Transglutaminase-Catalyzed Cross-Linking of Fibrils of Collagen V/XI in A204 Rhabdomyosarcoma Cells†

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ABSTRACT: Collagens V and XI are thought to form a core around which the major interstitial collagens, I and II, respectively, are organized during fibrillogenesis. We previously reported the presence of a heterotypic form of collagens V and XI, $[\alpha1(XI)]_2\alpha2(V)$, in cultures of A204 rhabdomyosarcoma cells [Kleman, J.-P., Hartmann, D. J., Ramirez, F., & van der Rest, M. (1992) *Eur. J. Biochem. 210,* 329−335]. This collagen forms a matrix which remains highly insoluble, even when cells were cultured in the presence of β-aminopropionitrile, an inhibitor of lysyl oxidase and thereby of "classical" collagen cross-linking. When the cells were cultured in the presence of putrescine, a competitive inhibitor of transglutaminase-catalyzed protein cross-linking, a drastic increase in collagen solubility was observed. This result indicates that a transglutaminase contributes to the covalent stabilization of the collagen matrix of these cells. A204 rhabdomyosarcoma cells express tissue transglutaminase as revealed by specific antibodies, and enzyme activity was detected in the cell layer during culture and in cell extracts. Both collagens V and XI are specific glutaminyl substrates for tissue transglutaminase *in vitro*, as shown by incorporation of [^{3}H]putrescine. The highly homologous α1 chains of collagens V and XI were the major targets for the cross-linking. Trypsin cleaved the [^{3}H] label from the α1 chain of collagen V, demonstrating that the cross-linking occurs in the non triple helical propeptide domains.

Transglutaminases are calcium-dependent enzymes that catalyze the formation of an isopeptide bond between the γ-carboxamide group of peptide-bound glutamine residues and the amino groups of a variety of primary amines, including those of putrescine, spermidine, and cadaverine, and, physiologically most relevant, the ε-amino group of peptide-bound lysine residues. The formation of N^{ϵ}-(γ-glutamyl)lysine cross-links is involved in a number of different physiological processes. Plasma transglutaminase (i.e., activated factor XIII a-subunit) is responsible for the covalent stabilization of the fibrin-clot in haemostasis, whereas keratinocyte and epidermal transglutaminase are involved in the formation of the cornified envelope in skin [for review see Aeschlimann and Paulsson (1994)]. The physiological function of tissue transglutaminase is less clear and might be diverse. Tissue transglutaminase has been implicated in intracellular cross-linking in programmed cell death (Fésus et al., 1991), but, in addition, there is plenty of evidence that tissue transglutaminase acts physiologically in the stabilization of extracellular matrices such as calcifying cartilage (Aeschlimann et al., 1993, 1995) and basal lamina (Aeschlimann & Paulsson, 1991; Martinez et al., 1994) during matrix assembly and in wound healing (Upchurch et al., 1991). An extracellular function for tissue transglutaminase is supported by, e.g., the finding that the enzyme cross-links numerous extracellular matrix proteins, such as fibronectin (Fésus et al., 1986; LeMosy et al., 1992), vitronectin (Sane et al., 1988; Skorstengaard et al., 1990), the N-propeptide of collagen III (Bowness et al., 1987), laminin−nidogen complexes (Aeschlimann & Paulsson, 1991; Aeschlimann et al., 1992), fibrin(ogen) (Achyuthan et al., 1988; Shainoff et al., 1991), and osteopontin (Prince et al., 1991; Sorensen et al., 1994), at least under *in vitro* conditions. Recently, osteonectin and collagen II were also shown to be specific tissue transglutaminase substrates and to be cross-linked during cartilage maturation *in vivo* (Aeschlimann et al., 1993, 1995).

Collagens V and XI are quantitatively minor components of extracellular matrices. They are thought to play a major role in collagen I and II fibrillogenesis, possibly by forming the core of the fibrils (van der Rest & Bruckner, 1993). Collagens V and XI from most sources are poorly soluble without pepsin treatment, indicating that they are cross-linked within the tissue. We have recently shown that an A204 rhabdomyosarcoma cell line synthesizes only a heterotypic form of collagen, $[\alpha1(XI)]_2\alpha2(V)$ (collagen V/XI), and deposits it into a highly insoluble fibrillar matrix that cannot be solubilized in acetic acid without proteolytic treatment (Kleman et al., 1992). This collagen remains insoluble even when cells are cultured in the presence of β-aminopropionitrile, a specific inhibitor of lysyl oxidase, the enzyme responsible for the formation of most lysyl-derived cross-links in collagens (Eyre, 1987). These findings suggest that a cross-linking mechanism is operative in these cells which specifically acts on the pepsin-sensitive, terminal, non triple helical domains of collagen V/XI. In the present study, we

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have investigated the possibility that a transglutaminase is responsible for the cross-linking of collagen V/XI fibrils.

MATERIALS AND METHODS

Cell Culture. The A204 rhabdomyosarcoma cell line (ATCC HTB 82) was obtained from Dr. F. Ramirez (Brookdale Center for Molecular Biology, Mt. Sinai School of Medicine, New York). The cells were grown in Dulbecco's modified Eagle medium as described previously (Kleman et al., 1992).

