Tissue (Type II) Transglutaminase Covalently Incorporates Itself, Fibrinogen, or Fibronectin into High Molecular Weight Complexes on the Extracellular Surface of Isolated Hepatocytes

USE OF 2-[(2-OXOPROPYL)THIO]IMIDAZOLIUM DERivATIVES AS CELLULAR TRANSGLUTAMINASE INACTIVATORS

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Rabbit hepatocyte surface-expressed tissue (type II) transglutaminase is shown to act as a binding site for fibrinogen or fibronectin and to covalently incorporate these glycoproteins, in addition to itself, into extracellular high molecular weight complexes. This concept is supported by the observation that a nonpeptidyl, active site-directed transglutaminase inactivator (L683685) elicited concentration-dependent (0.1–10 μM) decreases in the calcium-dependent binding and covalent cross-linking of 125I-fibrinogen, 125I-fibronectin, or [14C]putrescine by hepatocyte suspensions. In corroboration with these findings, an antiserum against rabbit liver transglutaminase, which did not cross-react with rabbit factor XIII, elicited concentration-dependent decreases in the calcium-dependent binding and covalent cross-linking of 125I-fibrinogen or [14C]putrescine by hepatocyte suspensions. Western blots of sodium dodecyl sulfate/Triton-insoluble hepatocyte fractions conducted with this antiserum, with a polyclonal antiserum against human erythrocyte transglutaminase, or with a monoclonal antibody (CUB-701) against guinea pig liver transglutaminase detected the 80-kDa tissue transglutaminase, as well as tissue transglutaminase-immunoreactive bands of higher molecular mass (range of 90 to >200 kDa). The higher molecular weight species were preferentially incorporated, in a time- and calcium-dependent manner, into very high molecular weight complexes which did not enter the stacking gel. Incorporation of these tissue transglutaminase-containing bands into the high molecular weight complexes was inhibited by L683685, indicating that cross-linking by the enzyme was responsible for the assembly of the complexes of which tissue transglutaminase was a component. Cellular integrins did not mediate ligand binding under the experimental conditions, as evidenced by the failure of the Arg-Gly-Asp-Ser tetrapeptide or anti-integrin antibodies to inhibit binding or cross-linking of 125I-fibrinogen or 125I-fibronectin, in the presence or absence of transglutaminase inactivators.

Transglutaminases mediate covalent cross-linking between proteins by forming amide bonds between the γ-carboxamide groups of appropriate peptide-bound glutamine moieties and the ε-amino groups of specific peptide-bound lysine residues (1). These calcium-dependent enzymes are ubiquitously distributed in mammalian tissues and body fluids (2, 3). Though many molecular forms of transglutaminase have been purified from a wide variety of tissues (1), cDNA analysis has thus far revealed only three distinct transglutaminase genes. The most recently described cDNA (4, 5) encodes for keratinocyte (type I) transglutaminase, a predominantly membrane-bound enzyme which shows homology to the previously sequenced tissue (type II) transglutaminase (6–8), as well as to the initially sequenced (9, 10) plasma transglutaminase (Factor XIIIa). A fourth transglutaminase, known as epidermal transglutaminase (11, 12), is an immunologically distinct enzyme previously referred to as hair follicle transglutaminase (13); however, a cDNA for this transglutaminase has not been reported.

Fibrinogen and fibronectin are adhesive glycoproteins found in plasma and extracellular fluids (14). These molecules share certain structural similarities, the most germane of which are the occurrence of the Arg-Gly-Asp integrin-binding sequence (14–16) and of tissue transglutaminase-sensitive cross-linking sites (2, 17–20) within their primary structures. This being the case, it is reasonable to assume that these macromolecules can interact with cell surfaces via their Arg-Gly-Asp sequences and, if transglutaminase is present, undergo covalent cross-linking reactions. In the course of studies on the interaction of these glycoproteins with hepatocytes, however, it was initially demonstrated (21) that when fibrinogen binding to hepatocyte suspensions is analyzed using a 3-h incubation at 4°C and a ligand concentration of 30 nM (which is below the Kd for fibrinogen binding to the platelet integrin, glycoprotein IIb/IIIa (Ref. 22)), an Arg-Gly-Asp-independent, transglutaminase-mediated interaction could be isolated and studied in detail (23). The same observation proved to be true for fibronectin binding to hepatocyte suspensions (23, 24). Under these experimental conditions, binding of fibrinogen and fibronectin to hepatocytes is Arg-Gly-Asp-independent, largely irreversible, and accompanied by cross-linking of the fibrinogen or fibronectin by tissue transglutaminase (23).

In light of the findings described above (21–24) and the observations that tissue transglutaminase specifically binds to fibrinogen (2), fibrin (25), and fibronectin (26–28), we hypothesized that an hepatocyte surface-expressed transglu-
Hepatocyte Surface-expressed Tissue Transglutaminase

EXPERIMENTAL PROCEDURES

Materials—Class II collagenase (lot 48702A) was obtained from Worthington. Carrier-free [221]T (Na in NaOH) was from Cambridge Medical Diagnostics, Inc. (Billerica, MA). Silicone oil (DC550) was purchased from Dow Corning Corp. through William K. Nye, Inc. (New Bedford, MA). Light mineral oil was from the pharmacy. NCS tissue solubilizer, OCS liquid scintillation mixture, and [1,4-3H]putrescine dihydrochloride (118 mCi/mmol) were obtained from Amer sham Corp. Sodium heparin (1000 USP units/ml) was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ). Hirudin, evidence and peptatin were obtained from Sigma. The nonpeptidyl transglutaminase inactivator was a gift of Drs. David Remy, David A. Claremon, and John J. Baldwin of Merck Sharp & Dohme Research Laboratories. Mechanistic compounds function as active site-directed transglutaminase inactivators.1 Goat antiserum against rabbit liver tissue transglutaminase (30) was generously provided by Dr. Soo Il Chung (National Institutes of Health). A polyclonal rabbit anti-human erythrocyte transglutaminase antiserum (31) and a monoclonal antibody (CUB-7401) against guinea pig liver transglutaminase (32) were obtained from Dr. Paul Birckbichler of the Samuel Roberts Noble Foundation (Ardmore, OK). The monoclonal antibody BC1 (33) against human keratinocyte (type I) transglutaminase was a gift from Dr. Robert Rice of the University of California (Davis, CA). Monoclonal antibody 7E3 (34), reactive with the human vitronectin receptor (αvβ3), was a gift of Dr. Barry Coller of New York University. The monoclonal antibody against the human fibronectin receptor (α5β1) was purchased from Telios Pharmaceuticals, Inc. (San Diego, CA). All other reagents were purchased from Sigma or Fisher.

