Cross-linking of Laminin-Nidogen Complexes by Tissue Transglutaminase

A NOVEL MECHANISM FOR BASEMENT MEMBRANE STABILIZATION*

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The laminin-nidogen complex, a major component of basement membranes, incorporates [3H]putrescine and monodansyleadaverine in the presence of guinea pig liver transglutaminase. Label was detected in nidogen in the isolated, as well as in the complexed form, but not in laminin. The incorporation proceeds in a time-dependent manner at a rate similar to that achieved with N,N-dimethylcasein, a well characterized transglutaminase substrate. Saturation of incorporation site(s), as well as comparison with the incorporation level in reference proteins, indicated the presence of one high affinity amine acceptor site in nidogen. Electron microscopy of the reaction products showed that the laminin-nidogen complexes become stabilized in a head-to-head arrangement, characteristic of Ca2+-induced self-aggregation. Indirect immunofluorescence and detection of transglutaminase activity on unfixed cryosections revealed an extracellular distribution of tissue transglutaminase. Intensive staining was observed in collagen-rich connective tissue. Codistribution with nidogen was not a ubiquitous feature, but was observed in many locations.

Transglutaminase (EC 2.3.2.13) is an enzyme that catalyzes a Ca2+-dependent acyl transfer reaction in which new γ-amide bonds are formed between γ-carboxamide groups of peptide-bound glutamine residues and various primary amines (for review, see Folk and Finlayson, 1977; Folk, 1980; Lorand and Conrad, 1984). A glutamine residue serves as acyl donor, and the most common acyl acceptor substrates are primary amino groups of some naturally occurring polyamines, like putrescine or spermidine, and ε-amino groups of peptide-bound lysine residues. In the latter case, the reaction results in the formation of γ-glutamyl-ε-lysine cross-links in or between proteins. The number of glutaminyl substrates is highly restricted, whereas the tolerance to structural differences in acyl acceptors is great. The specificity for different glutaminyl substrates varies, depending on the type of transglutaminase. Transglutaminases belong to a large protein family (Ichinose et al., 1990) and have a wide distribution among tissues and body fluids (Folk, 1980). Examples are the cytosolic tissue transglutaminase, often isolated from guinea pig liver (Folk and Finlayson, 1977; Korner et al., 1989; Ichinose et al., 1990); the plasma membrane-associated particulate transglutaminase (Chang and Chung, 1986; Cocuzzi and Chung, 1986), which is suggested to be closely related to the epidermal enzyme (Peterson and Wuepper, 1984; Thacher and Rice, 1985; Martinet et al., 1988); and the platelet/placental and plasma variants of factor XIII (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Ichinose et al., 1990). In some cases, the physiological functions are well established, like cross-linking of fibrin clots, e.g., in wound healing, by plasma factor XIII (Folk and Finlayson, 1977), cross-linking of the cytoplasmic protein involucrin (Simon and Green, 1988) by epidermal transglutaminase to form the cornified envelope (Thacher and Rice, 1985), and formation of the seminal plug by prostate transglutaminase in rodents (Williams-Ashman, 1984). In contrast, the physiological role of the abundant tissue transglutaminase remains unclear, although cytoskeletal proteins like actin (Takashii, 1988), β-crystallins (Berbers et al., 1984), spectrin and the erythrocyte membrane protein band 3 (Lorand and Conrad, 1984), as well as various extracellular proteins like fibrin(ogen) (Achurythan et al., 1988), fibronectin (Fésus et al., 1986), vitronectin (Sane et al., 1988), and collagen type III (Bowness et al., 1987) have been identified as specific glutaminyl substrates.

Tissue transglutaminase is a monomeric globular protein with a molecular mass of about 77 kDa and shows a high degree of sequence similarity to the a-subunit of factor XIII (Ikura et al., 1988). Factor XIII exists as a tetramer, αβ2 (M, ≈ 320,000), composed of a dimer of the globular catalytic subunit (M, ≈ 77,000 each) and two filamentous regulatory b-subunits (M, ≈ 80,000 each), when circulating in the blood plasma (Carrell et al., 1989), or as a dimer of only the a-subunit in, e.g., platelets, leukocytes, and placenta. Factor XIII is a zymogen, which is activated by thrombin cleavage to factor XIIIa, whereas tissue transglutaminase does not need proteolysis to achieve an active state.

Laminin is the most abundant noncollagenous protein of basement membranes and is present in the tissue as an equimolar complex with the protein nidogen (entactin) (Timpl et al., 1979; Paulsson et al., 1987). Laminin, isolated from the Engelbreth-Holm-Swarm (EHS) tumor, was found to have a cruciform shape with one long and three short arms (Engel et al., 1981). Each of the short arms contains an inner and a terminal globular domain connected by a rod-like structure composed of epidermal growth factor-like repeats (Sasaki