Collagen Extraction from Cell Culture. Confluent cultures were trypsinized, and the cell suspensions rinsed in serum-free Dulbecco's modified Eagle medium and seeded at twice the initial cell density. After 30 min, the remaining non-adherent cells were washed away. The resulting cultures were at that time fully confluent. To obtain an acid soluble collagen matrix, cells were cultured in serum-free medium (or Dulbecco's modified Eagle medium containing 2% fetal calf serum for harvesting the intact forms of collagen V/XI from the medium) supplemented with 0.3 mM sodium ascorbate, 0.2 mM β-aminopropionitrile (βAPN),[1] and 1 mM putrescine. After 18 h, cell layers were rinsed gently in cold phosphate-buffered saline (PBS) containing 10 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM EDTA as protease and transglutaminase inhibitors. The collagen was solubilized by gentle stirring of the cell layers in 1 mL of 0.5 M acetic acid per 25 cm^2 flask for 24 h. Cell layers were scraped away in the same solution, and the mixture was centrifuged at 20 000*g* for 15 min at 4 °C. The supernatant was lyophilized and dissolved in sample cocktail for electrophoresis (Laemmli, 1970).

Transglutaminase Extraction from Cell Culture. Transglutaminase was extracted from cells using a modification of the procedure described by Griffiths and co-workers (Griffiths et al., 1992). After several rinses in prewarmed PBS (37 °C), the cell layers were rapidly frozen at −20 °C in lysis buffer [10 mM Tris/HCl, pH 7.4, 10 mM dithiothreitol, 0.5 mM EDTA (3 mL/75 cm^2 flask)]. Cells were lysed by thawing slowly at 4 °C, and the cell homogenate was centrifuged at 20 000*g* and 4 °C for 15 min. The resulting supernatant (S1), containing soluble transglutaminases, was collected. The pellet was rinsed twice by resuspending in lysis buffer, followed by centrifugation as above. The washed pellet was resuspended in 1 mL of lysis buffer containing 1% Triton X-100, incubated for 5 min at 37 °C, and centrifuged as above. The resulting supernatant (S2) contains membrane-bound transglutaminases, if present. To determine the transglutaminase activity, the cell extracts were used directly. For SDS−PAGE and immunoblot analysis, proteins were recovered by precipitation in 20% (w/w) trichloroacetic acid, collected by centrifugation, rinsed in ethanol:ether (1:1, v/v), and lyophilized.

Determination of Transglutaminase Activity in Cell Extracts. Transglutaminase activity was measured using a modification of the method by Slaughter et al. (1992). The standard solution of tissue transglutaminase (Sigma) contained 1 mg (1.7 U)[2] enzyme in 1 mL 50 mM Tris/HCl, pH 7.5, 60 mM NaCl, 5 mM $CaCl_2$, and 10 mM dithiothreitol. The substrate stock solutions were 5 mg/mL *N,N'*-dimethylcasein (dissolved in 100 mM Tris/HCl, pH 8.3, 1 mM EDTA, and 10 mM $CaCl_2$) and 50 mM monodansylcadaverine (DNS-C) in methanol (stored at −20 °C). For the assay, microtiter plates were coated with 200 μL/well of *N,N'*-dimethylcasein stock solution, and nonspecific binding was blocked by incubation with 1% bovine serum albumin in TBS. In each well, 80 μL of cell extract or 0−5 μL of enzyme stock solution was added to 5 μL of DNS-C stock solution, 7 μL of 1 M $CaCl_2$ in TBS, and 100 μL of 100 mM Tris/HCl, pH 8.3. The volume was brought to 200 μL/well with deionized water. After 2 h at 37 °C, the reaction was stopped by rinsing with TBS containing 200 mM EDTA. Covalently bound DNS-C was subsequently detected with an antiserum to the dansyl moiety (diluted 1:500, for 30 min) and peroxidase-conjugated secondary antibodies (Biosys). Bound antibodies were visualized by adding 200 μL/well of 5 mg/mL 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) in 100 mM Na_2HPO_4, 60 mM citric acid, pH 4, and 0.01% H_2O_2, and quantitated by measuring the absorbance at 405 nm.

Preparation of Polyclonal Antisera. Antisera to guinea pig tissue transglutaminase (Aeschlimann & Paulsson, 1991), human pepsinized collagen V (Kleman et al., 1992), and dansylated (hapten) *Limulus polyphemus* hemocyanin (Aeschlimann et al., 1993) were raised in rabbits and purified and characterized as previously described.

Labeling of Cell Layers in Culture with DNS-C and Immunological Detection of Incorporated Dansyl Groups. Cells were seeded on glass cover slips at low density and were grown for 12 h in serum-free Dulbecco's modified Eagle medium in presence of 0.3 mM ascorbic acid, 0.2 mM βAPN, and either 0.1 mM DNS-C or 0.1 mM of the nonreactive analogue (dansylamido)pentanol (Aeschlimann et al., 1995), the acyl-accepting amino group of DNS-C in the transglutaminase reaction being replaced by a hydroxyl group). The cell layers were rinsed in PBS, fixed for 2 h in 4% paraformaldehyde-supplemented PBS, and incubated in methanol containing 1% H_2O_2 to block endogenous peroxidase and then in PBS containing 1% bovine serum albumin to block nonspecific antibody binding. After being rinsed, the cell layers were incubated for 1 h with the antiserum to the dansyl moiety (diluted 1:1000), followed by peroxidase-conjugated swine anti-rabbit IgG (Biosys). The reaction was developed by incubation with diaminobenzidine (1 mg in 15 mL PBS in the presence of 0.1% H_2O_2).