Purification and Labeling of Fibronectin and Fibronectin—Rabbit Factor XIII-free fibronectin was purified by DEAE-cellulose chromatography (35) as previously described (24). Human plasma fibronectin was purified by gelatin-Sepharose chromatography (36) as described previously (24). The proteins were labeled with [3H]I, to a specific activity of 100-300 cpm/μg, by the iodine monochloride procedure (37).

Hepatocyte Preparation—Stock suspensions of hepatocytes (5 × 106 cells/ml as determined using a Coulter counter) were prepared by a 5-10-min perfusion (60 ml/min) with collagenase (1 mg/ml) as previously described (22). Transglutaminase was prepared calcium-free “binding buffer” consisting of 0.15 M NaCl, 0.025 M NaI, 0.1 M CaCl2, (pH 7.4) containing hirudin (0.05 units/ml), aprotinin (200 kallikrein inactivating units/ml), leupepin (50 μg/ml), and peptatin (0.1 μg/ml), and kept at 4 °C on a horizontal shaker operated at 30 cycles/min for no longer than 15 min prior to the initiation of binding assays. The calcium-free conditions were used to prevent transglutaminase-mediated cross-linking of endogenous (unlabeled) proteins.

Binding Assays—Binding of [125I]labeled fibrinogen or fibronectin to suspended rabbit hepatocytes was measured as previously described (21). Briefly, ligands were incubated at 4 °C with isolated rabbit hepatocytes (2.5 × 106 cells/ml) suspended in binding buffer containing 5 mM calcium chloride or 0.5 mM EDTA which were added just prior to addition of radiolabeled ligands. Cell-bound ligands were separated from free ligands by centrifugation (12,000 × g for 1 min) of cells through an ice-cold oil mixture (4 parts silicone oil to 1 part mineral oil) and eluted by 2 min of 1-ml cycles with 1 ml of ethanol. Activity of 100-800 cpm/ng, by the iodine monochloride procedure (37).

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography—The molecular integrity of hepatocyte-bound [125I]Fgn, [125I]Fn, or 3H putrescine was examined by subjecting the solubilized cell pellet to SDS-polyacrylamide gel electrophoresis under reducing conditions, as described by Laemmli (38), using 4% polyacrylamide stacking gel and 10% (for [125I]Fgn or [125I]putrescine) or 7.5% (for [125I]Fn) polyacrylamide resolving gel. Gels were stained with Coomassie Brilliant Blue and autoradiography (or fluorography, in the case of [125I]putrescine incorporation) was performed using standard procedures. Scanning of autoradiograms was performed using a CAMAC TCC Scanner II on-line with a CAMAC SP4290 Integrator. Transmission was measured using a wavelength of 500 nm and a beam width equal to that of the entire lane. The lanes were scanned at 0.4 mm of the intensity maximum at the position of the major radioactive peak. Displacement was corrected using the intensity of the fibrinogen β chain (which did not undergo cross-linking) of each lane as an internal standard.
Western Blot—One-ml aliquots of hepatocyte suspension (≈5 × 10^9 cell/ml) were centrifuged through silicone oil, the buffer and oil were aspirated, and the cells solubilized in 250 μl of 0.01 M Tris (pH 7.5) containing 0.1% SDS, 2% Triton X-100, leupeptin (50 μg/ml), pepstatin (1 μg/ml), and phenylmethylsulfonyl fluoride (0.1 mM). The solubilized cells were kept at 4°C for 15 min and then centrifuged at 11,000 × g for 10 min. The supernatant was removed, the pellets resolubilized as before, and recentrifuged. The final pellets were suspended in 150 μl of 0.02 M Tris (pH 7.5) containing 4% SDS, 8 M urea, 10% β-mercaptoethanol, 2 mM EDTA, 2 mM N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride. Following the addition of bromphenol blue, the samples were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis using a 7.5% gel. Transfer of proteins to type HA 0.45-μm pore nitrocellulose paper (Millipore Corp., Bedford, MA) was performed with a Hoefer Transphor Model 7500 Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA). The transfer buffer consisted of 25 mM THAM, 192 mM glycine, 20% (v:v) methanol, and 400 mA for transfer of 0.02 M Tris, 0.02 M Tris-HCl, pH 7.4 for 3 h at 37°C in order to block nonspecific binding. The primary antibodies were diluted 1:250 (goat anti-rabbit liver transglutaminase), rabbit anti-human erythrocyte transglutaminase, and B.C1 monoclonal antibody (0.6 mg/ml) against human keratinocyte transglutaminase or 1:5000 (ascites fluid containing monoclonal antibody CUB-7401 against guinea pig liver transglutaminase) with Tris-buffered saline containing 1% gelatin and incubated with the nitrocellulose paper overnight at room temperature, followed by five 15-min washes with 0.05% Tween 20 in Tris-buffered saline. The secondary antibody, peroxidase-conjugated rabbit anti-goat IgG (Cappel Laboratories, Cochranville, PA), was diluted 1:10,000 with Tris-buffered saline containing 1% gelatin and incubated with the nitrocellulose paper for 3 h at room temperature. The color development reaction for visualization of transglutaminase-immunoactive bands was performed using the 4 CN membrane peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD), following the directions of the manufacturer.

