Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3152–3156, April 1993
Biochemistry

Affinity of human erythrocyte transglutaminase for a 42-kDa gelatin-binding fragment of human plasma fibronectin

(collagen-binding domain/ELISA/fluorescence polarization/thermolytic fragment)

JAMES T. RADEK*, JONG-MOON JEONG*, S. N. PRASANNA MURTHY*, KENNETH C. INGHAM†, AND LASZLO LORAND*‡

*Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208; and the †Holland Laboratory, American Red Cross, Rockville, MD 20855

Contributed by Laszlo Lorand, December 29, 1992

ABSTRACT **Complex formation between the human erythrocyte transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) and fibronectin or its fragments was examined by immunoanalytical procedures and by fluorescence polarization. A 42-kDa gelatin-binding structure, obtained from human plasma fibronectin by thermolytic digestion, showed as high an affinity for the cytosolic enzyme as the parent fibronectin chains themselves. A 21-kDa fragment comprising type I modules 8 and 9, the last two modules in the 42-kDa fragment, bound with an affinity 100-fold less than the 42-kDa fragment. Binding was remarkably specific and could be exploited for the affinity purification of transglutaminase directly from the hemoglobin-depleted erythrocyte lysate. In spite of the high affinity, it was possible to elute active enzyme from the 42-kDa fragment column with 0.25% monochloroacetic acid. This solvent might have general applicability in other systems involving separation of tightly bound ligands.**

Plasma fibronectin is thought to play an important homeostatic role by acting as a scavenger for cytosolic transglutaminases (TGs; protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13). Such enzymes occur in many different cell types and could pose the danger of polymerizing proteins if discharged freely into the circulation (1–3). We have focused on the interaction of human plasma fibronectin with the human erythrocyte (RBC) enzyme (4). Binding between the two proteins is instantaneous and very tight and occurs even in the absence of Ca^{2+}, which indicates that it does not depend on the unmasking of the active center of TG. Studies with chymotryptic fragments of fibronectin showed that the gelatin (collagen)-binding domain of the molecule was involved also in the binding of TG (5). However, the two sites seem to act independently because attachment to TG and gelatin can take place simultaneously in a ternary complex. Electron microscopic examination demonstrates that, along the contour lengths of the constituent chains of fibronectin, human RBC TG was bound at a distance of 5–10 nm from the N termini, frequently forming ring-like structures (6).

By using well-characterized thermolytic fragments of human fibronectin (7), it is now possible to further define the TG-associating domains of the plasma protein. It will be shown in the present paper that an ≈42-kDa gelatin-binding structure, which is sequentially composed of a type I, followed by two type II and three type I motifs (I_6-II_1-II_2-I_7-I_8-I_9), displays a full strength of binding for the human RBC enzyme. The high specificity for binding was exploited for the affinity chromatographic purification of TG directly from the hemoglobin-depleted RBC lysate.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Protein Preparations. The 42-kDa and 30-kDa gelatin-binding fragments of human plasma fibronectin were obtained by digestion with thermolysin as described by Borsi *et al.* (8). Further treatment of the 42-kDa fragment with pepsin was employed for producing a yet smaller 21-kDa gelatin-binding fragment (6). For use in immunoassays, fibronectin was purified as a by-product during the course of isolating factor XIII from outdated human CPDA-1 (1.9 mM citric acid/11 mM sodium citrate/20 mM dextrose/2 mM sodium phosphate/0.025 mM adenine) plasma (9). The protein was stored at −20°C in 50 mM Tris·HCl, pH 7.5/1 mM EDTA/Trasylol (FBA Pharmaceuticals, West Haven, CT; 10 units/ml). The 42-kDa thermolytic product (6.7 mg/ml) was taken up in 20 mM Tris·HCl, pH 7.4/0.02% NaN_3; the 30-kDa (5 mg/ml) and 21-kDa (2.3 mg/ml) fragments were dissolved in 20 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM EDTA/0.02% NaN_3. All products were stored at 4°C. Protein concentrations were estimated on the basis of the following $A_{280}^{1\%}$ values: 12.8 for fibronectin (10); 16.1, 16.7, and 15.3 for the 42-kDa, 30-kDa, and 21-kDa fragments, respectively (11).