Incorporation of Polyamine by Isopeptide Bond Formation in Vitro. The transglutaminase-catalyzed incorporation of [1,4-^{3}H]putrescine into potential substrate proteins was performed as previously described (Aeschlimann & Paulsson, 1991), except that the ionic strength and pH of the reaction buffer were set to physiological conditions (0.15 M NaCl, pH 7.4). Protein samples were cleared from particulate material by centrifugation (10 min, 10 000*g*, 25 °C) prior to the assay. Pepsinized human placenta collagen V was from Sigma, and intact collagen V was purified from human bone (Moradi-Ameli et al., 1994). Collagen XI, kindly provided

[1] Abbreviations: βAPN, β-aminopropionitrile; PBS, phosphate-buffered saline (10 mM sodium phosphate, pH 7.2, 137 mM NaCl, 2.7 mM KCl); DNS-C, monodansylcadaverine, N-(5'-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide; TBS, Tris-buffered saline (50 mM Tris/HCl, pH 8.0, 138 mM NaCl, 2.7 mM KCl).

[2] One unit of transglutaminase will catalyze the formation of 1.0 μmol of hydroxamate min^{-1} from N^{α}-carbobenzoxy-Gln-Gly and hydroxylamine at pH 6.0 and 37 °C.

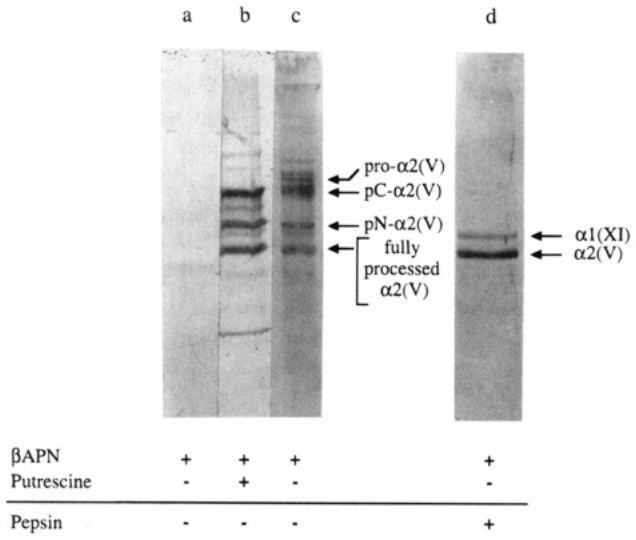

FIGURE 1: Solubility of collagens after culture of A204 rhabdomyosarcoma cells in the presence of inhibitors of lysyl oxidase and transglutaminases. Immunoblot of the proteins solubilized from the cell layer without proteolysis using an antibody to collagen V. Lanes a and b represent 0.5 M acetic acid extracts of cell layers which had been grown in the absence (a) or presence (b) of 1 mM putrescine (0.2 mM βAPN was included in both cultures). The collagen solubilized from putrescine-treated cultures (lane b) resembles the differentially processed forms of collagen V/XI seen in the cell medium of cultures performed in the absence of putrescine (lane c). Collagen V/XI extracted by pepsin cleavage from the cell-layer of untreated cultures (Kleman et al., 1992) is shown for comparison (lane d). The migration positions of the differentially processed forms of the α2(V) chain are indicated (Kleman et al., 1992; Moradi-Ameli et al., 1994).

by Lionel Labourdette (Institut de Biologie et Chimie des Proteines, Lyon, France), was extracted from fetal calf cartilage by pepsin digestion and further fractionated by salt precipitation (Labourdette & van der Rest, 1993). The radioactivity of the [^{3}H]putrescine-labeled samples was determined by scintillation counting or, after electrophoresis, visualized by fluorography as described previously (Aeschlimann et al., 1992).

Trypsin Digestion of [^{3}H]Putrescine-Labeled Collagen. [^{3}H]Putrescine labeled human placenta collagen V (30 μg) was subjected to partial cleavage by trypsin (1.25 μg; TPCK-treated, Sigma) in a final volume of 110 μL of 100 mM Tris/HCl, pH 7.4, 30 mM NaCl, 50 mM $CaCl_2$, and 1 mM EDTA. After digestion for 2, 5, or 12.5 min at room temperature, the reaction was stopped by precipitating the proteins with 70 μL of 50% (w/w) trichloroacetic acid (at 4 °C). The proteins were collected and rinsed as described, and the final pellets were dissolved in sample cocktail (Laemmli, 1970) containing 4 M urea and 1% (v/v) 2-mercaptoethanol for electrophoresis.

SDS−Polyacrylamide Gel Electrophoresis and Immunoblotting. Proteins were resolved by SDS−PAGE according to Laemmli (1970) and either stained with Coomassie brilliant blue R-250 or electrotransferred to poly(vinylidene difluoride) membranes as described (Kleman et al., 1992). Binding of primary antibodies was detected either with peroxidase-conjugated swine anti-rabbit IgG (Dakopatts) and the ECL-reagent kit (Amersham) (Figure 4) or with alkaline phosphatase-conjugated goat anti-rabbit IgG (Biosys) and the AP color reagent (Bio-Rad) (Figure 1) according to the manufacturer's specifications.

