Visualization of Purified Fibronectin-Transglutaminase Complexes*

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It has been reported previously (Turner, P. M., and Lorand, L. (1989) Biochemistry 28, 628-635) that human erythrocyte transglutaminase forms a noncovalent complex with human plasma fibronectin near its collagen-binding domain. In the present study, we show by nondenaturing electrophoresis that guinea pig liver transglutaminase, similarly to the erythrocyte enzyme, forms a complex with human fibronectin. Studies of anisotropic shifts of fluorescein-labeled liver and erythrocyte transglutaminases, upon addition of fibronectin, indicated that both transglutaminases bind to fibronectin with a stoichiometry of about 2:1. Polymerization of fibrinogen by human erythrocyte transglutaminase was inhibited after complex formation with fibronectin. Complexes of fibronectin with either erythrocyte or liver transglutaminase were isolated by gel gradient zone sedimentation and examined by rotary shadowing electron microscopy. The globular transglutaminase could be readily identified binding to the thin fibronectin strand. The binding site for transglutaminase was within 5-10 nm of the N terminus of fibronectin, consistent with its proximity to the collagen-binding domain. Under some experimental conditions, the complex of fibronectin with erythrocyte transglutaminase appeared as a ring-shaped structure in which two transglutaminase molecules had probably dimerized. The molecular weight of the erythrocyte transglutaminase was determined by sedimentation equilibrium to be 71,440 ± 630.

The interaction of human erythrocyte transglutaminase with human plasma fibronectin has been reported previously (Lorand et al., 1988; Turner and Lorand, 1989). Complex formation occurs rapidly upon mixing transglutaminase and fibronectin, and stable complexes can be cleanly separated from free transglutaminase by either nondenaturing gel electrophoresis or gel filtration chromatography. Complex formation is independent of Ca²⁺ and does not involve covalent cross-linking. Collagen-binding fragments of fibronectin also bind transglutaminase in the electrophoretic assay, and ternary complexes comprising transglutaminase, collagen-binding fibronectin fragment, and gelatin can be demonstrated. Both the electrophoretic assay and gel filtration show saturation of binding with the approximate ratio of 2 mol of transglutaminase (80 kDa) to 1 mol of fibronectin dimer (440 kDa). Together these data suggest the presence of a single transglutaminase binding site near the collagen-binding domain of each of the constituent chains of fibronectin.

In this paper, we examine the binding of human plasma fibronectin to guinea pig liver transglutaminase and show that the association is not entirely species- or tissue-specific. We describe the isolation of complexes by gel gradient zone sedimentation, a method which requires complexes to remain together over a 15-h period in a centrifugal field and thus could be assumed to be an even more rigorous test of complex stability than the methods previously used. Finally, we demonstrate the structure of the complexes by rotary shadowing electron microscopy and discuss the finding of some unique ring structures in mixtures of erythrocyte transglutaminase and fibronectin.

**EXPERIMENTAL PROCEDURES**

**Protein Preparations—**LTG¹ was a gift from Dr. Paul Turner of Northwestern University. ETG was prepared essentially as described by Brenner and Wold (1978). Human tenasin was purified from culture media conditioned by the U251 MG human glioma cell line as described (Aukhil et al., 1990).

Fn from fresh human plasma was eluted from a gelatin-agarose (Sigma) column with 4 M urea, 0.05 M Tris-HCl, 10 mM phenylmethylsulfonyl fluoride, pH 7.2 (Ruoslahti et al., 1982). Peak fractions were pooled and separated on a Sephacryl HR 500 gel filtration column (Pharmacia LKB Biotechnology Inc.) equilibrated in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.9. A 0.6-ml aliquot (1.0 mg/ml) of the Sephacryl-purified Fn was loaded onto a 12-ml 15-40% glycerol gradient containing 0.2 M ammonium formate, 1 mM Hepes, pH 7.6, and centrifuged for 18 h at 41,000 rpm in a Beckman SW 41 rotor at 20 °C. Fractions (0.5 ml) were collected from the bottom of the tube, and the peak fraction (0.4 mg/ml) was used in simple mixing experiments for EM. Fn used in fluorescence and activity studies was purified as a byproduct of human Factor XIII (Lorand et al., 1981).