RESULTS

To study the potential role of transglutaminase in mediating the interaction of fibrinogen or fibrinogen with suspended hepatocytes, we utilized several compounds which are members of a class of transglutaminase inactivators that have recently been shown to produce rapid and irreversible inhibition of human Factor XIIIa and, by analogy, of human erythrocyte transglutaminase. These compounds are highly specific transglutaminase inactivators as evidenced by observations that (a) acetylation of the active site cysteine of Factor XIIIa is the mechanism responsible for inactivation, (b) no other cysteine residues in the Factor XIIIa molecule are affected, (c) the inactivators are about 10^-1 to 10^-2 times less reactive against glutathione, a low molecular weight sulfhydryl-containing molecule, and (d) the compounds do not show detectable reactivity toward papain or several other sulfhydryl reagent-sensitive enzymes tested.

Initial screening experiments conducted with four different transglutaminase inactivators revealed that each of these low molecular weight compounds, when used at a concentration of 10 μM, markedly inhibited the calcium-dependent binding (data not shown) and the covalent cross-linking of 125I-Fgn (Fig. 1a) or 125I-Fn (Fig. 1b) by isolated hepatocytes. Since no marked selectivity of any compound was evident, L638685 was arbitrarily chosen for more extensive analysis in dose-response experiments.

Fig. 2 illustrates the concentration-dependent inhibitory effect of the transglutaminase inactivator L638685 on the calcium-dependent binding of 125I-Fgn or [14C]putrescine to the hepatocytes. The IC50 for inhibition of binding of either 125I-Fgn or [14C]putrescine was about 0.2 μM. When the hepatocyte-bound radioligands obtained from one of the experiments represented in Fig. 2 were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (fluorography in the case of [14C]putrescine), it was observed that L638685 also produced corresponding concentration-dependent inhibition in the covalent cross-linking of the bound 125I-Fgn (Fig. 3) and [14C]putrescine (Fig. 4). Scanning of the autoradiograms depicted in Fig. 3 revealed that the cross-linked 125I-Fgn which did not enter the stacking gel, when corrected for the amount of 125I-Fgn loaded in each lane, was decreased by L638685 with an IC50 of approximately 0.8 μM, somewhat higher than the value of 0.2 μM for inhibition of binding portrayed in Fig. 2.

Since fibronectin is a major component of the extracellular matrix of liver (40, 41), and since we previously demonstrated that fibronecin undergoes similar transglutaminase-mediated cross-linking upon interaction with rabbit hepatocytes (23, 24), we tested the ability of L638685 to inhibit binding and covalent cross-linking of fibronectin by the hepatocyte suspensions. As portrayed in Fig. 5, L638685 elicited concentration-dependent decreases in the binding of 125I-Fn to the hepatocytes. The IC50 of 0.4 μM for inhibition of 125I-Fn binding (Fig. 5) was comparable with the value of 0.2 μM for the inhibition of 125I-Fgn or [14C]putrescine binding (Fig. 2). Moreover, when the cell pellets from one of the experiments portrayed in Fig. 5 were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography, the transglutaminase inactivator was observed to produce corresponding decreases in the covalent cross-linking of the cell-bound 125I-Fn (Fig. 6), with total inhibition of cross-linking occurring at concentrations > 5 μM as evidenced by scanning of the autoradiograms. These data support the concept that fibronecin and fibrogen interact with hepatocytes via a very similar, if not identical, mechanism involving a transglutaminase as a crucial component of a binding locus on the extracellular surface of the cell.

Although the nonpeptidyl transglutaminase inactivators used in the present study are very specific and potent as transglutaminase inactivators, their potential effects on other cellular proteins have not yet been studied. For this reason, we also utilized another type of inhibitor, namely a goat antiserum against rabbit liver tissue transglutaminase (30). In corroboration with the results obtained with L638685, we observed that the antiserum, which recognized purified rabbit liver transglutaminase but not rabbit plasma Factor XIII, in Western blots (Fig. 7, inset), also elicited concentration-dependent inhibition in the calcium-dependent binding of 125I-Fgn or [14C]putrescine to the hepatocytes, while nonimmune goat serum did not (Fig. 7). Moreover, like L638685, the antiserum also decreased 125I-Fgn cross-linking at the hepatocyte surface (Fig. 8a), while nonimmune goat serum actually increased cross-linking of the bound 125I-Fgn (Fig. 8b). When the lanes in Fig. 8a were scanned with a TLC scanner and corrected for the amount of material loaded in each lane using the ω chain as an internal standard, the percent inhibition of 125I-Fgn cross-linking elicited by the antiserum (not shown) was found to be nearly superimposable with the inhibition curves for binding of 125I-Fgn or [14C]putrescine shown in Fig. 7. For example, at an antiserum concentration of 100 μl/ml, inhibition of cross-linking was 80% as was the inhibition in the calcium-dependent binding of either 125I-Fgn or [14C]putrescine. The anti-transglutaminase antiserum not only inhibited the binding and cross-linking of 125I-Fgn by hepatocytes, it also inhibited the enzymatic activity of tissue transglutaminase, purified from rabbit liver, as evidenced by a concentration-dependent decrease of [14C]putrescine incorporation into dimethylcasein (Table I). Taken
FIG. 1. Effect of transglutaminase inactivators on covalent cross-linking of $^{125}$I-Fgn and $^{125}$I-Fn by rabbit hepatocytes. $^{125}$I-Fgn (a, 30 nM) or $^{125}$I-Fn (b, 50 nM) were incubated at $4^\circ$C with rabbit hepatocytes (2.5 x 10$^6$ cells/ml) suspended in buffer containing 5 mM CaCl$_2$ (lane C), 0.5 mM EDTA (lane E), or 5 mM CaCl$_2$ plus 10 μmol/liter of the transglutaminase inactivators (chemical names defined in Footnote 1) L683696 (lane I), L722151 (lane 2), L683685 (lane 3), and L682777 (lane 4). The inactivators were added 5–10 min prior to addition of the $^{125}$I-Fgn or $^{125}$I-Fn. After 3 h of incubation, 1-ml aliquots of cell suspension were collected, as described under “Experimental Procedures,” and the cell-bound ligands were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Each lane was loaded with 10,000 cpm; therefore, approximately half as much cellular protein was loaded in lanes C, which had the highest level of binding, than in the other lanes. Molecular mass standards (kDa) are indicated on the left. An, Bo, and γ indicate individual fibrinogen chains; FN indicates reduced fibronectin.