1The abbreviations used are: EHS, Engelbreth-Holm-Swarm; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; EGF, epidermal growth factor; TBS, Tris-buffered saline.
et al., 1988), whereas the long arm consists of a long triple coiled-coil \( \alpha \)-helical structure with a larger terminal globule (Barlow et al., 1984; Paulsson et al., 1985). EHS laminin is composed of three polypeptide chains, which are associated with each other in the helical region of the long arm and form one of the short arms each. The structure is stabilized by interchain disulfide bonds at both ends of the helical domain (Paulsson et al., 1985; Sasaki et al., 1988). The terminal globular domain of the long arm is composed solely of the larger polypeptide chain, the A-chain of about 400 kDa. The two smaller chains, designated B1 and B2, with a molecular mass of about 220 kDa each, are homologous to each other and to the A-chain (Sasaki et al., 1988). Nidogen (entactin) is a dumbbell-shaped molecule of about 150 kDa, composed of one single polypeptide chain (Carlin et al., 1981; Paulsson et al., 1986). It is associated with one of the short arms of laminin with its carboxy-terminal globular domain (Paulsson et al., 1985; Mann et al., 1989). The middle portion of nidogen is a long rod-like structure made up of EGF-like repeats (Mann et al., 1989).

Interactions between the laminin-nidogen complex and collagen IV (Aumailley et al., 1989), heparin (Yurchenco et al., 1990), cell surface receptors (Edgar, 1989), as well as self-aggregation (Yurchenco et al., 1985) have been demonstrated. In addition to these more dynamic, noncovalent interactions, stable cross-links contribute to the scaffold structure, which ensures the mechanical strength of the tissue. One well-known mechanism is the formation of intra- and intermolecular cross-links in collagen molecules by deamination of lysine or hydroxylysine residues by the extracellular enzyme lysisoxidase to yield highly reactive aldehydes, which spontaneously form covalent bonds with each other or with other lysine or hydroxylysine residues (Eyre, 1987). The so-called pentosidine cross-links are formed by a pentose, most commonly ribose, which reacts with a lysine and an arginine residue of different sources, and are mainly detected in collagenous proteins (Sell and Monnier, 1989). The observation that it is more difficult to extract noncollagenous proteins, e.g. laminin, from adult tissues than from embryos or rapidly growing tumors, and the purification of covalently cross-linked laminin-nidogen complexes (Paulsson and Saladin, 1989), led us to look for additional cross-linking mechanisms also involving noncollagenous proteins. Thus, we decided to examine whether transglutaminases play a role in the stabilization of basement membranes.

In the present study, we show that nidogen, a component of the extracellular matrix, is a specific substrate for tissue transglutaminase. The complexes formed by this reaction show a predetermind supramolecular organization in which Ca\(^{2+}\)-induced aggregates of laminin-nidogen complexes become covalently stabilized. An extracellular localization of tissue transglutaminase, which in many cases showed a pattern similar to the tissue distribution of nidogen, was demonstrated.

**Experimental Procedures**

**Protein Purification**

Tissue transglutaminase was purified from guinea pig liver as previously described (Folk and Cole, 1986; Connellam et al., 1971) by means of DEAE-cellulose chromatography of liver homogenate supernatant fluid, protamine precipitation of the enzyme, selective extraction with ammonium sulfate solution, and rechromatography over a molecular sieve column. A protein fraction enriched in guinea pig factor XIII was obtained by molecular sieve chromatography of an ammonium sulfate precipitate of plasma. Blood was obtained by heart puncture, blood cells removed by centrifugation at 500 \( \times \) g for 20 min at 4 °C, and the plasma fraction cleared from remaining particulate material by a second centrifugation at 4200 \( \times \) g for 20 min at 4 °C. Saturated ammonium sulfate was slowly added with continuous stirring to a final concentration of 20%. The precipitate was allowed to form for 2 h at 4 °C without stirring and was collected by centrifugation as above. The pellet was resuspended in 0.15 M KCl solution of the original volume, 0.3-ml aliquots eluted with a Superox 6 column (HR 10/30, Pharmacia LKB Biotechnology, Inc.) with 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl and 2 mM EDTA, at a flow rate of 0.3 ml/min at room temperature, and 0.3-ml fractions were collected. Fractions enriched in factor XIII were pooled and subjected to SDS-PAGE and immunoblotting as described elsewhere. Laminin-nidogen complex was prepared from subcutaneously propagated EHS tumor in C57BL mice (Orkin et al., 1977), according to the method of Paulsson (1988), by use of molecular sieve chromatography and DEAE-cellulose chromatography of EDTA extracts of preserved tumor tissue. The tumor material was kindly provided by Dr. K. Berk, Institute of Biophysics, Linz, Austria. Laminin and nidogen were purified from the complex by dissociation in 2 M guanidine HCl and separation on a sizing column (Paulsson et al., 1987). Human plasma fibronectin was a kind gift of Dr. K. Ingham, American Red Cross. Guinea pig IgG was purchased from Sigma; bovine serum albumin (BSA) and N,N-dimethylcasein were from Serva Fine Biochemicals.