LTG and ETG were labeled with FITC (Sigma) according to the method of Freyssinet et al. (1978), by mixing a 6-fold molar excess of FITC (dissolved in acetone at 15 mM) with the protein (200-300 µg) for 90 min at 23 °C in the dark in a solution of 0.1 M Tris-HCl, 1 mM EDTA, pH 9.0. Unbound FITC was removed by dialysis on a Sephadex G-50 (Sigma) column (1 × 16 cm) equilibrated in 3.1 M Tris-HCl, 3 mM EDTA, pH 7.5. The LTG² or ETG² concentration was estimated on a PM30 ultrafiltration membrane (Amicon Corp., Lexington, MA) and dialyzed overnight against 0.075 M Tris-HCl, 2 mM EDTA, pH 7.5, containing 2% activated charcoal (NORIT A; MCB, Norwood, OH) at 4 °C. Using a molar extinction coefficient of 490 nm of 3.4 × 10⁶ M⁻¹ cm⁻¹ for FITC, the concentration of the peaks was estimated.

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§ The abbreviations used are: LTG, guinea pig liver transglutaminase; ETG, human erythrocyte transglutaminase; TG, transglutaminase; Fn, human plasma fibronectin; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy; FITC, fluorescein isothiocyanate; ETG², FITC-labeled ETG; LTG², FITC-labeled LTG; darmacel, 5-dimethylaminonaphthalene-1-sulfonyl.

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10° for fluorescein (Churchich, 1967), the extent of labeling was calculated as 1.5 mol of FITC/mole of LTG or ETG.

Bovine serum albumin (2 mg/ml) was purchased from Pierce Chemical Co. Human fibrinogen (plasminogen-free) was purchased from American Diagnostics (Greenwich, CT).

Binding conditions were determined using extinction coefficient values (E)_1%=280 nm 15.8 for LTG (Folk and Cole, 1966), 12.8 for Fn (Moessen and Umfleet, 1970), 15.1 for human fibrinogen (Mihalyi, 1968), and at 277 nm of 9.7 for human tenascin (Taylor et al., 1989). ETG, ETGF, and LTG' concentrations were measured by the method of Lowery et al. (1951) and are expressed using bovine serum albumin as standard.

Anisotropy Shifts—Fluorescence polarization measurements were carried out on a SLM 8000C double emission spectrofluorometer (SLM Aminco, Urbana, IL) with Glan-Thompson calcite prism polarizers in the excitation and emission channels (Lakowicz, 1983).

Protein samples (3–20 μg/ml) were placed in a 1 × 1 cm quartz fluorescence cuvette at 37 °C, with the contents (2 ml) stirred continuously. Wavelengths of excitation and emission were set at 490 and 530 nm, respectively. Polarization (P) was calculated using the following equation,

\[
P = \frac{(R_{nm}/R_{mm}) - 1}{[(R_{nm}/R_{mm}) + 1]}
\]

where \(R_{nm}\) is the ratio of emissions in the two channels when the excitation polarizer is in the vertical position (0°) and \(R_{mm}\) is the ratio when the excitation polarizer is in the horizontal position (90°). Anisotropy \((r)\) was derived from the \(P\) value by the following equation.

\[
r = 2P/(3 - P)
\]

All data were subjected to a smoothing routine with a 16-nm bandwidth, sharp cutoff, three-point low pass linear digital filter (10 passes) in order to reduce background noise. Data were collected and stored on an IBM Personal System 2 model 502 computer through the use of software provided by SLM Aminco.

Nondenaturing Electrophoresis of Complexes—Nondenaturing gradient polyacrylamide gel electrophoresis of LTG and Fn mixtures was performed using a modification of the procedure described by Morrow et al. (1981). A 3.8–14% gradient gel using 0.04 M Tris-acetate, 0.02 M sodium acetate, 1 mM EDTA, pH 7.5, with 3.6% stacking gel was used.

Inhibition of Fibrinogen Polymerization by the Complex—Cross-linking of fibrinogen was typically performed at 37 °C for 1 h in a reaction mixture (100 μl) containing approximately 8 μM human fibrinogen, 0.05 M Tris-HCl, 0.1 mM NaCl, 5 mM CaCl₂, pH 7.5, and 5–40 μg/ml ETG either as a free enzyme or complexed with Fn. ETG was complexed at 4 °C with Fn in a 1:1 molar ratio prior to use in the experiment. In controls, 5 mM EDTA was instead of CaCl₂. Cross-linked fibrinogen samples (-40 μg) were analyzed by SDS-PAGE of LTG and Fn mixtures was performed using a modification of the procedure described by Morrow et al. (1981). A 3.8–14% gradient gel using 0.04 M Tris-acetate, 0.02 M sodium acetate, 1 mM EDTA, pH 7.5, with 3.6% stacking gel was used.