Labeling of the 42-kDa (0.2 mg), 30-kDa (0.3 mg), and 21-kDa (0.3 mg) gelatin-binding fragments of fibronectin with fluorescein isothiocyanate (FITC; Sigma) was carried out as described by Freyssinet *et al.* (12). A 6-fold molar excess of FITC was mixed with the proteins in 0.1 M Tris·HCl/1 mM EDTA, pH 9.0 (room temperature, 1 hr, dark). Unreacted FITC was removed by gel filtration on a Sephadex G-50 (Sigma) column (1 × 16 cm) equilibrated in 100 mM Tris·HCl/1 mM EDTA, pH 7.5. The fluorescein-labeled proteins were concentrated on a PM10 membrane (Amicon) and dialyzed overnight against 75 mM Tris·HCl/1 mM EDTA, pH 7.5, containing 2% (wt/vol) activated charcoal (Norit-A; MCB Chemical) at 4°C. By using a molar extinction coefficient of 3.4×10^4 (13), the extent of labeling was found to be 1.5, 1.4, and 0.37 mol/mol of 42-, 30-, and 21-kDa fragments, respectively. Concentrations of the labeled derivatives were determined by the method of Lowry *et al.* (14) using bovine serum albumin (BSA; Pierce) as standard.

Human RBC TG was isolated either by a published procedure (15) or by the affinity chromatographic method of purification presented in *Results and Discussion*. For the latter purpose, blood (collected by Life Source, Glenview, IL, in CPDA-1 anticoagulant 2 weeks earlier) was centrifuged at 3000 × *g* for 10 min. The plasma and buffy coat were removed by aspiration, the RBCs were mixed with 2 vol of buffer K (0.1 M KCl/0.06 M NaCl/5 mM Tris·HCl, pH 7.4/0.01 M glucose), and the suspension was passed through a column of sterile absorbent cotton wool (3.5 × 7 cm,

Abbreviations: RBC, erythrocyte; TG, transglutaminase; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin.

‡To whom reprint requests should be addressed.

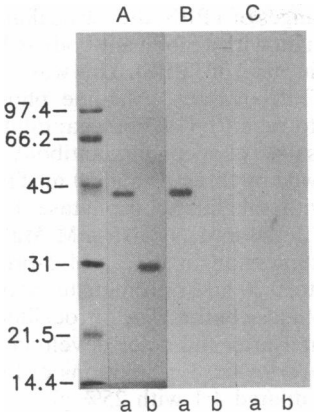

FIG. 1. Overlay assay demonstrates binding of human RBC TG to the 42-kDa fragment of human plasma fibronectin. (*A*) Protein stain. (*B*) Immunoblot for detecting the binding of TG with a monospecific antibody to the enzyme. (*C*) Control blot without overlaying of TG. Lanes: a, 42-kDa fragment; b, 30-kDa fragment. Molecular mass markers are shown on left in kDa.

Johnson & Johnson, New Brunswick, NJ). RBCs were separated by centrifugation (3000 × *g* for 10 min) and were washed three times with buffer K. Approximately 110 ml of packed RBCs was lysed by adding 10 vol of 5 mM imidazole hydrochloride, pH 7.5/1 mM EDTA/1 mM dithiothreitol/protease inhibitors [phenylmethylsulfonyl fluoride (20 μg/ml)/leupeptin (2 μg/ml)/pepstatin (2 μg/ml)/1 mM benzamidine/Trasylol (10 units/ml)]. Membranes were discarded after centrifugation (47,000 × *g* for 20 min) and 1045 ml of supernatant was mixed with DEAE-Sephadex A-50 [Sigma; 6 g, preswollen in 300 ml of 0.2 M imidazole hydrochloride (pH 7.5) and then equilibrated in 20 mM imidazole hydrochloride pH 7.5/1 mM EDTA] and stirred for 16 hr at 4°C. The suspension was poured into a column (2.5 × 40 cm) and washed with 300 ml of 0.2 M NaCl/1 mM EDTA/20 mM imidazole hydrochloride, pH 7.5, to ensure complete removal of hemoglobin. During subsequent elution with 0.5 M NaCl in 20 mM imidazole hydrochloride, pH 7.5/1 mM EDTA/10% (vol/vol) glycerol, protein was collected between 85 and