RESULTS

Solubility of the Collagen V/XI Matrix of A204 Rhabdomyosarcoma Cells Is Increased after Culture in the Presence of Putrescine. The action of transglutaminases leads to intermolecular cross-linking by the formation of isopeptide bonds between specific glutamine residues of substrate proteins and any accessible lysine-derived amino group in neighboring molecules. In the presence of excess free putrescine, the formation of intermolecular N^{ϵ}-(γ-glutamyl)lysine cross-links is competitively inhibited. While the collagen matrix deposited by A204 rhabdomyosarcoma cells cannot be solubilized without proteolytic cleavage when cells have been cultured in the presence of 0.2 mM βAPN alone (Figure 1, lane a), the simultaneous presence of 1 mM putrescine and 0.2 mM βAPN during culture (Figure 1, lane b) allows the solubilization of the collagenous components in acetic acid.[3] On the other hand, we did not observe differences in the amount of collagen precursors in the medium from cells cultured either in the absence or presence of putrescine (results not shown), indicating that the newly synthesized collagen molecules are assembled into the matrix also in the absence of cross-linking. When the electrophoretic pattern of the acid-solubilized collagen V/XI obtained from the cell layer of putrescine-treated cultures (Figure 1, lane b) is compared to that of collagen V/XI from the media of untreated cultures (Figure 1, lane c), it can be seen that the amount of collagen migrating in "high" molecular weight bands, corresponding to the pro- and pC chains (Kleman et al., 1992; Niyibizi & Eyre, 1993),[4] is strikingly diminished while the amount in "low" molecular weight bands is increased. Thus, the putrescine-solubilized collagen molecules most likely correspond to the processed forms of collagen V/XI found commonly in the extracellular matrix, e.g., in bone (Moradi-Ameli et al., 1994).

A204 Rhabdomyosarcoma Cells Express Pericellular Transglutaminase Activity. To further support the hypothesis that a transglutaminase is responsible for the insolubility of the collagenous matrix of these cells, we looked for transglutaminase activity in the cultures by studying incorporation of DNS-C, an amine-donor in transglutaminase-catalyzed cross-linking (Aeschlimann et al., 1995). The cell layers

[3] βAPN is a primary amine and can therefore act as an amine-donor in the transglutaminase-catalyzed reaction (Laszlo Lorand, personal communication). The concentration used to inhibit lysyl oxidase, however, is not sufficient to compete efficiently against transglutaminase-catalyzed protein cross-linking.

[4] The term "intact collagen" is used for the entire population of differentially processed collagen molecules extracted from tissues (or cell culture) without proteolytic treatment. From the deduced cDNA-sequences for procollagen V chains it was revealed that the α1(V) chain contains a large N-terminal propeptide domain and in addition a PARP (proline/argenine-rich protein)-like domain while an α2(V) chain contains only a relatively short N-terminal propeptide domain [for review see Prince et al. (1991), Yoshioka & Ramirez (1990), van der Rest & Garrone (1990) and Greenspan et al. (1991)]. Both gene products contain large C-terminal propeptide domains. In cultures of A204 rhabdomyosarcoma cells the pro-α2(V), pC-α2(V), pN-α2(V), and α2(V) forms of this molecule are observed, while the predominant form in tissues, pN-α2(V), lacks processing at the N-terminus (Kleman et al., 1992; Moradi-Ameli et al., 1994). Two major forms of collagen α1(V) with differential processing at the N-terminus are found in tissues. The larger form retains about 20 kDa of the N-propeptide domain while the shorter form is believed to be cleaved in the telopeptide region at $A^{541}-Q^{542}$ (Figure 7), in analogy to collagen I (Moradi-Ameli et al., 1994). The fully processed chains retain short non triple helical peptides at the N- and C-termini, the telopeptides.

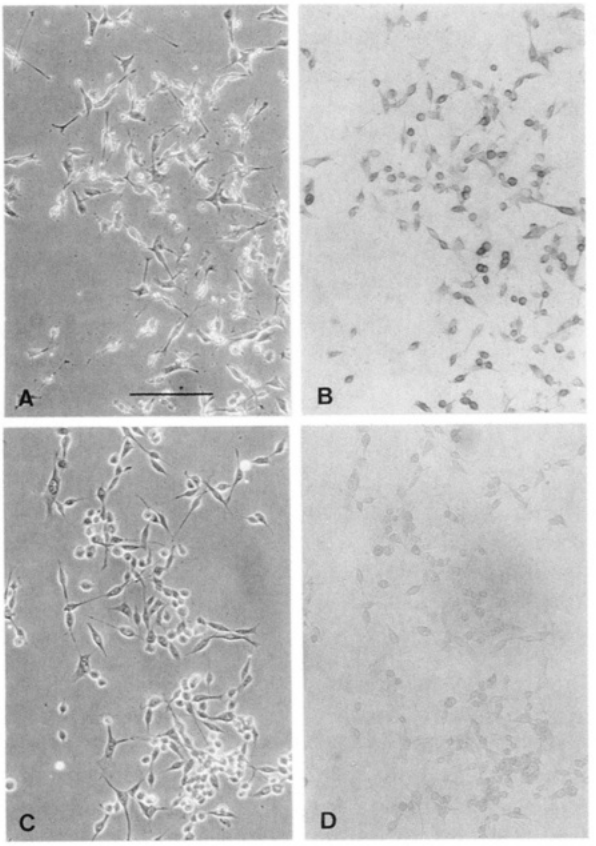

FIGURE 2: A204 rhabdomyosarcoma cells incorporate monodansylcadaverine in culture. Cells were grown for 18 h in the presence of 0.1 mM DNS-C (A and B) or the control derivative, (dansylamido)pentanol (C and D). Covalently incorporated DNS-C (or (dansylamido)pentanol) was revealed by immunoperoxidase staining using an antiserum directed to the dansyl group. The same field is shown in phase contrast (A and C) or in bright field (B and D) microscopy. Bar, 50 μm.