FIG. 2. Effect of L683685 on binding of $^{125}$I-Fgn or $[^{14}$C]putrescine to rabbit hepatocytes. $^{125}$I-Fgn (30 nM) or $[^{14}$C]putrescine (9.1 μM) were incubated at $4^\circ$C with rabbit hepatocytes (2.5 x 10$^6$ cells/ml) suspended in buffer containing 5 mM CaCl$_2$ and increasing concentrations of L683685 which had been added 5–10 min prior to addition of the radiolabeled ligands. After 3 h of incubation, calcium-dependent binding of $^{125}$I-Fgn or $[^{14}$C]putrescine was quantitated as described under “Experimental Procedures.” Each point represents the mean of four determinations obtained from two experiments.

FIG. 3. Effect of L683685 on covalent cross-linking of $^{125}$I-Fgn by rabbit hepatocytes. Hepatocyte pellets derived from 1-ml aliquots of the samples obtained from one of the experiments portrayed in Fig. 2 were collected as described under “Experimental Procedures,” and the cell-bound $^{125}$I-Fgn was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Each lane was loaded with 10,000 cpm. Molecular mass standards (kDa) are indicated on the left. An, Bo, and γ represent the chains of fibrinogen.

Together with the results obtained with L683685, these data support the idea that tissue transglutaminase, expressed on the extracellular surface of the cell, played an essential role in the calcium-dependent interaction of fibrinogen and fibronectin with hepatocytes.

To determine whether the transglutaminase molecule which mediated binding was itself covalently incorporated into the high molecular weight cross-linked complex, immunoblots of the hepatocyte pellets were performed using the polyclonal antiserum against rabbit liver transglutaminase (30), a polyclonal antiserum against human erythrocyte transglutaminase (31), or a monoclonal antibody (CUB-7401) against guinea pig liver transglutaminase (32). As illustrated in Fig. 9, immunoreactive material was minimally detectable at the top of the stacking or resolving gels after a 5-min incubation (lanes 1), but was clearly demonstrable after 3 h of further incubation of hepatocytes in the absence of L683685.
FIG. 4. Effect of L683685 on covalent cross-linking of [14C]putrescine by rabbit hepatocytes. Hepatocyte pellets derived from 1-ml aliquots of the samples obtained from one of the experiments portrayed in Fig. 2 were collected as described under "Experimental Procedures," and the covalently bound [14C]putrescine was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Each lane was loaded with 30,000 cpm. Molecular mass standards (kDa) are indicated on the left.

FIG. 5. Effect of L683685 on binding of 125I-Fn to rabbit hepatocytes. 125I-Fn (50 nM) was incubated at 4 °C with rabbit hepatocytes (2.5 x 10^6 cells/ml) suspended in buffer containing 5 mM CaCl_2 and increasing concentrations of L683685 which had been added 5-10 m before addition of the 125I-Fn. After 3 h of incubation, calcium-dependent binding of 125I-Fn was quantitated as described in "Experimental Procedures." Each point represents the mean of four determinations obtained from two experiments.

(lanes 2), but not in the presence of L683685 (lanes 3). These findings indicate that tissue transglutaminase itself was incorporated into these high molecular weight complexes, in a time-dependent fashion, via a mechanism which depended upon expression of transglutaminase cross-linking activity. Cells incubated with EDTA also lacked transglutaminase immunoreactive material at the top of the gels (not shown), indicating that the assembly of the transglutaminase-containing high molecular weight complexes required calcium, consistent with the known calcium-dependency of transglutaminase-mediated cross-linking events (1, 2). The L683685-treated (Fig. 9) and EDTA-treated (not shown) hepatocytes demonstrated immunoreactive bands at 80 kDa, but also bands with molecular masses of 90 to >200 kDa (TG_m) as seen in Fig. 9 (lanes 1 and 3). The temporally coordinated disappearance of these higher molecular weight forms of tissue transglutaminase antigen from the control hepatocytes, along with the concomitant appearance of very high molecular weight complexes which did not enter the gel (compare lanes 1 with lanes 2), indicate that the higher molecular mass forms of tissue transglutaminase, as opposed to the 80-kDa transglutaminase (TG), were preferentially assembled into covalently cross-linked high molecular weight complexes.

FIG. 6. Effect of L683685 on covalent cross-linking of 125I-Fn by rabbit hepatocytes. Hepatocyte pellets derived from 1-ml aliquots of the samples obtained from one of the experiments portrayed in Fig. 5 were collected as described under "Experimental Procedures," and the cell-bound 125I-Fn was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Each lane was loaded with 10,000 cpm. Molecular mass standards (kDa) are indicated on the left. FN indicates the reduced Fn monomer.

FIG. 7. Effect of anti-transglutaminase (TGase) antiserum on binding of 125I-Fn or [14C]putrescine to rabbit hepatocytes. 125I-Fn (30 nM) or [14C]putrescine (9.1 pM) were incubated at 4 °C with rabbit hepatocytes (2.5 x 10^6 cells/ml) suspended in buffer containing 5 mM CaCl_2 and increasing concentrations of goat antiserum against rabbit liver transglutaminase or nonimmune goat serum which had been added 30 min prior to addition of the radiolabeled ligands. After 3 h of incubation, calcium-dependent binding of 125I-Fn or [14C]putrescine were quantitated as described under "Experimental Procedures." Each point represents the mean of four determinations obtained from two experiments. Inset, Western blot of 10 pg of either purified rabbit plasma Factor XIII (lane 1) or rabbit liver transglutaminase (lane 2) using the goat antiserum against rabbit liver transglutaminase (30).
linked very high molecular weight complexes which accumulated at the top of the gel. Keratinocyte (type I) transglutaminase (M, 90,000-92,000), which is not immunohistochemically detectable in liver sections (42), was also undetectable in hepatocytes by Western blotting (not shown) with monoclonal antibody B.C1 (33).