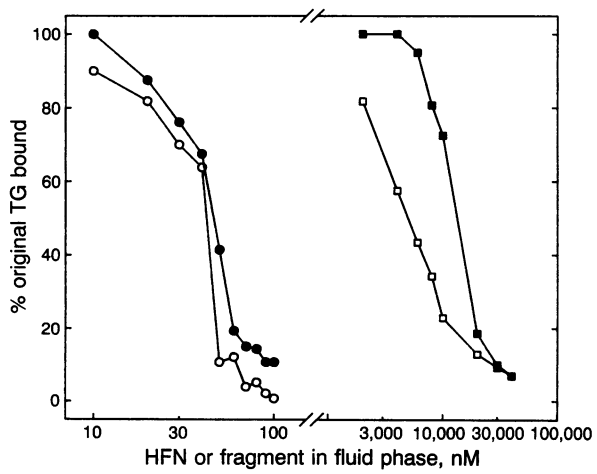

FIG. 2. Inhibition of binding of RBC TG to solid-phase human fibronectin (HFN) by fluid-phase HFN or its gelatin-binding fragments. Mixtures of fixed amounts of TG (5 pmol) were prepared in the fluid phase with various concentrations of fibronectin (●) or its fragments [i.e., 42 kDa (○), 21 kDa (□), or 30 kDa (■)], and the free TG remaining was allowed to bind to microtiter plates coated with fibronectin (1 μg). Binding was detected with a monospecific antibody to the enzyme. The ordinate represents the calculated percent of TG left unbound in the fluid-phase mixtures of the enzyme with fibronectin (given as a single-chain equivalent) or its fragments.

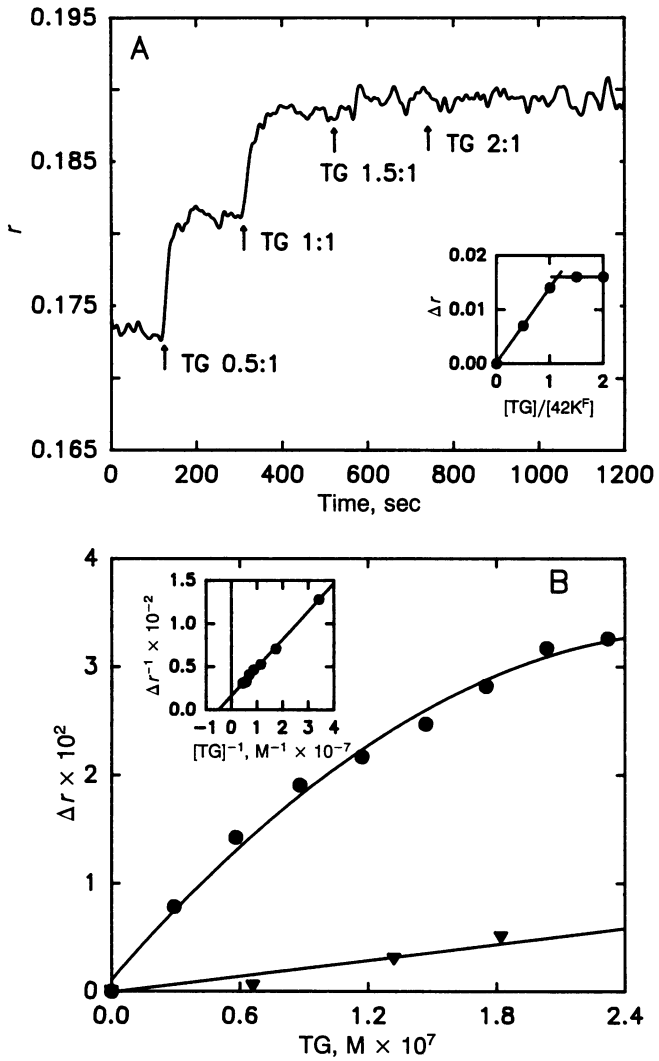

FIG. 3. Binding of RBC TG to fluorescein-labeled fragments of fibronectin as examined by the technique of fluorescence polarization. (*A*) Anisotropy values (*r*) observed in a solution of 41 nM fluorescein-labeled 42-kDa fragment ($42K^F$) upon injection of TG to increasing mole proportions of TG to $42K^F$ (0.5:1, 1:1, 1.5:1, and 2:1). (*Inset*) Changes in anisotropy (Δ*r*) as a function of the [TG]/[$42K^F$] ratio are plotted. (*B*) Changes in fluorescence anisotropy of 30 nM solutions of fluorescein-labeled 21-kDa (●) and 30-kDa (▼) fragments of fibronectin with various concentrations of TG. (*Inset*) Double-reciprocal plot of the data for the 21-kDa fluorescent fragment.