Where \(M_r\) is the ratio of emissions in the two channels when the excitation polarizer is in the vertical position (0°) and \(M_m\) is the ratio when the excitation polarizer is in the horizontal position (90°). Anisotropy \((r)\) was derived from the \(P\) value by the following equation.

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RESULTS AND DISCUSSION

Measurements of anisotropy can yield information on the decrease of rotational mobility of a fluorescently labeled protein upon forming a complex with another protein (see Lakowicz (1983)). Fig. 1 shows the change in anisotropy of LTG (Fig. 1A) or ETG (Fig. 1B) when Fn was added in the approximate mol ratios of 2:1 TG:Fn. The initial addition of Fn to LTG or ETG (at 600 s) caused the anisotropy values to increase significantly, but subsequent additions of Fn caused barely detectable increases. In control experiments where bovine serum albumin or human fibrinogen was substituted for Fn, no change in anisotropy was observed (data not shown). Thus, the increase in anisotropy with either LTG or ETG and Fn may be taken as a specific index of complex formation between these two proteins.

Protein profiles for various mixtures of Fn and LTG, as revealed by nondenaturing polyacrylamide gel electrophore-
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Previous work (Turner and Lorand, 1989; Radek et al., 1991) demonstrated that 42-, 46-, and 56-kDa collagen (gelatin)-binding fragments of Fn bound TG and could compete with intact Fn for TG binding. We turned to electron microscopy in an effort to define more closely the TG-binding region of Fn. In rotary-shadowed specimens, Fn appears as a thin, flexible strand with length 120–160 nm (Erickson et al., 1981). The C termini of the two constituent chains are disulfide-linked at the center of the strand and the chains extend separately to their N termini at the ends. We expected that bound TG should be visible as a globular protrusion along the length of the Fn strand and it would be possible to map its binding position.

Initially we rotary-shadowed simple binary mixtures of components which had been found individually to be homogeneous by EM. Mixtures of ETG and Fn demonstrated nearly complete saturation of a single TG-binding site near the N terminus of each chain of the Fn molecule (Fig. 4A). Whereas native Fn is a uniform strand thinly tapered at the ends (Erickson et al., 1981) (Fig. 4C), almost every strand in the mixture had a globular domain, corresponding to the ETG, at each end. Complexes of LTG and Fn (Fig. 4B) were identical in appearance to ETG-Fn complexes, but, in contrast to the analyses by gel electrophoresis and gel filtration, the binding of the LTG appeared to be of lower affinity than that of ETG. Complex formation increased somewhat with increasing LTG-Fn ratios such that at the highest molar ratio tested (40:1), Fn molecules were most frequently complexed with one, and often with two, LTG molecules. In spite of the fact that the Fn never appeared to be saturated, the frequency of Fn molecules with a globular LTG at one or both ends was nevertheless convincing that specific binding between the two proteins occurred. We believe that the low frequency of intact complexes is partially explained by the dissociation of complexes upon contact with mica, presumably because of distortion of the bonding interfaces. Such dissociation has been reported for Factor XIII A3 dimers (Carrell et al., 1989) and has also been observed for the tetrameric proteins catalase and ß-galactosidase. The high ratios of TG to Fn used...
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FIG. 4. Representative fields of rotary-shadowed simple mixtures. A, ETG-Fn (12:1 molar ratio to Fn dimer); B, LTG-Fn (12:1 molar ratio); C, Fn alone; D, LTG-tenascin (20:1 molar ratio to tenascin subunit). In panel A, examples of bound globular ETG molecules are indicated by solid arrowheads, while in panel B, all bound LTG molecules are similarly marked. The globular structure of free TG molecules is shown by open arrows. Bar, 50 nm.

Throughout the EM study (4:1-40:1) were dictated by the relative difficulty in demonstrating LTG-Fn complexes, but the micrographs consistently supported the stoichiometry of binding (2 TG molecules/Fn dimer) found by other techniques.