We initially demonstrated (21) that when fibrinogen binding to hepatocyte suspensions is analyzed using a 3-h incubation at 4 °C and a ligand concentration of 30 nM, which is below the Kd for fibrinogen binding to the platelet integrin glycoprotein IIb/IIIa (22), that an Arg-Gly-Asp-independent, transglutaminase-mediated interaction could be isolated and studied in detail (23). The same observation proved to be true for fibronectin binding to hepatocyte suspensions (23, 24). However, since integrins play such a prominent role in mediating the interaction of various cytoadhesive glycoproteins with cells (43), and because it is possible that the rapid transglutaminase-mediated cross-linking of the glycoproteins precluded binding to an integrin, we determined whether the binding of 125I-Fgn or 125I-Fn to hepatocytes treated with transglutaminase inactivators could be mediated by cellular integrins. As seen in Table II, neither the Arg-Gly-Asp-Ser tetrapeptide, a monoclonal antibody (7E3) reactive with the vitronectin receptor (α5β3), or a fibronectin receptor (α5β1) monoclonal antibody (FnR-Ab) were able to inhibit either total binding (control binding in buffer containing 5 mM calcium chloride) or the L683685-resistant component of the calcium-dependent binding of 125I-Fgn or 125I-Fn. Moreover, binding of 125I-Fgn or 125I-Fn to hepatocytes in the presence of EDTA was also resistant to inhibition by these anti-integrins and by L683685 (data not shown), indicating that the inhibitory effect of L683685 on binding was on the cal-

**Fig. 8.** Effect of anti-transglutaminase (TGase) antiserum on covalent cross-linking of 125I-Fgn by rabbit hepatocytes. Hepatocyte pellets derived from 1-ml aliquots of the samples obtained from one of the experiments portrayed in Fig. 7 were collected as described under "Experimental Procedures," and the cell-bound 125I-Fgn was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Each lane was loaded with 10,000 cpn. Molecular mass standards (kDa) are indicated on the left. Aa, Bb, and γ represent the constituent chains of fibrinogen.

**Table I**

Effect of anti-transglutaminase antiserum on the enzymatic activity of purified rabbit hepatocyte transglutaminase

Increasing concentrations of goat antiserum against rabbit liver transglutaminase were incubated for 1 h at 37 °C with purified rabbit liver transglutaminase in Tris buffer (pH 7.5) containing 1% dimethylcasein and [14C]putrescine (9.1 μM) and either 5 mM CaCl2 or 5 mM EDTA. After incubation, the calcium-dependent covalent incorporation of [14C]putrescine into casein was determined as described under "Experimental Procedures."

| Antiserum dilution | Percent of calcium-dependent [14C]putrescine incorporation |
|--------------------|----------------------------------------------------------|
| Buffer             | 100                                                      |
| 2 μl/ml            | 72                                                       |
| 10 μl/ml           | 35                                                       |
| 20 μl/ml           | 26                                                       |

* Results of a single experiment.

**Fig. 9.** Western blots of tissue transglutaminase in covalently cross-linked, high molecular weight protein complexes. 125I-Fgn (30 nM) or 125I-Fn (50 nM) were incubated at 4 °C with rabbit hepatocytes (2.5 × 106 cells/ml) suspended in buffer containing 5 mM CaCl2 with or without L683685 (10 μM) which had been added 5-10 min prior to addition of the radioligands. After either 5 min or 3 h of incubation, 1-ml aliquots of cell suspension were collected, as described under "Experimental Procedures," and the cell-bound radioligands extracted with 0.1% SDS, 2% Triton X-100. The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting with goat antirabbit liver transglutaminase (panel A) or rabbit antihuman erythrocyte transglutaminase (panel B). Lanes 1, control cells after 5 min of incubation; lanes 2, control cells after 3 h of incubation; and lanes 3, L683685-treated cells after 3 h of incubation. TGm represents a tissue transglutaminase-immuno-reactive major band which migrated with a molecular mass > 200 kDa. TG represents native liver transglutaminase.

**Table II**

Effect of RGDS and anti-integrin antibodies on binding of 125I-Fgn or 125I-Fn to L683685-treated rabbit hepatocytes

125I-Fgn (30 nM) or 125I-Fn (50 nM) were incubated at 4 °C with rabbit hepatocytes (2.5 × 106 cells/ml) suspended in buffer containing 5 mM CaCl2 (control), 0.5 mM EDTA, or 5 mM CaCl2 plus the listed agents which had been added prior to the 125I-Fgn or 125I-Fn. After 3 h of incubation, ligand binding was quantitated as described under "Experimental Procedures." FnR, fibronectin receptor.

| Treatment group | Ligand binding (of control) |
|-----------------|-----------------------------|
|                  | 125I-Fgn*                   | 125I-Fn*                   |
| EDTA            | 43 ± 11                     | 47 ± 7                     |
| L683685         | 51 ± 12                     | 59 ± 9                     |
| RGDS            | 98 ± 8                      | 92 ± 20                    |
| 7E3             | 104 ± 20                    |                             |
| FnR-Ab          | 95 ± 8                      |                             |
| L683685 + RGDS  | 50 ± 17                     | 59 ± 10                    |
| L683685 + 7E3   | 52 ± 12                     |                             |
| L683685 + FnR-Ab| 62 ± 12                     |                             |

* Control is defined as binding in buffer with 5 mM CaCl2.

**Mean ± S.D. of 9-17 determinations from 3-6 experiments.

**Mean ± S.D. of 8-14 determinations from 3-5 experiments.
cium-dependent component, and that integrins did not participate in the binding process as analyzed using the conditions described above. That functional integrins are present in rabbit hepatocytes was evident by the observations that the Arg-Gly-Asp-Ser tetrapeptide, as well as both antibodies, impaired attachment and spreading of hepatocytes to plastic dishes coated with fibronectin or fibrinogen (not shown).