cultured in the presence of 0.1 mM DNS-C showed a cell-bound incorporation of dansyl groups as detected with a specific antiserum (without prior cell permeabilization) (Figure 2, panels A and B). Dansyl groups were absent in control cultures grown in the presence of 0.1 mM (dansylamido)pentanol, a nonreactive substrate analogue (Figure 2, panels C and D). These results demonstrate that a transglutaminase is present and active in the pericellular area of the cells. It further indicates the presence of specific glutaminyl substrate(s) for the transglutaminase in close contact with the cells. In agreement with an earlier study using Chinese hamster ovary cells (Cornwell et al., 1983), DNS-C did not show effects on the cultures other than being incorporated by the cellular transglutaminase when used at low concentration (0.1 mM). At higher concentrations ($\geq$0.5 mM) of DNS-C or (dansylamido)pentanol, cellular adhesion and/or spreading were markedly reduced.

Demonstration of Transglutaminase Activity and Tissue Transglutaminase Protein in Cell Extracts. Transglutaminase activity was determined as incorporation of DNS-C in *N,N′*-dimethylcasein in an enzyme-linked immunosorbent assay according to Slaughter et al. (1992). Activity could be detected only in the supernatant (S1) of the cell lysate which contains largely cytosol-derived proteins (Figure 3). No significant activity was present in the membrane protein fraction (S2). From a comparison to guinea pig tissue transglutaminase, we estimate that the transglutaminase

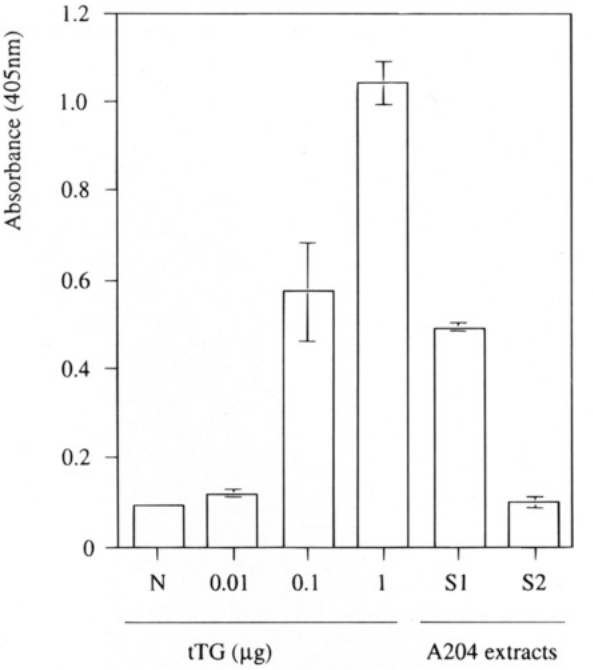

FIGURE 3: Transglutaminase activity in cell extracts. Transglutaminase activity was quantified as the incorporation of DNS-C into *N,N′*-dimethylcasein (coated to microtiter plates). Extract S1 represents a cell lysate, and extract S2 represents detergent-solubilized proteins. The results obtained with the cell extracts were compared to the signal obtained with 0 (lane N) to 1 μg of purified guinea pig tissue transglutaminase (tTG). The values represent the mean $\pm$ standard deviation of two independent determinations.

activity recovered in the extract S1 equals approximately 5 mU/10^6 cells (see footnote 1).

Tissue transglutaminase appeared as a likely candidate for catalyzing collagen cross-linking as it has been shown to be involved in the cross-linking of a variety of extracellular matrix proteins (Aeschlimann & Paulsson, 1991, 1994; Aeschlimann et al., 1993, 1995; Bowness et al., 1987). An antiserum raised against guinea pig tissue transglutaminase (Aeschlimann & Paulsson, 1991, 1994) was used to detect the enzyme in the cell extracts (Figure 4). Standard guinea pig tissue transglutaminase (Figure 4, lane a) was immunolabeled as a major band migrating at the expected molecular mass of ~77 kDa. In extract S1, which was shown to contain transglutaminase activity, a band comigrating with guinea pig tissue transglutaminase at ~77 kDa was detected (Figure 4, lane b) and is likely to be the human homologue. Indeed, preadsorption of the antiserum (100 μL) with guinea pig tissue transglutaminase (150 μg) significantly diminished the labeling of guinea pig tissue transglutaminase and the comigrating band in extract S1 (results not shown).

Incorporation of [^{3}H]Putrescine into Collagens V and XI by Tissue Transglutaminase. Human placenta collagen V and bovine cartilage collagen XI were used to establish whether these molecules are specific glutaminyl substrates for tissue transglutaminase since the purification of intact heterotypic collagen V/XI from A204 rhabdomyosarcoma cells in sufficient amounts did not appear feasible. Time-dependent [^{3}H]putrescine incorporation into collagen V and XI in the presence of guinea pig tissue transglutaminase and Ca^{2+} was determined as described previously (Aeschlimann & Paulsson, 1991) (Figure 5). When the quantity of [^{3}H]-putrescine incorporated into collagens V and XI is compared to the radioactivity in a sample of collagen I, a collagen

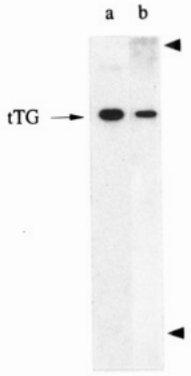

FIGURE 4: Immunoblot of the cell lysate with an antiserum to tissue transglutaminase. Guinea pig tissue transglutaminase (2 μg, lane a) and cell extract S1 (20 μg of total extract, lane b) were electrophoresed side by side on a 12% polyacrylamide gel under nonreducing conditions, electrotransferred to poly(vinylidene difluoride) membrane, and labeled using an antiserum to tissue transglutaminase (lanes a and b). The migration position of the ~77 kDa tissue transglutaminase (tTG) is indicated on the left. Bound antibodies were visualized using the ECL reagent kit and varying exposure times, and the results are shown for an exposure of 10 s (lane a) and 3 min (lane b). Top and bottom of the separating gel are indicated by arrowheads.