**Preparation and Purification of Polyclonal Antibodies**

Antisera directed against murine EHS tumor laminin-nidogen complex (serum 143) and guinea pig tissue transglutaminase (serum 144) were raised in rabbits by multiple intradermal injections of antigens (200 \( \mu \)g of laminin-nidogen complex or 100 \( \mu \)g of transglutaminase on two occasions at an interval of 1 mon) three times. The first injections were done with complete Freund's adjuvant, and the second, with incomplete adjuvant. Antibodies were purified by affinity chromatography. Laminin, nidogen or tissue transglutaminase (1 mg/ml gel) was coupled to preswollen and prewashed CNBr-activated Sepharose 4B (Pharmacia), as described by the supplier. Serum (1 ml/ml gel) was applied to the columns, equilibrated in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl (TBS), and incubated for 30 min at 4 °C on the column without flow. The columns were then washed with at least 3 bed volumes of TBS before elution with 3 M KSCN. The eluted antibodies were immediately dialyzed against a 1000-fold volume of TBS at 4 °C. Specific antibodies directed against nidogen were purified by passing serum 143 three times over the laminin column, followed by collecting the eluting nidogen antibodies on the nidogen column. The affinity-purified antibodies were concentrated to a final concentration of 0.16 mg/ml by use of ultrafiltration in Centricron cups (Amicon). Antibodies against tissue transglutaminase were purified in an analogous way and concentrated to 0.10 mg/ml. An ammonium sulfate-precipitated anti-fibronectin IgG fraction (λ-CIG 1803, rabbit) was kindly provided by Dr. M. Chiquet, Biocenter, University of Basel. A polyclonal antisera against human platelet factor XIII was purchased from Calbiochem.

**Protein Concentration Determination**

Protein concentrations were determined using the bichinchoninic acid reagent (Pierce Chemical Co.) standard protocol as described by the supplier, with BSA as a standard. Protein concentration was determined before each experiment to account for losses due to protein precipitation during freezing and thawing.

**Transglutaminase Assays**

Radioactive—Incorporation of \([1,4-\text{\textsuperscript{3}}\text{H}]\)putrescine (Amersham Corp., 30 Ci/mmol) into various proteins was determined according to a modification of the method by Bowness et al. (1987). The reaction was performed by incubation of samples (160 \( \mu \)l in 0.10 M Tris/HCl, pH 8.3, containing 5 mM CaCl\(_2\)) of 2 \( \mu \)Ci (417 nM) of \([\text{\textsuperscript{3}}\text{H}]\)putrescine (\([\text{\textsuperscript{3}}\text{H}]\)putrescine/putrescine, 1:4), 1.56 \( \mu \)M putrescine (Serva Fine Biochemicals), 625 nM protein substrate, and 125 nM tissue transglutaminase in a 37 °C water bath. The reaction was stopped by precipitating the protein by addition of 100 \( \mu \)l of 50% trichloroacetic acid and incubating on ice for 1 min. Precipitates were washed by centrifugation in a Heraeus Biofuge A for 10 min at 10,000 rpm, and the resulting pellet was washed once with 200 \( \mu \)l of 10% trichloroacetic acid and twice with ethanol/ether (v/v, 1:1). The final pellet was dissolved in 35 \( \mu \)l of 0.10 M Tris/HCl, pH 6.8, containing 4 \( \mu \)l urea, 2% SDS, 5 mM EDTA, and 2 mM 2-mercaptoethanol, by boiling for 2 min and vortex mixing at a maximum speed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with 10 ml of scintillation fluid (Insta Gel, Packard Instrument Co.).
and analyzed in a Tri-Carb liquid scintillation spectrometer.

Fluorescent—Monodansylcadaverine (N-(6'-aminoptyl)-5-di-
methylamino-1-naphthalenesulfonamide, Serva Fine Biochemicals) was used as an amine donor for enzyme activity detection, as sug-
gested by Lorand and Campbell (1971). Samples (180 μl in 0.10 M Tris/HCl, pH 8.3, containing 5 mM CaCl₂) of 39 μM monodansylca-
daverine, 40 μg of protein substrate and 4 μg of tissue transglutamin-
ase were processed as above, except that the final pellet was dissolved in sample fluid, containing 4 μa and 0.5 mM 2-mercaptopeto-
enthal, and subjected to SDS-PAGE. Gels were fixed in 25% isopropanol, 10% acetic acid and stained with 1% Coomassie Brilliant Blue R (Bio-Rad) or by immunoblotting on nitrocellulose membranes (Schleicher & Schuell) according to the method of Towbin et al. (1979). Blots were developed by the peroxi-
dase-anti-peroxidase procedure (Imhof et al., 1983).

Electron Microscopy
Laminin-nidogen complex was cross-linked with tissue transglu-
taminase, as described for the radioactive transglutaminase assay, except that putrescine was excluded from the reaction mixture. The reaction was stopped by addition of EDTA to a final concentration of 25 mM, and the samples were extensively dialyzed into 50 mM ammonium bicarbonate at 4 °C. Small aliquots of 20-50 μg of protein/ ml were mixed with an equal volume of glycerol, sprayed onto freshly cleaned grid, and finally covered with one drop of 90% glycerol and 10% 0.2 M Na-
H₂PO₄, pH 7.4. Several pictures were taken at random (magnification, 30,000×) from each grid. The experiment was performed twice with the same results.