**DISCUSSION**

In the present study, we demonstrate that rabbit hepatocyte surface-expressed tissue transglutaminase can serve as a crucial component of a binding site for exogenously added fibrinogen or fibronectin, covalently incorporating these glycoproteins, in addition to itself, into high molecular weight complexes on the outside of the cell. That the transglutaminase activity is not Factor XIIIa-related is based on the demonstration that the goat antiserum against rabbit liver transglutaminase (30) used in these studies did not cross-react, in Western blots, with purified rabbit Factor XIII, and by our prior observations (23) that, characteristic of the action of tissue transglutaminase (2, 18, 44), the Az chain of fibrinogen is cross-linked much more rapidly and extensively than is the γ chain, and no γγ-dimers are formed. The participation of keratinocyte (type I) transglutaminase in these phenomena was ruled out based on the failure of the monoclonal antibody B.C1 (33), which recognizes type I transglutaminase in Western blots of epidermal keratinocytes (13, 45), to detect any type I transglutaminase in Western blots of hepatocyte proteins. Moreover, immunohistochemical analysis conducted with this antibody has revealed the absence of type I transglutaminase in rat liver sections (42).

The superimposability of the binding inhibition curves for 125I-Fgn or [14C]putrescine obtained with the low molecular weight active site-directed transglutaminase inactivator L683685 (Fig. 2) is suggestive of a mechanism whereby alteration of the active site cysteine residue of the enzyme was responsible for the decreases in the binding of 125I-Fgn or 125I-Fn. The important role of the active site in the binding process was corroborated by the demonstration that the anti-transglutaminase antisem which inhibited 125I-Fgn binding and cross-linking also produced corresponding decreases in hepatocyte-expressed transglutaminase activity as evidenced by concentration-dependent inhibition of [14C]putrescine incorporation into the hepatocytes. These data suggest that the antisem probably recognized epitopes located near the active site of the hepatocyte surface-expressed transglutaminase. This conclusion is supported by the previous demonstration that the antisem inhibited the catalytic activity of purified rabbit liver transglutaminase measured by quantitating putrescine incorporation into casein (30), an observation which was reproduced in the present investigation (Table 1). We have also previously shown (23) that, depending on the divalent cation employed in the assays, the extent of binding and cross-linking of fibrinogen or fibronectin to hepatocytes is directly proportional to the level of enzymatic activity, with calcium supporting maximum levels of enzymatic activity which correlate with the degree of ligand binding and cross-linking. These data (23), along with those presented in the present report, indicate that accessibility of the ligands to the active site of the transglutaminase molecule is an important step for the binding of these glycoproteins to the hepatocyte surface.

Although accessibility of the active site of the surface-expressed transglutaminase is important for the binding of fibrinogen or fibronectin to hepatocytes, it is unclear whether the active site cysteine residue itself directly participated in the binding process or whether conformational changes induced in the enzyme as a result of inactivation precluded binding of 125I-Fgn or 125I-Fn to some other domain of the transglutaminase molecule. In this regard, it is important to note that the interaction of fibrinogen with hepatocyte surface-expressed transglutaminase described in the present investigation differs from the binding of fibrinogen (2), fibrin (25), or fibronectin (27, 28) to tissue transglutaminase in purified systems. In these systems, the binding process has been shown to be calcium-independent and therefore not related to the ability of transglutaminase to express catalytic activity. Moreover, a region of the enzyme distinct from the active site appears to mediate this mode of interaction, since tissue transglutaminase retains catalytic activity following binding to fibronectin, and the transglutaminase and fibronectin molecules can be completely dissociated from each other by SDS-polyacrylamide gel electrophoresis (27, 28).

Multiple molecular weight forms of transglutaminase have been detected in liver (46, 47), but the biochemical identification of these higher molecular weight forms has not been resolved. In the present study, the 80-kDa form of tissue (type II) transglutaminase, along with tissue transglutaminase-related antigens with molecular masses of 90 to >200 kDa, were detected in Western blots of SDS/Triton-insoluble hepatocyte proteins (26, 31), and guinea pig liver transglutaminase (32). Each of these antisera also immunoblotted tissue transglutaminase present in very high molecular weight cross-linked complexes isolated from cells incubated with fibrinogen or fibronectin in the absence, but not in the presence, of the transglutaminase inactivator L683685 or EDTA, indicating that the enzyme itself was also covalently incorporated, in a calcium-dependent manner, into high molecular weight complexes on the extracellular surface of the hepatocytes. These findings suggest the possibility that the 80-kDa tissue transglutaminase molecule may be cross-linked to other hepatocyte proteins, accounting for the 90- to >200-kDa forms, and that these tissue transglutaminase-containing structures mediated the binding and/or cross-linking of 125I-Fgn or 125I-Fn to form very high molecular weight complexes. It is interesting to note that the particulate transglutaminase of rat liver has recently been demonstrated to cross-react, in Western blots, with monoclonal and polyclonal antibodies against the soluble form of tissue transglutaminase (48), suggesting that, under certain conditions, the 80-kDa cytosolic tissue (type II) transglutaminase of liver can be associated with cellular membranes, thus accounting for the transglutaminase activity associated with the particulate fraction of liver which has been observed by many investigators (46–63).