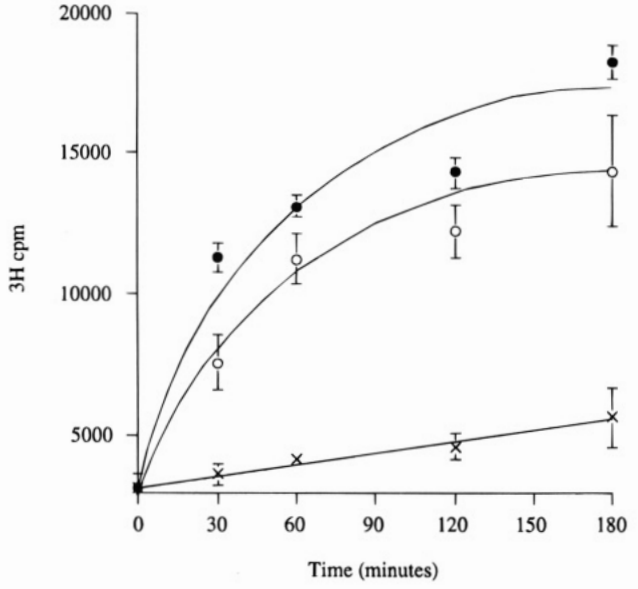

FIGURE 5: Kinetics of tissue transglutaminase-catalyzed [^{3}H]-putrescine incorporation into collagens V and XI. The time course of the reaction was determined using guinea pig tissue transglutaminase, with human placenta collagen V (●) and bovine cartilage collagen XI (○) as substrate proteins. Calf skin collagen I, assayed in the same experiment (×), was not specifically labeled (the small amount of [^{3}H] incorporation is due to the autocatalytic activity of tissue transglutaminase; Aeschlimann & Paulsson, 1991). The values represent three independent determinations and are shown as the mean ± standard deviation.

which is not a substrate for this enzyme (Bowness et al., 1987), it is clear that both collagens V and XI are specific glutaminyl substrates for tissue transglutaminase.

Major Transglutaminase Cross-linking Site is Present in the Non Triple Helical Telopeptides of the Collagen α1(V) chain. Isolated collagen V (Figure 6, lane a) was incubated with tissue transglutaminase in the presence of [^{3}H]putrescine and Ca^{2+}, resolved by SDS−PAGE, and further examined by Coomassie blue staining (Figure 6, lane b) and fluorography (Figure 6, lane c). Covalently attached [^{3}H]putrescine was detected in the α1 chain of collagen V (Figure 6, lane c). However, a substantial amount of the collagen molecules became cross-linked into high molecular mass complexes which were retained in the stacking gel. Tissue transglutaminase was also labeled and detected by fluorography as the enzyme acts autocatalytically on itself (Aeschlimann & Paulsson, 1991).

To further investigate the location of the target glutamine residue(s) for transglutaminase cross-linking in collagen V, we took advantage of the differential sensitivity of the triple helical and terminal non triple helical domains of the collagen to proteolysis (Bruckner & Prockop, 1981). Trypsin digestion of collagen V resulted in a truncated molecule which consists of almost the entire triple helical domain (Figure 6, lane f). Fluorography of the truncated collagen V molecules showed that cleavage of the flanking regions removed the [^{3}H] label in the α1 chain completely, demonstrating that the cross-linking occurs primarily in the N- or C-terminal telopeptide and trypsin-sensitive domains of the α1 chains (Figure 6, lanes d−g).

DISCUSSION

We investigated the possibility that the collagen V/XI fibrils deposited by A204 rhabdomyosarcoma cells in culture and previously shown to be highly insoluble (Kleman et al., 1992), are anchored in the matrix by transglutaminase-mediated cross-linking. We demonstrate that the collagen matrix of these cells can be solubilized without proteolytic treatment when the cells are grown in medium containing putrescine (Figure 1), a competitive inhibitor of transglutaminase-catalyzed cross-linking, suggesting that N^{ϵ}-(γ-glutamyl)-lysine cross-links contribute to the insolubility of this collagen matrix. We further demonstrate that a transglutaminase is present (Figure 4) and active (Figures 2 and 3) in A204 rhabdomyosarcoma cells. This transglutaminase is human tissue transglutaminase on the basis of the immunological similarity and comigration with a guinea pig tissue transglutaminase standard (Ikura et al., 1988) in SDS−PAGE. Moreover, the labeling of the ~77 kDa band in the cell extract with the antiserum to tissue transglutaminase was significantly diminished after preadsorption of the antiserum with guinea pig tissue transglutaminase, demonstrating the specificity of the immunological reaction (results not shown). However, as all known mammalian transglutaminases are homologous and show about 35% amino acid identity (Aeschlimann & Paulsson, 1994), it is still possible that our antibody raised against the guinea pig tissue type enzyme cross-reacts with another transglutaminase, although it has been shown to not recognize guinea pig factor XIII a-subunit (Aeschlimann & Paulsson, 1991). Similarly, an antibody to the human placenta factor XIII a-subunit did not recognize the A204 rhabdomyosarcoma cell transglutaminase (results not shown).