Indirect Immuno- and Direct Fluorohistochemistry
12-14-μm thick unfixed cryosections were adsorbed on gelatin-
coated glass slides and used either for indirect immunohistochemical analysis according to standard procedures (Chiquet and Fambrough, 1984) or transglutaminase assay by incubation with the fluorescent amine donor monodansylcadaverine. All steps in the processing of the sections were carried out in a wet chamber at room temperature in the dark. Free binding sites were blocked by treatment with 1% BSA/TBS solution. Pictures were taken on Ilford HP 5 film with a 300-nm UV screen using a standard fluorescent lamp together with an UV light filter.

RESULTS
Putrescine Incorporation into the Laminin-Nidogen Com-
pact and Its Constituents Compared with the Incorporation into Reference Proteins—The formation of large protein ag-
gregates by the action of tissue transglutaminase can strongly influence assay performance. As the extent of aggregation is dependent on the nature of the protein substrate, we decided to examine the course of the reaction rather than only measuring limit values of incorporation. [³H]Putrescine incorporation into murine EHS laminin-nidogen complex and its constituents was determined with BSA and guinea pig IgG as negative controls (Clarke et al., 1959), and with bovine N,N-
dimethylcasein (Gorman and Folk, 1960) and human plasma fibronectin (Fész et al., 1986), two well characterized transglutaminase substrates, as positive controls. In contrast to the commonest procedures, we performed the assays without addition of reducing agent, since addition of dithiothreitol, 2-
mercaptopetoanil, or glutathione at the normally used concen-
tration of 1 mM did not affect, or even reduce, the extent of the reaction (results not shown). Thus, it appears that the reducing agent may be omitted if oxidation of the enzyme during purification is prevented. Incubation of isolated lamin-
in with tissue transglutaminase gave only background levels of incorporation, comparable with the reaction with BSA or IgG (Fig. 1a). Purified nidogen, in contrast, showed an initial reaction rate and a limit incorporation similar to that obtained with N,N-dimethylcasein. On a molar basis, only about half as much label was incorporated into the intact laminin-
nidogen complex as into isolated nidogen, possibly due to steric hindrance. The possibility that the molar ratio of lamin-
in to nidogen differed from the previously estimated 1:1 ratio (Paulson et al., 1987) was excluded by densitometry of nidogen on SDS-PAGE gels to which either the isolated or complexed form had been applied at similar concentrations (results not shown).

It was previously described that tissue transglutaminase itself may act as a glutamine donor (Birckbichler et al., 1977b). Therefore, the incorporation of label into the enzyme itself was determined, with transglutaminase at 125 nM used before or at 750 nM to mimic the situation of enzyme plus substrate. The label detected at the lower enzyme concentration, but without addition of any other protein substrate, was equally as low as that with BSA, IgG, or laminin (Fig. 1b). The limit incorporation obtained at the higher transglutaminase con-

![Fig. 1. Comparison of tissue transglutaminase-catalyzed [³H]putrescine incorporation into various proteins.](attachment:image)

Fig. 1. Comparison of tissue transglutaminase-catalyzed [³H]putrescine incorporation into proteins. [³H]Putres-
cine incorporation into EHS laminin-nidogen complex (LN/ND) and its constituents was compared with that into human plasma fibro-
nectin, BSA, and guinea pig IgG (a). Similarly, incorporation into isolated EHS nidogen was compared with that into bovine milk N, N-dimethylcasein and autoantibody incorporation in guinea pig liver tissue transglutaminase (TG) at either the normal enzyme concen-
tration (125 nM) or at the same total protein concentration (750 nM) as in the other samples (b). All values are the average of four to seven independent measurements. The bars indicate standard deviation from mean values.
Saturation of the Reactive Glutamine Residues in Nidogen with Label—The similarities in the amount of label incorporated into nidogen, N,N-dimethylcasein and tissue transglutaminase indicated a comparable number of high affinity substrate sites (Fig. 1b). To determine the number of sites in nidogen in absolute terms, the incorporation of label was driven to saturation by incubation with successively increasing amounts of unlabeled putrescine, whereas the [3H]putrescine concentration was left constant (Fig. 2). The actually incorporated amount of putrescine was calculated from the [3H]putrescine/putrescine ratio used. By comparison of these data with those obtained by incorporation with [3H]putrescine alone or at the normally used [3H]putrescine/putrescine ratio of 1:4, it was shown that neither the dilution of [3H]putrescine with unlabeled putrescine nor calculation based on the dilution ratio influenced the results. The saturation curve for self-incorporation of putrescine into tissue transglutaminase was determined as a control (Fig. 2). At saturating conditions, both nidogen and tissue transglutaminase incorporated about 1 mol of putrescine/mol of protein substrate.

Structure of the Reaction Products Formed by Tissue Transglutaminase Catalyzed Cross-linking of the Laminin-Nidogen Complex—Transglutaminase-catalyzed incorporation of monodansylcadaverine into the laminin-nidogen complex, its constituents, and the various reference proteins, followed by SDS-PAGE analysis, corroborate the results obtained with radioactive putrescine (Fig. 3). The bands at 130 and 100 kDa, in addition to the major 150 kDa band in the nidogen sample, could be shown, by immunoblotting with an affinity-purified antibody against nidogen, to be the previously described endogenous degradation fragments of nidogen (Dziadek et al., 1985; Paulsson et al., 1987). The fact that these fragments also incorporate label indicates that the cross-linking site(s) is located either in the rodlike region or the COOH-terminal domain of nidogen, as these fragments correspond to NH2-terminally truncated nidogen molecules (Mann et al., 1989).