The detection of 90- >200-kDa tissue transglutaminase-immunoreactive bands in Western blots of the SDS/Triton-insoluble fraction of hepatocytes, and the coincident incorporation of these materials into very high molecular weight complexes which did not enter the stacking gel, is a novel finding of the present investigation. However, such a result should not have been entirely unexpected, in light of the earlier report demonstrating that purified guinea pig liver transglutaminase can both incorporate [14C]putrescine into itself and autocatalytically cross-link itself into high molecular weight polymers (54). Furthermore, evidence has been presented (49) which suggests that the transglutaminase present in liver cytosolic and particulate fractions may serve as both the enzyme and acceptor molecule. Further evidence that transglutaminase may be capable of cross-linking substrates to itself was provided by the observation that purified guinea pig liver transglutaminase and purified β2-microglob-
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ulin, a 12-kDa protein found on the surface of a variety of mammalian cells, comigrate to the top of the gel when incubated together in the presence of calcium (55). Similarly, murine leukemia macrophage cell lines, following differentiation, express transglutaminase activity present in a high molecular weight complex which elutes in the void volume following Sepharose 4B chromatography (56). Whether this high molecular weight complex contained covalently incorporated transglutaminase, as shown in the present study using hepatocytes, or whether the transglutaminase was noncovalently associated with cellular proteins, such as fibronectin (27, 28), was not determined (56). Apart from or in addition to the cross-linking of tissue transglutaminase to hepatocyte proteins, it is possible that some of the higher molecular weight forms of tissue transglutaminase observed in the present investigation may have been polymers of the enzyme formed by autocatalytic cross-linking, as previously reported to occur with the purified enzyme (49, 54, 55). Additional work will be required to address this issue and to determine the functional significance of the assembly of surface-expressed tissue transglutaminase into high molecular weight complexes on the extracellular surface of the hepatocyte.

Finally, the involvement of integrins in the binding process is highly unlikely, since neither the calcium-dependent nor the calcium-independent components of fibrinogen and fibronectin binding could be inhibited by the Arg-Gly-Asp-Ser tetrapeptide or by monoclonal antibodies reactive with the vitronectin or fibronectin receptors. These observations were true even when binding was analyzed under conditions where cross-linking did not occur (i.e. in the presence of L-683685). Thus, by using appropriate experimental conditions (21), we have isolated and characterized (21, 23, 24) an Arg-Gly-Asp-independent transglutaminase-mediated pathway for the interaction of fibrinogen or fibronectin with hepatocytes. We have also shown this system to be operative for fibrinogen binding by human umbilical vein endothelial cells (57), while others have demonstrated a strikingly similar, Arg-Gly-Asp-independent interaction of fibrinogen with a murine melanoma cell line (58). Both with the endothelial cells (57) and the melanoma cells (58), the transglutaminase-mediated covalent cross-linking of the cell-bound fibrinogen suggest the possibility that surface-expressed transglutaminase may also serve as a binding site for the glycoproteins in these cells, as presently shown for hepatocytes. The low level of the calcium-dependent components of fibrinogen (15%) and fibronectin (25%) binding which was not inhibited by the transglutaminase inactivators or the anti-integrins may have involved cellular proteins distinct from tissue transglutaminase or integrins, and this possibility is presently under investigation.

The functional significance of tissue transglutaminase-mediated cross-linking of fibrinogen, fibronectin, or itself at the hepatocyte surface is presently unknown. However, based on the accumulated findings by many investigators, a role for the extracellular expression of the enzyme in mediating hepatocyte-hepatocyte interactions (59-63) or the covalent restructuring of extracellular matrix proteins (19, 20, 26, 64) are possibilities. The extracellular expression of tissue transglutaminase activity by hepatocytes (21, 23, 24, 65) or endothelial cells (57) may possibly play a role in the formation of the highly insoluble matrix found in cirrhotic liver (41). Moreover, fibronectin (40, 66) and tissue transglutaminase (66) colocalize to the space of Disse, and cirrhosis increases fibronectin deposition at this location (41). Hypothetically, transglutaminase may mediate the covalent cross-linking of fibronectin, forming a scaffold upon which other extracellular components may deposit leading to the collagenization of the space of Disse. The recently developed transglutaminase inactivators used in the present investigation have previously been shown to inhibit fibrin cross-linking by Factor XIIIa (29, 67); however, the potential for modulation of tissue transglutaminase-mediated physiologic or pathologic processes awaits further experimentation.