Collagens are usually synthesized as a pro-form. The processing of the fibrillar collagens, as studied for collagen I [for review see van der Rest and Garrone (1990)], is characterized by the cleavage of the N- and C-terminal globular propeptides and is required for proper assembly into fibrils. The processed collagen molecules retain at least short telopeptides at both the N- and the C-terminal ends of the triple helix. Even though putative cleavage sites have been reported within the cDNA-deduced sequences for the N-telopeptides of collagen V and XI chains (Yoshioka &

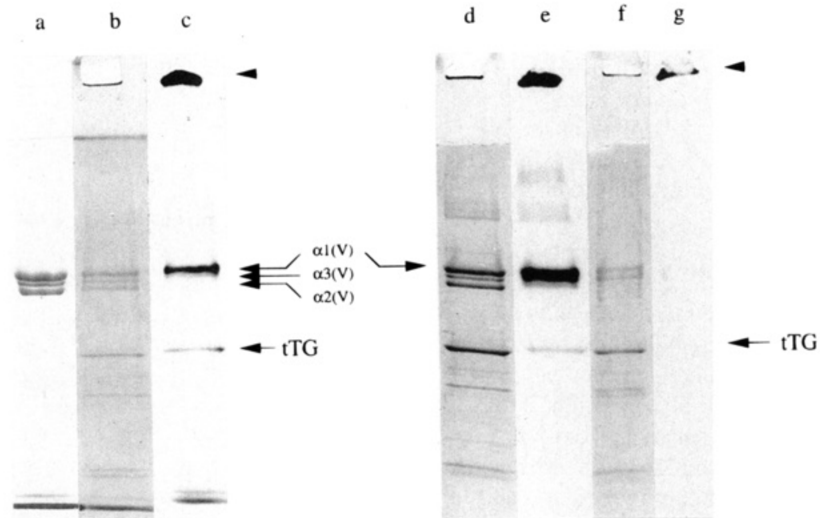

FIGURE 6: SDS−PAGE separation of [^{3}H]putrescine-labeled collagen V before and after trypsin digestion. Tissue transglutaminase-catalyzed incorporation of [^{3}H]putrescine into human placenta collagen V was performed for 3 h at 37 °C in Ca^{2+}-containing buffer. Samples of isolated collagen V before (lane a) and after (lanes b and c) cross-linking were separated on a linear 4%−20% gradient gel under reducing conditions, stained with Coomassie blue (lane b), and then subjected to fluorography (lane c). The migrations of the collagen chains [α1(V), α2(V), α3(V)], the enzyme (tTG), and the high molecular mass reaction products (arrowhead) are indicated on the right. [^{3}H]Putrescine-labeled collagen V (lanes d and e) was digested with trypsin for 5 min at room temperature (lanes f and g), separated by SDS−PAGE as described above, and stained with Coomassie blue (lanes d and f) or detected by fluorography (lanes e and g). After trypsin treatment, no [^{3}H] label is detected in the position of the collagen α1(V) chain. Note: the collagen α3(V) chain reproducibly incorporates a small amount of [^{3}H]putrescine (<10%, as compared to the α1(V) chain), which is not cleaved off by trypsin digestion (lane f). While most of the collagen molecules consist of the entire triple helical domain after trypsin cleavage (lane f), some of the collagen molecules are overdegraded, likely due to the presence of microunfolded regions in the collagen molecules (Morris et al., 1990; Morris & Bächinger, 1987). Heat denaturation of the proteins before performing the trypsin cleavage resulted in a complete degradation of also the triple helical domain of the collagen molecules (results not shown).

N-telopeptides

α1(XI) - - **Q** E A **Q** A **Q** A I L **Q** **Q** A R I A L R *COL1*
α1(V) - - **Q** E S **Q** A **Q** A I L **Q** **Q** A R L A L R *COL1*
α2(V) **Q** M A G L D E K S G L G S **Q** V G L M P *COL1*

C-telopeptides

α1(XI) *COL1* I **Q** P L P I L S S K K T R R H T E G M **Q** A D - - -
α1(V) *COL1* I **Q** P L P I **Q** A S R T R R N I D A S **Q** L L D - - -
α2(V) *COL1* T A A L G D I M G H Y D E S M P D P L P E F T E D

FIGURE 7: Comparison of the N- and C-terminal telopeptide sequences of human α1(V), α1(XI), and α2(V) collagen chains. The sequences of the telopeptides were taken from the complete amino acid sequences reported for the α1(XI) (Yoshioka & Ramirez, 1990; Bernard et al., 1988), α1(V) (Takahara et al., 1991), and α2(V) (Woodbury et al., 1989; Myers et al., 1985) collagen chains and are aligned for a correct staggering of the main triple helical domain (COL1). Glutamines are printed in boldface.

