system.

The finding that fibronectin and its fragments can form tight complexes with transglutaminase also has significant practical implications. On the one hand it should aid in the isolation of transglutaminases from tissues, and on the other, it will make it possible to selectively remove these enzymes from plasma. In some clinical specimens, assays for the fibrin stabilizing factor (i.e., factor XIII) activity are thought to yield ambiguous results because of the presence of tissue transglutaminase in plasma (Lorand et al., 1969). This difficulty could now be obviated by the absorption of plasma to solid-phase fibronectin ligands (such as gelatin, heparin, or anti-fibronectin antibodies) prior to testing for factor XIII or perhaps simply by cold precipitation (Mossesson & Amrani, 1980) of the transglutaminase–fibronectin–heparin complex.

References

Brenner, S. C., & Wold, F. (1978) *Biochim. Biophys. Acta 522*, 74–83.

Feinman, R. D., Ed. (1983) *Ann. N.Y. Acad. Sci. 421*.

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

Hynes, R. (1985) *Annu. Rev. Cell Biol. 1*, 67–90.

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

Lorand, L., & Conrad, S. M. (1984) *Mol. Cell. Biochem. 58*, 9-35.
Lorand, L., Urayama, T., De Kiewiet, J. W. C., & Nossel, H. L. (1969) *J. Clin. Invest. 48*, 1954-1064.
Lorand, L., Dailey, J. E., & Turner, P. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1057-1059.
Lowe, M. E., & Ashwell, G. (1982) *Arch. Biochem. Biophys. 216*, 704-710.
Miller, G. L. (1959) *Anal. Chem. 31*, 964.
Molnar, J., Gelder, F. B., Lai, M. Z., Siefring, G. E., Jr., Credo, R. B., & Lorand, L. (1979) *Biochemistry 18*, 3909-3916.
Mosesson, M. W., & Amrani, D. L. (1980) *Blood 56*, 145-158.
Putnam, F. W., Ed. (1975) *The Plasma Proteins* Vol. 2, pp 2-50, Academic, New York.
Saba, T. M., Blumenstock, F. A., Weber, P., & Kaplan, J. E. (1978) *Ann. N.Y. Acad. Sci. 312*, 43-55.
Salonen, E.-M., Vartio, T., Hedman, K., & Vaheri, A. (1984) *J. Biol. Chem. 259*, 1496-1501.
Turner, P. M., & Lorand, L. (1988) *FASEB J. 2*, A1783 (Abstr. 8635).
Upchurch, H. F., Conway, E., Patterson, M. K., Jr., Birkbichler, P. J., & Maxwell, M. D. (1987) *In Vitro Cell. Dev. Biol. 23*, 795-800.
Vuento, M., & Vaheri, A. (1979) *Biochem. J. 183*, 331-337.
Yamada, K. M. (1983) *Annu. Rev. Biochem. 52*, 761-799.

The Single-Chain Form of Tissue-Type Plasminogen Activator Has Catalytic Activity: Studies with a Mutant Enzyme That Lacks the Cleavage Site†

Jeri Ann Boose,‡ Esa Kuismanen,§ Robert Gerard,∥,⊥ Joe Sambrook,∥ and Mary-Jane Gething*,‡,∥

Howard Hughes Medical Institute, Department of Biochemistry, and Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235, and Department of Biochemistry, University of Helsinki, Unioninkatu 35, 00170 Helsinki, Finland

Received February 29, 1988; Revised Manuscript Received August 15, 1988

ABSTRACT: Tissue-type plasminogen activator (t-PA), the serine protease responsible for catalyzing the production of plasmin from plasminogen at the site of blood clots, is synthesized as a single-chain polypeptide precursor. Proteolytic cleavage at the C-terminal side of Arg_{275} generates a two-chain form of the enzyme whose subunits are held together by a single disulfide bond. We have measured the activities of both forms of the wild-type enzyme, as well as that of a mutant enzyme ($Arg_{275} \rightarrow Gly$), created by oligonucleotide-directed mutagenesis, that cannot be cleaved into a two-chain form. Both types of single-chain t-PAs are enzymatically active and exhibit identical V_{max} and K_m values when assayed with synthetic peptide substrates, indicating that the single amino acid change had no effect on the amidolytic activity of the enzyme. However, cleavage of wild-type t-PA into the two-chain form results in increased activity both on a peptide substrate and on the natural substrates Lys- and Glu-plasminogen in the absence or presence of stimulation by soluble fibrin. The enhanced activity is due to a 3-5-fold increase in the V_{max} of the cleaved enzyme, rather than to any change in the K_m values for the various substrates. During incubation with plasminogen, the single-chain form of wild-type t-PA is converted to the two-chain form by plasmin generated during the reaction. This conversion, from the less active to the more active form of the enzyme, results in a reaction that displays biphasic kinetics. Both the single-chain, cleavage-minus t-PA and the two-chain, wild-type t-PA are stimulated by soluble fibrin, although the single-chain form requires higher levels of fibrin to achieve maximum activity. Finally, both forms of the enzyme can be inhibited to the same extent by the serpin plasminogen activator inhibitor 1 (PAI-1).

Tissue-type plasminogen activator (t-PA), a serine protease that plays a key role in fibrinolysis, converts the zymogen plasminogen to its active form, plasmin, by the specific cleavage of a single peptide bond. Plasmin is a serine protease of broad specificity that degrades the fibrin network of blood clots [reviewed in Collen (1980) and Dano et al. (1985)]. The rate of activation of plasminogen by t-PA increases dramatically in the presence of fibrin (Camiolo et al., 1971), so that high concentrations of plasmin are generated on the surface of a clot (Bergmann et al., 1983; Hoylaerts et al., 1982). This property has led to the clinical application of t-PA as a thrombolytic agent (Matsuo et al., 1981; Collen & Linjen, 1984; Collen, 1987), and the enzyme has become a major focus of scientific attention.

Several groups have cloned cDNA copies of the mRNA coding for t-PA (Pennica et al., 1983; Edlund et al., 1983; Fisher et al., 1985; Kaufman et al., 1985; Harris et al., 1986; Sambrook et al., 1986). Sequencing of these cDNAs and genomic clones has shown that the precursor form of t-PA is 562 amino acids in length (Pennica et al., 1983; Edlund et al., 1983; Pohl et al., 1984; Fisher et al., 1985; Friezner-Degan et al., 1986; Harris et al., 1986). Removal of a hydrophobic signal sequence and a hydrophilic pro sequence from the precursor generates the mature polypeptide of 527 amino acids,

† This work was supported in part by grants from the American Cancer Society (M.J.G.), the Bugher Fund of the American Heart Association (J.S.), and the Texas Affiliate of the American Heart Association (R.G.). E.K. was supported by an EMBO fellowship.

* Address correspondence to this author at the Howard Hughes Medical Institute, University of Texas Southwestern Medical Center.
‡ Howard Hughes Medical Institute, University of Texas Southwestern Medical Center.
§ University of Helsinki.
∥ Department of Biochemistry, University of Texas Southwestern Medical Center.
⊥ Department of Internal Medicine, University of Texas Southwestern Medical Center.

0006-2960/89/0428-0635$01.50/0