Moreover, additional fluorescent bands appeared at higher apparent Mr, values than those of the original substrate proteins, indicating that covalent complexes are formed. While the amount of complexed material increased with time, the amount of material in the original position of both substrate and enzyme decreased (Fig. 3), suggesting that the aggregates formed contain tissue transglutaminase, as well as substrate protein. The loss of material in the position of laminin indicates that laminin becomes part of the aggregates as well, although it does not serve as a glutamine donor in the reaction. The observed preferential loss of laminin A-chain was not due to proteolytic degradation, as A-chain fragments could neither be seen on Coomassie Blue-stained gels or on immunoblots with anti-laminin-nidogen complex antibodies; nor was the same loss observed in identically treated samples in

![Figure 2: Saturation of the amine incorporation site(s) in nidogen. (a) Putrescine incorporation into nidogen by tissue transglutaminase-catalyzed reaction was driven to saturation by addition of increasing amounts of putrescine. Pure [3H]putrescine (A), [3H]putrescine/putrescine at a ratio of 1:4 (X), or increasing amounts of unlabeled putrescine at a constant [3H]putrescine concentration (C) was used as the amine substrate. Saturation of autocatalytic [3H]putrescine incorporation into tissue transglutaminase itself was done by addition of increasing amounts of unlabeled putrescine at a constant [3H]putrescine concentration (A). As the relation between glutaminy substrate concentration and putrescine incorporation is nonlinear (results not shown), it is not adequate to correct for the autocatalytic incorporation of label into the enzyme in the presence of nidogen by only subtracting the label incorporated in the absence of nidogen. Since the limit levels of autocatalytic label incorporation into the enzyme and incorporation into nidogen were comparable (Fig. 1), the putrescine incorporation was calculated based on the total protein concentration instead of the nidogen concentration. The results are shown as either a linear (a) or a semilogarithmic plot (b). The curve fits were calculated with a nonlinear regression program (Enzfitter, Elsevier-Biosoft) with the assumption of a single class of binding sites.](image)

![Figure 3: SDS-PAGE analysis of tissue transglutaminase-catalyzed incorporation of fluorescent amine into the laminin-nidogen complex. Monodansylcadaverine incorporation by tissue transglutaminase (TG) into the laminin (LN)-nidogen complex (a) and isolated nidogen (b) was performed for the indicated time periods at 37°C in a Ca++-containing buffer. Left panels, Coomassie Brilliant Blue staining; right panels, the fluorescence pattern of 3-15% polyacrylamide gradient gels run under reducing conditions. Samples at 0 min incubation represent the original protein preparations directly applied to the gel. Mr standards are indicated on the left.](image)
the absence of the enzyme. Therefore, structural factors like the potential presence of particularly reactive lysine residues in the A-chain or the spatial orientation of the A-chain in respect to the substrate site on nidogen are likely to be the cause. The reason that the observed losses in the original position of the proteins are not quantitatively recovered as complexes on top of the stacking gel is that some aggregated material is lost during the staining procedure.

Attempts to evaluate the composition of the complexes by immunoblotting with affinity-purified antibodies against tissue transglutaminase or against nidogen were often not conclusive due to the broad and diffuse nature of the bands obtained for the complexes. The broad bandwidth reflects the structural diversity of the cross-linking products resulting from a reaction in which different lysine residues present on the protein surface serve as amine donor. Even though the results did not allow definite conclusions about the nature of the single bands, the overlap of staining indicates that most likely some are composed of tissue transglutaminase, as well as nidogen molecules, as could be shown by immunoblotting of the predominant band at approximately 200 kDa (results not shown), which occurs as an intermediate cross-linking product of nidogen. The size fits a heterodimeric structure composed of a 77-kDa tissue transglutaminase and a 150-kDa nidogen molecule.

To get more detailed information about the structure of the complexes, we prepared samples by rotary shadowing for electron microscopy. The cross-linking reaction of the laminin-nidogen complex and of the enzyme alone was performed under standard conditions for different time periods and stopped by addition of EDTA together with immediate chilling to 4 °C. As it is known that laminin polymerizes in the presence of Ca²⁺ to large aggregates and that this reaction is largely reversible upon removal of Ca²⁺ and lowering the temperature (Yurchenco et al., 1985, 1986; Paulsson, 1988), this procedure also served to distinguish between aggregates formed spontaneously and those stabilized by transglutaminase cross-links. Aliquots of the samples used for electron microscopy were subjected in parallel to SDS-PAGE to demonstrate that the complexes formed actually were covalently cross-linked (as in Fig. 3). The globular tissue transglutaminase particles form large aggregates, which show no clear supramolecular organization. The transglutaminase-catalyzed cross-linking of the laminin-nidogen complex gave a reasonably well defined organization within the large complexes similar to the one observed for Ca²⁺-induced self-aggregation (Fig. 4). The laminin molecules were often oriented with the three short arms pointing toward the center of the aggregates and the long arm extended. Ca²⁺-induced aggregation of laminin has been shown to be mediated through the short arm structures of the laminin molecule in studies using proteolytic fragments (Paulsson et al., 1988; Bruch et al., 1989). Nidogen is associated with one of the short arms of laminin and is therefore located in the center of the aggregates. The reversibility of the Ca²⁺-induced aggregation of the laminin-nidogen complex was lost by tissue transglutaminase cross-linking and increasing cross-linking was accompanied by a pronounced condensation of the aggregates.