Ramirez, 1990; Dion & Myers, 1987; Bernard et al., 1983; Greenspan et al., 1991), the tissue processing of collagen V and XI molecules remains controversial. Recent results suggest that the N-terminal processing in heteromeric collagen V molecules [α1(V)α2(V)α3(V)] occurs within the globular propeptide extension (Moradi-Ameli et al., 1994; Niyibizi & Eyre, 1993). This manner of processing excludes the classical cleavage at an N-proteinase cleavage site within the N-telopeptide. On the other hand, the homomeric $[\alpha1(V)]_3$ collagen may retain only a short N-telopeptide (Moradi-Ameli et al., 1994). We have previously shown that a part of the heterotypic collagen V/XI molecules of A204 rhabdomyosarcoma cells is fully processed but retain the telopeptide ends (Kleman et al., 1992).

We demonstrate that collagens V and XI are both specific glutaminyl substrates for tissue transglutaminase *in vitro* (Figure 5), further substantiating the indirect evidence for cross-linking of these collagens in the matrix of A204 rhabdomyosarcoma cells in culture (Figure 1). The genes encoding collagens V and XI are highly related, the homology being most pronounced between the two α1 chains (Yoshioka & Ramirez, 1990; Bork, 1992), which show more than 84% amino acid identity within the triple helix and share highly homologous N-propeptide domains and N-telopeptides (Figure 7). Therefore, it appears likely that conserved regions of the α1(V) and the α1(XI) chains contain the transglutaminase cross-linking site(s). Incorporation of [^{3}H]putrescine into collagen V by tissue transglutaminase *in vitro* (Figure 6) showed indeed almost exclusive labeling of the α1 chain. While the α2 chain of collagen V does not appear to be a major glutaminyl substrate for the enzyme (Figure 6), it is retained in the insoluble matrix deposited by the cells (Figure 1, lane a) because it is part of a stable triple helix with the cross-linked collagen α1(XI) chains (Kleman et al., 1992). On the other hand, we cannot exclude that the α2(V) chain does also contribute lysine site(s) to the cross-links.

So far, it has not been possible to derive a consensus sequence around the specific glutamine residues from the numerous transglutaminase substrates characterized. However, the reactive Gln residue(s) are almost always present in a flexible part of the molecule, very often at the N- or C-terminal end [for discussion see Aeschlimann and Paulsson (1994) and Aeschlimann et al. (1992)], like Gln-3 at the N-terminus of fibronectin (Mc Donagh et al., 1981) or Gln-14 in the N-propeptide of collagen III (Bowness et al., 1987). The common lysyl-aldehyde-derived cross-linking of fibrillar collagens (I, II, and III) occurs primarily within the telopeptides (Bailey et al., 1980). The accessibility of these terminal regions together with the fact that, in collagen V and XI α1 chains, they are rich in glutamine residues (Figure 7) makes them likely to be the target for transglutaminase cross-linking. Indirect evidence that the transglutaminase is primarily acting on the telopeptides and/or propeptide domains in collagen V/XI fibrillogenesis is provided by the fact that the highly cross-linked matrix of A204 rhabdomyosarcoma cells can be solubilized by extensive pepsin digestion, *i.e.*, cleavage of the terminal globular regions (Figure 1).

It is important to note that the human placenta collagen V used to demonstrate [^{3}H]putrescine incorporation in the presence of the transglutaminase (Figures 5 and 6) was pepsin solubilized (Niyibizi et al., 1984) and that a portion of the collagen molecules solubilized under these conditions retain the telopeptides (Bruckner & Prockop, 1981). We have verified the results with intact collagen V purified from human bone (Moradi-Ameli et al., 1994) and found that collagen V isolated from this source is also a specific tissue transglutaminase substrate *in vitro*. It incorporates [^{3}H]-putrescine to a similar extent as the pepsinized collagen V, demonstrating that the latter retains the major cross-linking site(s) (results not shown). While the incorporated [^{3}H]-putrescine-label in the collagen α1(V) chain was sensitive to further digestion with trypsin, the triple helix remained stable (Figure 6). This confirms that the major transglutaminase cross-linking site in the α1(V) chain is located within the telopeptide domains, probably in the N-terminal telopeptide as the degree of conservation between α1(V) and α1(XI) is higher in this region (Figure 7).

The detailed organization of the collagen V and XI molecules within the fibrils is still controversial (Linsenmayer et al., 1993). Several lines of evidence indicate that collagens V and XI are assembled in tissues as cores of fibrils that contain mainly collagens I and II, respectively (van der Rest & Bruckner, 1993; Birk et al., 1988). Immunoelectron microscopy on cartilage fibrils supports the view that this collagen type is located in the centre of the fibrils (Mendler et al., 1989). The analysis of peptides bonded by lysine-aldehyde-derived cross-links has shown that collagen XI in cartilage and collagen V in bone are preferentially cross-linked to themselves (Mayne & Brewton, 1993; Niyibizi & Eyre, 1994), suggesting a microfibrillar organization. The heterotypic collagen V/XI matrix of the A204 rhabdomyosarcoma cell line is also of a microfibrillar nature (Kleman et al., 1992). A similar heterotypic collagen composed of α1(XI) and α2(V) chains has recently been isolated from bovine vitreous (Mayne et al., 1993), and a study by Brown et al. (1991) strongly suggests its presence in primary cultures of smooth muscle cells. The α1(XI) collagen chains have also been demonstrated in bone (Niyibizi & Eyre, 1989), suggesting that heterotypic collagen molecules are indeed part of the collagen matrix in tissues. The irreversible cross-linking of collagen V/XI fibrils by tissue transglutaminase observed in cell culture raises the possibility that, in tissues, tissue transglutaminase may play a role in the stabilization of extracellular matrices in the early steps of collagen fibrillogenesis.

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