138 ml of effluent and was pooled for an affinity purification step employing the 42-kDa fragment of fibronectin. The affinity chromatographic column (1 × 2 cm; 2.6 mg of the 42-kDa fragment coupled to 2 ml of Affi-Gel-15 according to instructions of Bio-Rad) was equilibrated with 20 mM imidazole hydrochloride, pH 7.5/1 mM EDTA/10% glycerol. The protein (representing an input of ≈144 A_{280} units) was passed through the column (flow rate, 8 ml/hr). The bulk of the unbound proteins (≈130 A_{280} units) was removed by washing with 80 ml of 0.5 M NaCl/1 mM EDTA/20 mM imidazole hydrochloride, pH 7.5/10% glycerol. When the A_{280} of the effluent dropped to near zero (20 ml of additional wash buffer), a solution of 0.25% monochloroacetic acid in 0.2 M NaCl and 10% glycerol was applied. In the course of collecting, the pH of each fraction (1 ml) was raised to pH ≈6.3 by addition of 0.1 ml of 0.75 M imidazole hydrochloride, pH 7.2/10% glycerol/5 mM EDTA. TG activity was measured (on 2-μl aliquots of fractions in a total reaction volume of 40 μl) by the incorporation (37°C for 60 min) of [^{14}C]putrescine

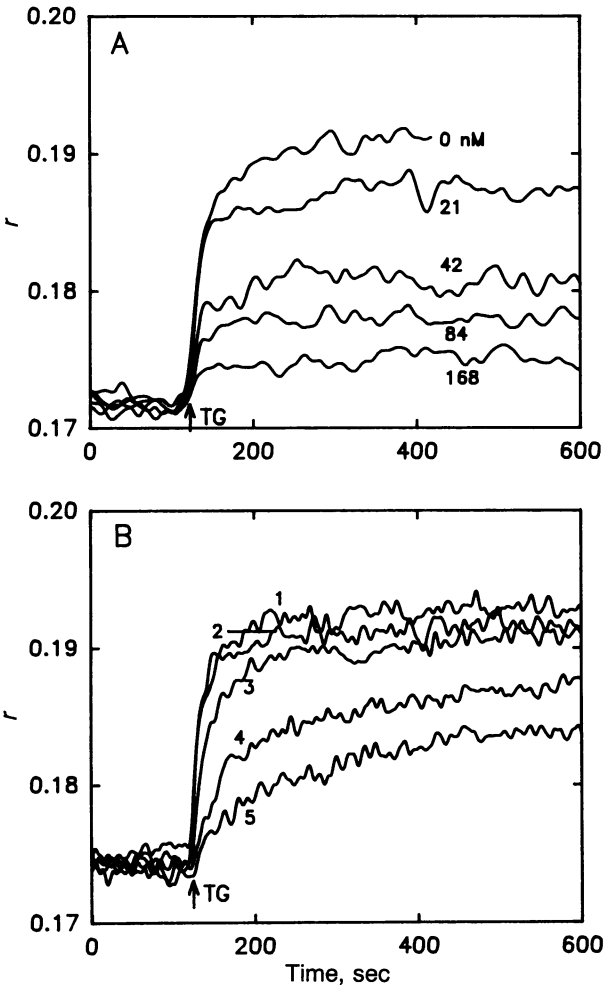

FIG. 4. Competition of unlabeled fragments of fibronectin against the fluorescein-labeled 42-kDa fragment for binding to TG. (*A*) TG was added (50 nM; 120 sec, arrow) to solutions of the 42-kDa fluorescent fragment (41 nM) premixed with various concentrations of unlabeled 42-kDa fragment (0–168 nM). Fluorescence anisotropy values (*r*) were measured (ordinate). (*B*) Results for similar experiments of adding TG to 41 nM 42-kDa fluorescent fragment in mixtures with unlabeled 30-kDa fragment (3200 nM, line 2) or unlabeled 21-kDa fragment (400 nM, line 3; 800 nM, line 4; and 1600 nM, line 5). Line 1 is a control with the 42-kDa fluorescent fragment (41 nM) alone.

(0.5 mM, 4.8 μCi/μmol; 1 Ci = 37 GBq) into *N,N*-dimethylcasein (2 mg/ml) in the presence of 10 mM dithiothreitol and 5 mM $CaCl_2$, by using the filter paper assay described by Lorand *et al.* (16). Ten active fractions were pooled and concentrated on a Centricon-30 microconcentrator (Amicon) by centrifuging at 3000 × *g*. The concentrated enzyme (≈3 A_{280} units in 0.45 ml) was stored at −80°C. Purity of the enzyme preparation was also assayed with one- and two-dimensional polyacrylamide gel electrophoresis (17, 18) and by activity staining with dansylcadaverine after nondenaturing electrophoresis in agarose (19).