Comparison of the Distribution of Tissue Transglutaminase and Extracellular Matrix Proteins in Tissues—So far we have shown that nidogen is a specific substrate for tissue transglutaminase in vitro, but the question whether this reaction is of physiological importance remains, in particular as secretion of tissue transglutaminase has not been demonstrated and the enzyme neither contains a signal sequence nor any other characteristics indicating conventional secretion. Thus, we examined by immunohistochemistry if tissue transglutaminase and nidogen show, at least in some cases, a codistribution in the extracellular space, which would allow this type of reaction to occur in vivo.

The polyclonal affinity-purified antibodies against tissue transglutaminase were shown to be highly specific in immunoblotting of a sucrose extract of guinea pig liver tissue (Fig. 5a). Furthermore, cross-reactivity with the catalytic a-subunit of the plasma transglutaminase, factor XIII, which has been shown to be highly homologous to tissue transglutaminase (Ikura et al., 1988), was excluded by comparison of our antibodies against tissue transglutaminase with an antisemur against human platelet factor XIII in immunoblots of a factor XIII-enriched fraction of guinea pig plasma. No cross-reactivity of the antibodies against tissue transglutaminase with factor XIII could be detected, whereas the antisemur against factor XIII showed a slight cross-reactivity with tissue transglutaminase (Fig. 5b). The polyclonal antisemur directed against murine EHS laminin-nidogen complex has a broad cross-reactivity between species. It cross-reacts with EDTA extracts of heart tissue from various mammals, including guinea pig (results not shown). Antibodies directed against nidogen were purified from the anti-EHS laminin-nidogen complex serum using affinity chromatography and were shown to be specific (Fig. 6).

Cryosections of guinea pig organs, e.g. liver, heart, kidney (Fig. 7) and brain (Fig. 8) were stained for laminin-nidogen complex, fibronectin, and tissue transglutaminase by use of a rhodamine-conjugated secondary antibody. Staining with the antisemur against laminin-nidogen complex was largely restricted to basement membranes, which were seen underlying the endothelial and epithelial cell layers, as well as surrounding muscle fibers and peripheral nerves within tissue structures like triads and sinusoids in liver (Fig. 7a); myocardium and vessels in heart (Fig. 7b); glomeruli, ducts, and capillaries in kidney (Fig. 7c); and choroid plexus and capillaries in brain (Fig. 8b). An identical staining pattern was observed with the affinity-purified antibodies against nidogen (results not shown). With the antisemur against fibronectin, widespread matrix staining was seen (Fig. 7, d–f). In blood vessels, intense staining of the endothelium and weaker staining in the tunica media was observed. The adventitia or tunica externa, which is largely composed of collagen fibers, was completely negative. The capillary coil of the glomeruli in kidney appeared strongly positive, and staining similar to that of the laminin-nidogen complex was found in the choroid plexus of the brain (Fig. 8c). Tissue transglutaminase also showed a widespread distribution in the pericellular matrix, but the fine structure of the staining pattern differed from that of fibronectin (Fig. 7, g–i). In blood vessels, tissue transglutaminase was detected mainly in the adventitia, which was completely negative for laminin-nidogen complex and fibronectin. In heart muscle, the perimysium, which is rich in fibrillar collagen as is the vascular adventitia, was intensely stained. In agreement with its absence from the vascular intima, tissue transglutaminase was not detected either in the glomeruli in kidney or in brain capillaries. The clear colocalization of the fluorescence pattern of laminin-nidogen complex and tissue transglutaminase in the choroid plexus and in several types of pericellular matrix was in accordance with nidogen being a protein substrate for tissue transglutaminase (Fig. 8, b and d).

The widespread extracellular distribution of tissue transglutaminase was surprising, as it is considered to be primarily an intracellular enzyme. Specific binding of the enzyme to the extracellular matrix after cell rupture has been described (Upchurch et al., 1987). As this could occur by sectioning, we
tested if preincubation of sections with exogenously added tissue transglutaminase would give rise to similar staining patterns. In such preincubated sections, broad unspecific staining of all tissue compartments was seen (results not shown), indicating that the distinct patterns seen without preincubation is due to tissue transglutaminase present in extracellular matrix structures in vivo (see below).

To corroborate these results by an independent method and to exclude cross-reactivity of the antiserum against tissue transglutaminase with another extracellular matrix component, a new staining procedure based on the catalytic activity of transglutaminase was developed. Sections were stained with monodansylcadaverine as a fluorescent marker for transglutaminase activity (examples are shown in Fig. 8, e–h). A perfect colocalization between immunolabeling and fluorescent amine labeling was observed, except that some structures like the tunica media in blood vessels were stained comparatively more intensely by fluorescent amine incorporation (results not shown). This indicates that, in addition to the tissue enzyme, other forms of transglutaminase are present in these structures. Treatment of the sections with exogenous tissue transglutaminase resulted in a similarly unspecific staining as seen by immunofluorescence after preincubation with enzyme (Fig. 8g). The fact that the incorporation of monodansylcadaverine could be inhibited by addition of EDTA (Fig. 8h) demonstrates that the staining is dependent on the presence of enzymatically active transglutaminase.