Fig. 4D shows a field from a mixture of LTG and human tenascin (20:1 ratio to tenascin subunit). We tested tenascin as a probable negative control for TG binding, because tenascin bears no homology to the collagen-binding region of fibronectin, though it does have homology to fibronectin type III domains (reviewed by Erickson and Bourdon (1989)). No association could be detected between tenascin and LTG (Fig. 4D) nor between tenascin and ETG (not shown) by EM of binary mixtures. The lack of association was also demonstrated by gradient sedimentation (see below).

Glycerol gradient sedimentation provided a stringent test of complex stability as well as a means of separating TG-Fn complexes from free TG. In separate experiments ETG and LTG were combined with Fn and sedimented through glycerol gradients. Silver-stained gels (Fig. 5, A and B) clearly show the presence of TG in fractions containing Fn. Parallel gradients of either TG run alone show TG only in fractions 14-19 (data not shown), demonstrating that the TG in fractions 6-13 was not composed of TG aggregates. Again, LTG showed much less complex formation with Fn than did ETG. Fig. 5C illustrates, as expected, that tenascin did not form a complex with ETG (or with LTG, not shown).

TG-Fn complexes at high magnification are shown in Fig. 6. Complexes containing either ETG or LTG are indistinguishable from each other. In most complexes the TG appears to be bound close to the N terminus, but in some complexes a small fibronectin tail is visible beyond the bound TG. Fibronectin is not a rigid molecule and indeed often displays a forked or curled end (Erickson et al., 1981) (Fig. 6, row 4D). TG binds close enough to the N terminus that most complexes land on the mica surface in a conformation unfavorable for seeing the protruding end segment. Photographed complexes with measurable N-terminal tails (n = 10) gave values between 5 and 10 nm for the N-terminal tail, corrected for 1 nm of platinum thickness. We conclude that the center of the bound transglutaminase lies about 10 nm from the N terminus of Fn. The N terminus of Fn comprises five Type I domains, followed by two Type II domains and three addi-

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**FIG. 5.** Silver-stained SDS-PAGE gels of mixtures fractionated on 15-40% glycerol gradients. Alternate gradient fractions are numbered except in C, where fractions 10 and 12 were not run on the gel. A, ETG-Fn (8:1 molar ratio to Fn dimer); B, LTG-Fn (8:1 molar ratio); C, ETG-tenascin (6:1 molar ratio to tenascin subunit).
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Fig. 6. Selected examples of complexes. Row A, 1–4, ETG-Fn, mixing experiment as in Fig. 4. Row B, 1–4, ETG-Fn gradient fraction 7. Row C, 1–4, and Row D, 1–4, LTG-Fn gradient fractions 8 and 9. Row D, 2 and 3, LTG-Fn, mixing experiment as in Fig. 4. Row D, 4, uncomplexed fibronectin. Bar, 40 nm.

Tional Type I domains. Since each of these domains is 5–6.5 kDa and is estimated to be about 2.3–2.6 nm in diameter, the TG would appear to bind to the fourth or fifth Type I domain. However, the resolution is not sufficient to exclude binding to the Type II domains.

The dominant conformation of ETG-Fn complexes from glycerol gradient fractions was not the linear form shown in Figs. 4 and 6, but rather a ring figure consisting of a flexible, thin strand (Fn) with a single globular thickening along its length (Fig. 7A). Approximately 60% of the ETG-Fn complexes in rotary shadowed fields were present as ring structures. The closure of Fn into a ring shape was dependent on the presence of ETG, as Fn specimens shadowed under identical conditions did not contain ring structures. The peak of Fn distribution in gradient centrifugation centered in fractions 8–10 (~11 S) when run alone but shifted to fractions 7–8 (~12.5 S) when run with ETG, consistent with the presence of a more compact conformation of Fn during the centrifugation (see Erickson and Carrell (1983)). This shift did not occur with LTG-Fn complexes. Measurements of the globular domains in both ring and linear ETG-Fn complexes showed the dimensions of the globular domain in the ring forms (18 ± 2 nm by 11 ± 1 nm) to be significantly larger than the dimensions of ETG globules in linear complexes (11 ± 1 nm by 9 ± 1 nm).