Immunoanalytical procedures. *Dot-blot assay.* One microliter of fibronectin or its proteolytic fragments (42, 30, and 21 kDa) was applied in various dilutions, containing from 4 to 1000 ng of protein, to nitrocellulose [BA83, 0.2 μm (pore size); Schleicher & Schuell], which was then washed in 0.05% Tween 20/10 mM sodium phosphate/0.145 M NaCl, pH 7.4 (TPBS; three 10-min 50-ml washes), and immersed in a solution of purified human RBC TG (120 μg in 50 ml of TPBS for 1 hr at room temperature). The control was incubated in TPBS alone. After further washing with three 10-min 50-ml changes of TPBS, the nitrocellulose sheets were incubated overnight with a rabbit antibody to human RBC TG (1:2000 dilution in 50 ml of TPBS). This was followed by three 10-min 50-ml TPBS washes. Alkaline phosphatase-conjugated goat IgG to rabbit IgG (Promega; diluted 1:5000 in 50 ml of TPBS) was used as secondary antibody during a 90-min treatment, followed by three 10-min 50-ml TPBS washes and a final rinse with alkaline phosphatase buffer (100 mM Tris·HCl, pH 9.5/100 mM NaCl/5 mM $MgCl_2$). Color was developed by immersion in 0.37 mM 5-bromo-4-chloro-3-indolyl phosphate/0.39 mM nitro blue tetrazolium (Sigma) in the above phosphatase buffer. The nitrocellulose sheets were washed with water after full color development.

SDS/PAGE overlay assay. Solutions of fibronectin or its fragments were diluted 4:1 with 25% glycerol/5% (wt/vol) SDS/5 mM EDTA/50 mM Tris·HCl, pH 6.8, and electrophoresed (3 μg of proteins per lane) by the procedure of Laemmli (17) on 12.5% polyacrylamide gels. Transfer to nitrocellulose (20) was accomplished in an LKB transfer electroblotting unit (Bromma, Sweden) in 25 mM Tris/192 mM glycine/20% methanol, at pH 8.3 (2 hr, 0.6 A, 4°C). Overlaying with human TG and probing of the attached enzyme by antibody were carried out as described for the dot-blot assay.

ELISA competition assay. Microtiter wells (Dynatech) were coated with a solution of human plasma fibronectin (1 μg in 100 μl of PBS per well) for a period of 2 hr at room temperature on an orbit shaker (Lab-Line, Melrose Park, IL) and were washed for three 5-min periods with 0.5% BSA (Sigma) in 200 μl of PBS per well. Parallel to this blocking procedure, 50 μl of RBC TG (5 pmol in PBS) was mixed with 50 μl of fibronectin or the 42-kDa (0–10 pmol), 30-kDa, or 21-kDa fragments (0–4 nmol) in various dilutions with PBS; after a 30-min incubation, 900 μl of PBS with 0.5% BSA was admixed, and 100 μl was pipetted into the wells to allow for the free TG remaining in the solutions to bind to the fibronectin-coated wells. This step was followed by three 5-min washings with 0.5% BSA in 200 μl of PBS per well and then by the addition of the rabbit antibody to human RBC TG (200 μl per well, 1:2000 dilution with 0.5% BSA in PBS; 1 hr) and by three 5-min washings with 200 μl of 0.5% BSA in PBS. Alkaline phosphatase-conjugated goat IgG to rabbit IgG (Promega) was used as secondary antibody (200 μl per well, 1:5000 dilution with 0.5% BSA in PBS; 1 hr). The plates were washed for two 5-min periods with 0.5% BSA in PBS and, finally, with the alkaline phosphatase buffer (5 min) prior to initiating color development by adding *p*-nitrophenyl phosphate (Sigma; 1 mg/ml in the alkaline phosphatase buffer) at 100 μl per well. A_{410} values were read after 30 min with a Dynatech plate reader (Chantilly, VA). A calibration curve was obtained for human RBC TG (0–6 pmol per well) on a fibronectin-coated plate and was employed for computing the amounts of TG that were left unbound in the fluid-phase mixtures of the enzyme with fibronectin or its fragments.