**DISCUSSION**

In the present study, the potential role of tissue transglutaminase in cross-linking of laminin-nidogen complex was investigated. Incubation of laminin-nidogen complex from murine EHS tumor (Paulsson, 1988) with tissue transglutaminase from guinea pig liver (Folk and Cole, 1966; Connellan et al., 1971), resulted in incorporation of $[^{1,4}-H]$putrescine...
A Novel Mechanism of Basement Membrane Stabilization

FIG. 5. Specificity of affinity-purified antibodies against tissue transglutaminase. SDS-PAGE of a guinea pig liver extract on a 3-15% polyacrylamide gradient gel was performed under reducing conditions, and the gel was stained either with Coomassie Brilliant Blue or after electrophoretic transfer to nitrocellulose by incubation with affinity-purified antibodies against tissue transglutaminase (diluted 1:200) (a). Guinea pig plasma factor XIII was partially purified by ammonium sulfate precipitation at 20% saturation, followed by molecular sieve chromatography on a Superose 6 fast protein liquid chromatography column. Reduced samples of tissue transglutaminase (TG, left sample in each panel) and plasma factor XIII (FXIII, right sample in each panel) were subjected to SDS-PAGE on a 3-15% polyacrylamide gradient gel and stained with Coomassie Brilliant Blue or by immunoblotting with a dilution series of antibodies against either human placenta factor XIII or guinea pig liver tissue transglutaminase, as indicated (b). The dilution series of the two antibodies were tested on pairs of samples run next to each other on a single blot. Identical samples from the same gel are shown after Coomassie staining. M, standards are indicated on the left.

FIG. 6. Comparison of the antiserum against laminin-nidogen complex and the affinity-purified antibodies against nidogen by immunoblotting. Specific antibodies against nidogen were purified from a serum raised against laminin-nidogen complex by means of affinity chromatography. Blots of purified laminin-nidogen complex were stained either with laminin-nidogen complex antiserum (diluted 1:200) (a) or with affinity-purified antibodies against nidogen (diluted 1:100) (b).

or monodansylcadaverine into the complex. Thus, laminin-nidogen complex was shown to contain glutamine residue(s), which act as amine acceptor site(s) in tissue transglutaminase-catalyzed cross-linking. Reaction with the isolated constituents of the complex revealed that the site(s) are located in nidogen alone. The suggestion by Fésus et al. (1986) that laminin acts as a glutaminyl substrate in this type of reaction could not be confirmed, and we assume that their results were due to nidogen contamination of their laminin preparation.

A comparison of putrescine incorporation in nidogen with that in several reference proteins revealed a level equal to that seen with N,N-dimethylcasein. This protein has been shown to contain one primary amine acceptor site for the plasma factor XIII-catalyzed reaction (Gorman and Folk, 1980), and was shown to also act as a glutaminyl substrate for guinea pig liver tissue transglutaminase (Gorman and Folk, 1981, 1984). Tissue transglutaminase itself, at a higher concentration (equal to substrate plus enzyme), also showed a putrescine incorporation level comparable with that of nidogen, but was previously suggested by Birckbichler et al. (1977b) to contain two to three amine acceptor sites. In order to obtain more conclusive data on the stoichiometry of the reaction of putrescine with nidogen, we attempted to reach saturation of the binding site(s) by performing the reaction with increasing amounts of putrescine as described previously (Bowness et al., 1987; Sane et al., 1988). At saturation, 1 mol of putrescine/mol of protein was incorporated, suggesting that nidogen contains one high affinity amine acceptor site. A similar saturation experiment with tissue transglutaminase as amine acceptor gave an incorporation level that was slightly lower than the one for nidogen but clearly in the range of one amine acceptor site/protein molecule.

Surprisingly, the laminin-nidogen complex was found to incorporate only half as much amine as isolated nidogen. Determination of the laminin to nidogen ratio in our preparation of the complex confirmed that the proteins were present in a perfect equimolar ratio. Thus, we are so far not able to explain this discrepancy, but suspect that the steric accessibility of the amine acceptor site in nidogen may differ between the isolated and the complexed forms. We are currently pursuing the identification of the reactive glutamine residue by proteolytic fragmentation of labeled nidogen and sequencing of the labeled peptides, which may allow us to answer this question.