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REFERENCES

1. Folk, J. E. (1980) *Annu. Rev. Biochem.* 49, 517-531
2. Chung, S. I. (1972) *Ann. N. Y. Acad. Sci.* 202, 240-255
3. Thomazy, V., and Fesus, L. (1986) *Cell Tissue Res.* 255, 215-224
4. Floyd, E. E., and Jetten, A. M. (1989) *Mol. Cell. Biol.* 9, 4846-4851
5. Kim, H., Idler, W. W., Kim, L. G., Han, J. H., Chung, S. I., and Steinert, P. M. (1991) *J. Biol. Chem.* 266, 536-539
6. Ikura, K., Nasu, T., Yokota, H., Sasaki, R., and Chiba, H. (1987) *Agric. Biol. Chem.* 51, 957-961
7. Ikura, K., Nasu, T., Yokota, H., Tsushima, Y., Sasaki, R., and Chiba, H. (1988) *Biochemistry* 27, 2988-2995
8. Gentle, V., Saydak, M., Chioce, A. E., Akande, O., Birkchichler, P. J., Lee, K. N., Stein, J. P., and Davies, P. J. A. (1991) *J. Biol. Chem.* 266, 478-483
9. Grundmann, U., Amann, A., Zettelmeissl, G., and Kupper, H. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 8024-8028
10. Ichinose, A., Hendrickson, L. E., Fujikawa, K., and Davies, E. W. (1986) *Biochemistry* 25, 6900-6906
11. Ogawa, H., and Goldsmith, L. A. (1976) *J. Biol. Chem.* 251, 7281-7288
12. Martinet, N., Kim, H., Girard, J. E., Nigra, T. P., Strong, D. H., Chung, S. I., and Folk, J. E. (1988) *J. Biol. Chem.* 263, 4236-4241
13. Kim, H. C., Lewis, M. S., Gorman, J. J., Park, S. C., Girard, J. E., Folk, J. E., and Chung, S. I. (1990) *J. Biol. Chem.* 265, 21971-21978
14. Pytel, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F., and Ruoslahti, E. (1986) *Science* 231, 1558-1562
15. Pierschbacher, M. D., and Ruoslahti, E. (1984) *Nature* 309, 30-33
16. Yamada, K. M., and Kennedy, D. W. (1984) *J. Cell Biol.* 99, 29-36
17. Farrell, J., and Laki, K. (1970) *Blood* 35, 804-808
18. Doolittle, R. F. (1973) *Adv. Protein Chem.* 27, 1-109
19. Mosher, D. F. (1975) *J. Biol. Chem.* 250, 6614-6621
20. Feus, L., Mertis, M. L., Munsbek, L., and Koteliavsky, V. E. (1986) *Eur. J. Biochem.* 154, 371-374
21. Barsigian, C., Fellin, F. M., Base, W., Schaeffer, A., Fish, S., and Martinez, J. (1987) *J. Biol. Chem.* 262, 3674-3679
22. Peerschke, E. I. B. (1985) *Semin. Hematol.* 22, 241-259
23. Barsigian, C., Fellin, F. M., Jain, A., and Martinez, J. (1988) *J. Biol. Chem.* 263, 14015-14022
24. Fellin, F. M., Barsigian, C., Rich, E., and Martinez, J. (1988) *J. Biol. Chem.* 263, 1791-1797
25. Acharuman, K. E., Mary, A., and Greenberg, C. S. (1988) *J. Biol. Chem.* 263, 1436-1439
26. Upchurch, H. F., Conway, E., Patterson, M. K., Jr., Birkchichler, P. J., and Maxwell, M. D. (1987) *In Vitro Cell. Dev. Biol.* 23, 795-800
27. Lorand, L., Dailey, J. E., and Turner, P. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 1057-1059
28. Turner, P. M., and Lorand, L. (1989) *Biochemistry* 28, 628-635
29. Leidy, E. M., Stern, A. M., Friedman, P. A., Bush, L. R. (1990) *Thorax* 59, 15-26
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Abe, T., Chung, S. I., DiAugustine, D. P., and Folk, J. E. (1977) Biochemistry 16, 5495–5501
Birckbichler, P. J., and Patterson, M. K., Jr. (1978) Ann. N. Y. Acad. Sci. 312, 354–365
Birckbichler, P. J., Upchurch, H. F., Patterson, M. K., Jr., and Conway, E. (1985) Hybridoma 4, 179–186
Thacher, S. M., and Rice, R. H. (1985) Cell 40, 685–695
Coller, B. S. (1985) J. Clin. Invest. 76, 101–108
Mosesson, M. W., and Finlayson, J. S. (1963) J. Lab. Clin. Med.
Engvall, E., and Ruoslahti, E. (1977) Int. J. Cancer 20, 1-5
McFarlane, A. S. (1958) Nature 182, 53
Laemmli, U. K. (1970) Nature 227, 680–685
Towbin, H., Staehelin, T., and Gordon, J. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5072–5076
Martinez-Hernandez, A. (1984) Lab. Invest. 51, 57–74
Martinez-Hernandez, A. (1985) Lab. Invest. 53, 166–186
Parenteau, N. L., Pilato, A., and Rice, R. H. (1986) Differentiation 33, 130–141
Albelda, S. M., and Buck, C. A. (1990) FASEB J. 4, 2868–2880
Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1973) J. Biol. Chem.
George, M. D., Vollberg, T. M., Floyd, E. E., Stein, J. P., and Chang, S. K., and Chung, S. I. (1986) J. Biol. Chem. 261, 8112–8121
Barsigian, C., Rich, E., Finner, K., and Martinez, J. (1989) FASEB J 3, A999 (Abstr. 4490)
Knight, C. R. L., Rees, R. C., Elliott, B. M., and Griffin, M. (1990) FEBS Lett. 265, 93–98
Birckbichler, P. J., Orr, G. R., and Patterson, M. K. (1976) Cancer Res. 36, 2911–2914
Barnes, R. N., Bungay, P. J., Elliott, B. M., Walton, P. L., and Griffin, M. (1985) Carcinogenesis 6, 459–463
Slife, C. W., Dorsett, M. D., Bouquet, G. T., Register, A., Taylor, E., and Conroy, S. (1985) Arch. Biochem. Biophys. 241, 329–336
Tyrrell, D. J., Sale, W. S., and Slife, C. W. (1986) J. Biol. Chem. 261, 14833–14836
Hand, D., Elliott, B. M., and Griffin, M. (1988) Biochim. Biophys. Acta 970, 137–145
Birckbichler, P. J., Orr, G. R., Carter, H. A., and Patterson, M. K. (1977) Biochem. Biophys. Res. Commun. 78, 1–7
Fesus, L., Falus, A., Erdé, A., and Lakó, K. (1981) J. Cell Biol. 89, 706–710
Kannagi, R., Teshigawara, K., Noro, N., and Masuda, T. (1982) Biochem. Biophys. Res. Commun. 105, 164–171
Martinez, J., Rich, E. D., and Barsigian, C. (1989) J. Biol. Chem. 264, 20502–20508
Cardinali, M., Uchino, R., and Chung, S. I. (1990) Cancer Res. 50, 8019–8026
Schmell, E., Slife, C. W., Kuhlenschmidt, M. S., and Roseman, S. (1982) J. Biol. Chem. 257, 3171–3176
Slife, C. W., Zhou, R.-L., and Roseman, S. (1982) Fed. Proc. 41, 784
Slife, C. W., Dorsett, M. D., and Tillotson, M. L. (1986) J. Biol. Chem. 261, 3451–3456
Tyrrell, D. J., Sale, W. S., and Slife, C. W. (1988) J. Biol. Chem. 263, 1946–1951
Tyrrell, D. J., Sale, W. S., and Slife, C. W. (1988) J. Biol. Chem. 263, 8464–8469
Bowness, J. M., Folk, J. E., and Timpl, R. (1987) J. Biol. Chem. 262, 1022–1024
Piazza, G. A., Callanan, H. M., Mowery, J., and Hixson, D. C. (1988) Biochem. J. 262, 327–334
Greenberg, C. S., Achyuthan, K. E., Borowitz, M. J., Shuman, M. A. (1987) Blood 70, 702–709
Shebuski, R. J., Sitko, G. R., Claremon, D. A., Baldwin, J. J., Remy, D. C., and Stern, A. M. (1990) Blood 75, 1455–1459