Fluorescence Polarization. Experiments were performed on a SLM 8000C double-emission spectrofluorometer (SLM Aminco, Urbana, IL) with Glan-Thompson calcite prism polarizers in the excitation and emission channels (21). Protein solutions (≈2 ml) of 0.6–3.0 μg/ml in 75 mM Tris·HCl/150 mM NaCl/0.5 mM EDTA, pH 7.5, were placed in a 1 × 1 cm quartz cuvette with continuous stirring (37°C). Rapid additions were made with a microsyringe (Hamilton) through the port of the sample chamber. Excitation and emission wavelengths were set to 490 and 530 nm, respectively. Polarization was calculated as

$$P = [(R_{vert}/R_{horiz}) - 1]/[(R_{vert}/R_{horiz}) + 1]$$

$$= (R_{corr} - 1)/(R_{corr} + 1),$$

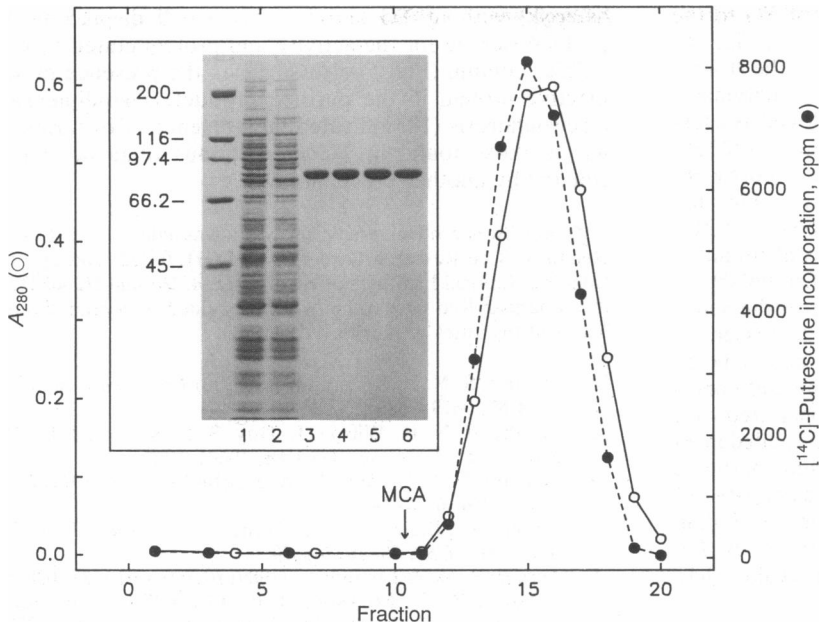

FIG. 5. Affinity purification of human RBC TG on a column of the 42-kDa gelatin-binding fragment of human fibronectin. Hemoglobin-depleted cell lysate was applied to the affinity column. After extensive washing, the enzyme was eluted with 0.25% monochloroacetic acid (MCA). Fractions were neutralized and analyzed for protein [A_{280}, left ordinate (○)] and enzyme activity [right ordinate (●); corresponding to a 40-μl reaction mixture containing 2 μl of eluted fraction]. (*Inset*) Samples were analyzed by SDS/PAGE and stained with Coomassie brilliant blue R. Lanes: 1, hemoglobin-depleted lysate; 2, unbound fraction from 42-kDa fragment affinity column; 3–6, fractions 14–17 eluted from affinity column. Molecular masses in kDa are indicated.

where R_{vert} is the ratio of emissions in the two channels when the excitation polarizer is in the vertical (0°) direction and R_{horiz} when the excitation polarizer is in the horizontal (90°) direction. Anisotropy (r) is defined as $r = 2P/(3 - P)$. R_{corr} is R_{vert}/R_{horiz}. Data were subjected to a smoothing routine to reduce background noise with a 16-nm-bandwidth sharp-cutoff three-point low-pass linear digital filter (10 passes) and were stored in an IBM Personal System 2, model 50Z, computer with software provided by SLM Aminco.

RESULTS AND DISCUSSION

The dot-blot assay was employed for the preliminary screening in which fibronectin derivatives, immobilized on nitrocellulose, were allowed to bind to purified RBC TG. The bound enzyme was detected by a specific antibody. Of the three gelatin-binding fragments of human fibronectin (i.e., 42, 30, and 21 kDa), only the 42-kDa material gave a positive result for binding TG (data not shown). Amido black-stained transblots of SDS/PAGE gels showed well-defined bands for the 42- and 30-kDa fragments (Fig. 1*A*, lanes a and b, respectively), but only a diffuse and poorly staining area at 21 kDa (data not shown). Binding of the overlaid enzyme was evident only at the position of the 42-kDa band (Fig. 1*B*, lane a). It is important to point out that SDS/PAGE for the overlay procedure was performed in the absence of reducing agent, because we found that treatment of the 42-kDa fragment with dithiothreitol (40 mM, 37°C, 30 min) abolished its ability to bind TG. This indicates that some retention of the disulfide-bonded character of this structure (I_6-II_1-II_2-I_7-I_8-I_9; ref. 7) was critical in the binding of TG.