Detailed studies of the reaction products formed by the tissue transglutaminase-catalyzed reaction with intact laminin-nidogen complex and its constituents by SDS-PAGE and by electron microscopy after rotary shadowing showed that homogeneous, as well as heterogeneous, oligomers are formed, with the reservation that, in the presence of proteins without an amine acceptor site, e.g. laminin, enzyme homo-oligomers are formed predominantly (results not shown). Moreover, the reversibility of the Ca2+ and temperature-dependent polymerization of laminin-nidogen complexes into large aggregates (Yurchenco et al., 1985, 1986; Paulsson, 1988) was lost by the action of tissue transglutaminase. The covalently cross-linked aggregates formed were organized in a head-to-head arrangement similar to that described for Ca2+-mediated aggregation. Thus, it is likely that tissue transglutaminase action leads to a covalent stabilization of the laminin-nidogen complex in the Ca2+-induced organization. The head-to-head arrangement of the complex in the aggregates is consistent with the finding that the reactive glutamine residue is located in nidogen, as nidogen is associated with one of the short arms of the laminin molecule.

We have preliminary evidence that additional basement membrane components, e.g. BM-40 (SPARC, osteonectin) and collagen type IV, act as high affinity transglutaminase substrates (results not shown). In addition, nidogen from other sources than the EHS tumor, such as bovine and murine heart, is an effective substrate. This may be a common feature of many extracellular matrix proteins.

Indirect immunofluorescence and enzyme assays on unfixed cryosections revealed an extracellular distribution of tissue...
Fig. 7. Comparison of the staining pattern obtained for tissue transglutaminase, laminin-nidogen complex and fibronectin by indirect immunofluorescence on sections of a liver triad, heart muscle, and kidney marrow. Cryosections of a liver triad (14 μm) (a, d, and g), heart muscle (12 μm) (b, e, and h), and kidney marrow (12 μm) (c, f, and i) were stained with antiserum against laminin-nidogen complex (1:50) (a-c), antiserum against fibronectin (1:100) (d-f), or affinity-purified antibodies against tissue transglutaminase (1:5) (g-i) using a rhodamine-conjugated second antibody. The fields on the left show the same sections in phase contrast. The bar represents 200 μm.

Transglutaminase, mainly in tissues rich in fiber-forming collagen such as the vascular adventitia and the perimysium in heart muscle. This observation supports the relevance of the finding that collagen type III is a specific in vitro substrate for tissue transglutaminase (Bowness et al., 1987) and points to the possibility of an important physiological function of this reaction. In addition, codistribution with laminin-nidogen complex and fibronectin was often seen, particularly clearly in the choroid plexus in brain and in the pericellular matrix of heart muscle, kidney marrow, and cortex. This indicates a role for tissue transglutaminase cross-linking of the laminin-nidogen complex in vivo.

The observation that the expression of the cytosolic tissue transglutaminase is reduced severalfold in transformed cells compared with normal cells (Birckbichler et al., 1977a, 1981), in contrast to the unchanged expression level of the particulate enzyme (Byrd and Lichti, 1987; Piacentini et al., 1988; Korner et al., 1989; George et al., 1990), may correlate with the fact that basement membrane components are more easily extracted from tumor tissue than from normal tissue (Paulsson and Saladin, 1989) due to a lower degree of covalent cross-linking.

The observed extracellular distribution of the enzyme is not consistent with tissue transglutaminase being a purely intracellular enzyme. It does not have a signal sequence (Ikura et al., 1988) and it could not be detected in the medium of preconfluent cell cultures (Birckbichler and Patterson, 1978). Activation of the enzyme, occurring at the elevated Ca++ concentration in the endoplasmic reticulum and the Golgi apparatus, could have deleterious consequences. We speculate that one mechanism for tissue transglutaminase release may be cell lysis due to physiologically occurring cell death. It was shown by Fesus et al. (1987) that in lead nitrate-induced liver hyperplasia, tissue transglutaminase-catalyzed protein cross-linking is part of the biochemical pathway of programmed cell death. In addition, the enzyme was detected in low amounts in the medium of confluent cell cultures, as well as in close vicinity to the plasma membrane (Birckbichler and Patterson, 1978). High affinity binding to the extracellular matrix, after perturbation of the cell membrane with detergent, was found in cell cultures (Upchurch et al., 1987). In light of these observations and the fact that an increasing number of proteins lacking a signal sequence and other typical characteristics of secreted proteins such as glycosylation and disulfide bonds, e.g. interleukin 1β (Rubartelli et al., 1990), acidic and basic fibroblast growth factor (Rifkin and Moscatelli, 1989), the lactose-binding muscle lectin L-14 (Cooper and Barondes, 1990) and, most importantly, the α-subunit of plasma factor XIII (Ichinose et al., 1990), have been shown to have an extracellular function, the possibility of secretion of
these proteins, as well as tissue transglutaminase, by an alternative pathway cannot be excluded.

Cell-cell and cell-matrix interactions may be influenced by transglutaminase action, as it has been reported that epithelial and fibroblastic cells prefer to migrate on cross-linked fibronectin substrates (Grinnell et al., 1980) and that cross-linking occurs between fibronectin and fibrin(ogen) and the surface of hepatocytes, fibroblasts, and endothelial cells (Barsigian et al., 1988; Barry and Mosher, 1989; Martinez et al., 1989). In conclusion, we propose that tissue transglutaminase, besides its potential role in intracellular processes during terminal differentiation of cells (Lorand et al., 1981; Lorand and Conrad, 1984; Nara et al., 1989), may also play an important role in matrix processing during postnatal organ development (Ikura et al., 1990) and in aging of tissues.

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