Our interpretation is that the ring form represents a trimolecular complex (two ETG:one Fn) in which the ETG molecules bound near the two ends of the Fn strand are dimerized, bringing the N termini of the two chains of Fn together to form a circle. This structure is not stabilized by covalent cross-linking (Fig. 5A). Since the catalytic subunit of the plasma transglutaminase, Factor XIII, exists as a dimer (Schwartz et al., 1973) and bears sequence homology to tissue transglutaminases (Ikura et al., 1988; Ichinose et al., 1990; Phillips et al., 1990), it is not unreasonable to suppose that under the conditions of our experiments ETG could have formed dimers as well. An alternative interpretation of the data is the formation of a circular bimolecular complex (one ETG:one Fn) in which a second binding site for Fn is exposed when the ETG-Fn complex is formed in the presence of glycerol. Although we cannot rule out this possibility, we favor the dimerization hypothesis for the following reasons. 1) Complexes formed in glycerol initially show the 2:1 stoichiometry demonstrated by other techniques (Fig. 4A). 2) The size of the globular domain is consistent with the presence of two ETG molecules in the ring forms. 3) There is not evidence of sufficient ETG trailing behind the ETG–Fn peak in the gradient (Fig. 5A, fractions 9–13) to support a conclusion that an ETG molecule is displaced from most trimolecular com-

Fig. 7. A ring form is the predominant structure seen in ETG–Fn gradient fractions. A, examples of figures found in ETG–Fn gradient fraction 7. Bar, 40 nm. B, following sedimentation equilibrium analysis of ETG, the ETG concentration across the sedimentation cell is plotted as ln A_{280} versus R². Absence of curvature in the plot indicates the presence of a single form of ETG. This species is monomeric ETG with a molecular weight of 71,440 ± 830.
plexes by formation of a bimolecular ETG-Fn complex over the course of the centrifugation.

In order to determine if ETG can form dimers independently of Fn, we performed sedimentation equilibrium analysis on purified ETG. The resultant plot of ln A_{o} versus R^{2} (Fig. 7B) is linear across the cell indicating that ETG does not form dimers in the 20-fold concentration range generated by the gradient. This range includes concentrations at least double that of the total ETG concentration (150–300 μg/ml) in the ETG-Fn mixtures loaded in gradient centrifugation experiments, as well as that of the calculated effective concentration of two ETG molecules bound to the ends of a single Fn molecule (115–240 μg/ml, using an average separation distance of 75–100 nm). Concentration effects thus cannot be solely responsible for the observed dimerization because ETG does not form dimers independently in this concentration range. Fibronectin must be present for this to take place. Binding to Fn may expose a cryptic dimerization site on TG by causing a conformational change, or it may be that the bound ETG molecules are oriented such that surfaces required for dimerization are favorably disposed. In the latter case, each time the ends of the Fn arms approach each other (assuming a hinge-like motion with limited lateral flexibility), the bound ETG molecules are oriented correctly for a dimerization event to occur. We have so far observed the ring forms only in gradient-purified samples, perhaps because the prolonged incubation times before preparation of EM specimens (18–72 h, including 15 h of centrifugation) allowed a slow but stable association to occur.

The sedimentation analysis also yielded a molecular weight (M_{w}) of 71,440 ± 830 for ETG. This value is lower than the 82,000 ± 5000 estimated from electrophoresis and gel filtration (Brenner and Wold, 1978).

CONCLUSIONS

Tissue transglutaminase binds a single high affinity site approximately 10 nm from the N terminus of fibronectin. Formation of this complex does not inhibit enzyme activity on small substrates (e.g. dansylcadaverine) but, in the case of erythrocyte transglutaminase, there is strong inhibition of cross-linking activity with the large protein substrate, fibrinogen. This in vitro inhibition provides evidence that sequestration of the enzyme by plasma fibronectin in vivo may afford protection from indiscriminate cross-linking by transglutaminase accidentally released into the bloodstream. The mechanism by which transglutaminase activity is downregulated following binding to fibronectin remains unclear, but the dependence of inhibition on substrate size suggests that steric blockage of the transglutaminase active site by fibronectin may be important. Future study of the structure of the complex formed in plasma (e.g. linear, ring, or cross-linked polymer), as well as its cross-linking activity and interactions with scavenger cells, will provide further insight into the significance of transglutaminase-fibronectin binding following tissue injury.

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