An ELISA proved to be the most sensitive immunological procedure for demonstrating the binding of TG also to the 21- and 30-kDa fragments, but the measured affinities were 100- to 300-fold weaker than for the 42-kDa fragment (Fig. 2). On a single-chain equivalency basis, the 42-kDa fragment displayed as high a binding affinity for the RBC enzyme as the constituent chains of human plasma fibronectin themselves; 50% inhibition occurs near 50 nM of either species. As of now, the 21-kDa fragment is the smallest fragment of fibronectin capable of binding TG. It represents the two type I motifs (I_8-I_9) at the C-terminal end of the 42-kDa domain. The 30-kDa structure, with a still weaker ability to bind TG, contains the N-terminal type I motif of the 42-kDa fragment followed by the two type II and one type I motifs of this fragment (I_6-II_1-II_2-I_7). It is tempting to suggest that a fragment corresponding to the three C-terminal type I units of the 42-kDa fragment (i.e., I_7-I_8-I_9) would be the minimum structure capable of binding TG with high affinity.

The technique of fluorescence polarization offered an even more rigorous approach for probing the interaction of human RBC TG with fragments of fibronectin. These fragments were labeled with FITC to yield the 42-, 30-, and 21-kDa fluorescent derivatives. Binding to TG was evaluated by measuring the change in fluorescence anisotropy (r) occurring with the addition of TG. Fig. 3*A* illustrates the increase in the anisotropy value of a solution of the 42-kDa fluorescent fragment (41 nM) upon the incremental addition of TG. As the mole ratio of the enzyme to the 42-kDa fluorescent fragment exceeds 1:1, no significant further increase of anisotropy was recorded, suggesting very tight binding for a one-to-one stoichiometric complex between the 42-kDa fluorescent fragment and TG (see Fig. 3*A* *Inset*). The same approach could

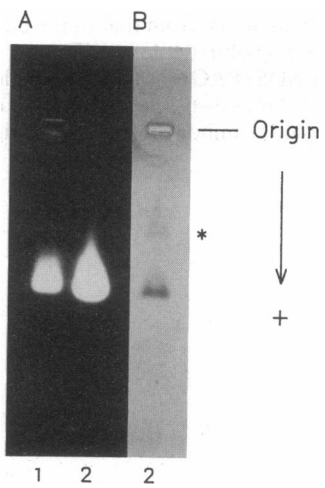

FIG. 6. RBC TG isolated by affinity chromatography has the same mobility as the enzyme in the cell lysate, as detected by activity staining (14) after nondenaturing electrophoresis. (*A*) Activity staining with dansylcadaverine photographed under UV light (366 nm). (*B*) Protein staining (Coomassie brilliant blue R) of sample electrophoresed in parallel. Lanes: 1, human RBC lysate; 2, TG purified on 42-kDa fragment affinity column. A minor band, representing an inactive slower moving protein, is marked by an asterisk.

be used to demonstrate a fairly tight binding of TG to the 21-kDa fluorescent fragment ($K_{d,app} = 1.8 \times 10^{-7}$; Fig. 3*B*, upper curve and *Inset*). Binding of the enzyme to the 30-kDa fluorescent fragment (Fig. 3*B*, lower curve) was marginal.

Fluorescence polarization could also be employed as a test for competition of unlabeled fibronectin fragments (42, 30, and 21 kDa) against the binding of the 42-kDa fluorescent fragment to TG. In the experiment presented in Fig. 4*A*, increasing amounts (0–168 nM) of the unlabeled 42-kDa fragment were mixed with the 42-kDa fluorescent fragment (41 nM) prior to the injection of TG (50 nM). The unlabeled fragment was found to be an equal competitor in the sense that the anisotropy value of the system was depressed by ≈50% when near equal amounts of the labeled and unlabeled fragments were present. Under similar experimental conditions, no significant competition could be demonstrated with the unlabeled 30-kDa fragment, even when it was added in an ≈76-fold excess over the 42-kDa fluorescent fragment (line 2 in Fig. 4*B*). The unlabeled 21-kDa fragment was capable of competing with the 42-kDa fluorescent fragment for binding to TG (lines 3–5 in Fig. 4*B*), albeit competition by the unlabeled 21-kDa fragment was nearly 40-fold weaker than with the unlabeled 42-kDa fragment.

Binding of the RBC TG to the 42-kDa fragment of human plasma fibronectin was also found to be of remarkably high specificity. A single passage of the hemoglobin-depleted RBC lysate (110 ml) through an affinity column with the covalently coupled 42-kDa fragment (2.6 mg) as ligand caused a near-total removal of TG from among the many proteins of the lysate. The most obvious difference between the protein profiles of the material applied to the column and the pass-through fraction was the absence of an ≈80-kDa band corresponding to TG (Fig. 5 *Inset*, compare lanes 1 and 2). Desorbing the enzyme with fair retention of activity proved to be difficult because, possibly on account of the tight binding, conventional methods of elution [e.g., with 10% (vol/vol) acetic acid, 50 mM Tris·HCl, pH 7.5/1 M NaCl, 0.5 M $MgCl_2$, or 4 M urea; 4°C] were ineffective. However, satisfactory results were obtained in a single step of purification with 0.25% monochloroacetic acid (Fig. 5 *Inset*, lanes 3–6), allowing an ≈18% recovery of enzyme activity, measured by the [^{14}C]putrescine incorporation assay (16).

Mobility of the purified TG in nondenaturing electrophoresis, as observed by activity staining with dansylcadaverine (19), was indistinguishable from that of the enzyme in the cell lysate prior to hemoglobin depletion (Fig. 6). However, even though reduced SDS/PAGE showed a single protein of 80 kDa (Fig. 5 *Inset*, lanes 3–6), nondenaturing electrophoresis in agarose revealed a minor slower-migrating band (Fig. 6, asterisk) without TG activity. The small displacement of peaks observed for the activity and protein eluted from the affinity column (Fig. 5) also suggests the presence of some inactive protein in the purified product. Two-dimensional electrophoresis (18) indicated the presence of isoforms similar to those found in TG preparations (data not shown) isolated by another procedure (15).

At Northwestern University, this work was aided by a U.S. Public Health Service Research Career Award (HL-03512) and by grants from the National Institutes of Health (HL-45168 and HL-02212); at the American Red Cross, this work was aided by a grant from the National Institutes of Health (HL-21791).

1. Murthy, S. N. P. & Lorand, L. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9679–9682.
2. Murthy, S. N. P., Wilson, J., Guy, S. L. & Lorand, L. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10601–10604.
3. Shainoff, J. R., Urbanic, D. A. & DiBello, P. M. (1991) *J. Biol. Chem.* **266**, 6429–6437.
4. Lorand, L., Dailey, J. E. & Turner, P. M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1057–1059.
5. Turner, P. M. & Lorand, L. (1989) *Biochemistry* **28**, 628–635.
6. LeMosy, E. K., Erickson, H. P., Beyer, W. F., Jr., Radek, J. T., Jeong, J. M., Murthy, S. N. P. & Lorand, L. (1992) *J. Biol. Chem.* **267**, 7880–7885.
7. Ingham, K. C., Brew, S. A. & Migliorin, M. M. (1989) *J. Biol. Chem.* **264**, 16977–16980.
8. Borsi, L., Castallani, P., Balza, E., Siri, A., Pellecchia, C., DeScalzi, F. & Zardi, L. (1986) *Anal. Biochem.* **155**, 335–345.
9. Lorand, L., Credo, R. B. & Janus, T. J. (1981) *Methods Enzymol.* **80**, 333–341.
10. Mosesson, M. W. & Umfleet, R. A. (1970) *J. Biol. Chem.* **245**, 5728–5736.
11. Litvinovich, S. V., Strickland, D. K., Medved, L. V. & Ingham, K. C. (1991) *J. Mol. Biol.* **217**, 563–575.
12. Freyssinet, J.-M., Lewis, B. A., Holbrook, J. J. & Shore, J. D. (1978) *Biochem. J.* **169**, 403–410.
13. Churchich, J. E. (1967) *Biochim. Biophys. Acta* **147**, 511–517.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
15. Brenner, S. C. & Wold, F. (1978) *Biochim. Biophys. Acta* **522**, 74–83.
16. Lorand, L., Campbell-Wilkes, L. K. & Cooperstein, L. (1972) *Anal. Biochem.* **50**, 623–631.
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
18. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
19. Lorand, L., Siefring, G. E., Jr., Tong, Y. S., Bruner-Lorand, J. & Gray, J. A., Jr. (1979) *Anal. Biochem.* **93**, 453–458.
20. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
21. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy* (Plenum, New York), pp. 111–153.