Detection of Substrates of Keratinocyte Transglutaminase in Vitro and in Vivo Using a Monoclonal Antibody to Dansylcadaverine†

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ABSTRACT: A method providing more sensitive detection of transglutaminase substrates was developed to localize transglutaminase activity in tissue and to identify in vivo substrates in epidermal extracts. The enhanced sensitivity of this method was achieved via the generation of a monoclonal antibody (designated E7) made to dansylcadaverine. Transglutaminase substrates were visualized by western blot after a 1-min incubation with dansylcadaverine in contrast to the 2 h required when [^{14}C]putrescine incorporation was measured by autoradiography of SDS–polyacrylamide gels. In addition, putative substrates not apparent using conventional methods were readily detected by western analysis. An ELISA assay to measure transglutaminase activity showed similar sensitivity to the traditional radiometric assay (Lorand et al., 1972). The correlation between the ELISA procedure and the radiometric assay was high (r^2 = 0.924). Strips of neonatal human and mouse skin incubated in dansylcadaverine-supplemented culture medium were used to localize enzyme activity and to detect substrates in vivo. Transglutaminase activity was demonstrated at the cellular periphery in the upper spinous and granular cell layers of the epidermis. Substrates detected in epidermal extracts were similar to those detected using the in vitro assay. This technique allows for highly sensitive and nonradiometric analysis of both enzymatic activity and the substrates involved. The extension of this methodology to an in vivo system is the first demonstration of a system in which the dynamics of cornified envelope assembly may be further studied.

Transglutaminases are a class of enzymes, widely distributed in a number of eukaryotic cells, which share in common the ability to catalyze an acyl transfer between the γ-carboxyl group of peptide-bound glutamine and an acceptor amine [for reviews see Folk (1980) and Lorand and Conrad (1984)]. The binding site for the glutamine- (Gln-) containing moiety is highly selective, requiring peptide-bound Gln as a substrate. In contrast, the amine acceptor site is less selective, with a variety of short-chain primary aliphatic amines serving as suitable substrates. The resulting isopeptide bond is extremely stable; the occurrence of this linkage in nature is associated with highly cross-linked, lattice-type structures which are highly insoluble. Numerous examples of such structures have been described in a variety of systems (Folk & Finlayson, 1977; Williams-Ashman, 1984; Lorand, 1988; Velasco & Lorand, 1987) including the keratinocyte cornified envelope, which provides strength and resilience to the stratum corneum of epidermis (Rice & Green, 1977, 1978).

The keratinocyte cornified envelope forms in the upper spinous and granular layers of epidermis as a product of terminal differentiation (Sun & Green, 1976; Rice & Green, 1977, 1978; Green, 1977). The cross-links in the cornified envelope are catalyzed by a membrane-associated transglutaminase (Thacher & Rice, 1985). It has thus been axiomatic that a putative cornified envelope precursor must be a transglutaminase substrate. The understanding of the assembly and composition of this structure is important since it represents a unique aspect of cellular differentiation while simultaneously functioning physiologically to provide the insoluble matrix to which the lipid barrier adheres. The isopeptide cross-links give the cornified envelope resistance to harsh external stimuli such as detergents and organic solvents. This aspect of epidermal structure and function is unique among epithelia. The investigation of the composition of the envelope has been hampered because of its highly insoluble nature. Standard protein chemistry and immunological approaches have been only partially successful in elucidating envelope composition. With the exception of immunoelectron microscopy, all evidence implicating cellular proteins as cornified envelope precursors has been of an indirect nature. Further, there is a complete lack of technology allowing for in vivo examination of cornified envelope composition and the kinetics of assembly.

The broad specificity of the transglutaminases for the acyl acceptor group has historically been exploited in designing assays both for enzyme activity and for the detection of putative substrates. Short-chain aliphatic amines with either fluorescent or radioactive labels have traditionally been used. In both cases, radiometric assays have become the standard procedure since they are considered more sensitive than fluorescence assays. These assays are not particularly sensitive, however, especially in the detection of putative substrates. Crude mixtures of proteins must be incubated for periods of 1–2 h at 37 °C before labeling can be detected, which may promote proteolytic activity and/or aggregation of proteins. In recent years, investigators have used alternative approaches to detect both enzyme activity and substrates. A recently described assay for enzyme activity involves the use of a biotinylated amine as the acyl acceptor and subsequent detection of label using an enzyme conjugated to streptavidin (Jeon et al., 1989). Also both polyclonal and monoclonal antibodies to dansylcadaverine have been used to detect enzyme activity by enzyme-linked immunosorbent assay (ELISA) (Velasco et al., 1988) and substrates by western analysis (Lorand et al., 1986, 1988; Velasco & Lorand, 1987).

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Our interest in cornified envelope composition and assembly has led to the development of a method to assess both transglutaminase activity and substrates which is dramatically more sensitive than traditional methods. We extended the technology to an organ culture system in which in vivo substrates and activity can be examined. The latter methodology is the first demonstration of such a system and provides a way in which the dynamics of cornified envelope assembly can be studied.

Materials and Methods

Cross-Linking of Homogenates with Labeled Aliphatic Amines. The skin of Balb/c mice (age 3–5 days, sacrificed by decapitation) was removed and placed on ice. The skin was cut into 5 × 20 mm slices, incubated for 60 s at 60 °C, and plunged into ice-cold water to separate epidermis from dermis. The epidermis was homogenized in 50 mM Tris, 2 mM EDTA, and 2 mM phenylmethanesulfonyl fluoride (PMSF), pH 8.3 (TE buffer), using a Dounce homogenizer (20 strokes). Cultured human keratinocytes (NH-1 cells) were grown as described (Baden et al., 1987a,b). Confluent cultures were rinsed with PBS and homogenized in TE buffer. Dansylcadaverine was added to homogenates of both epidermis and cultured cells to a final concentration of 1.5 mM. The labeling reaction was initiated by the addition of $CaCl_2$ and dithiothreitol (DTT) to final concentrations of 12 and 50 mM, respectively. The reaction mixtures were incubated at 37 °C for the times indicated in the text and figure legends and quenched by EDTA addition (final concentration of 20 mM). The homogenates were centrifuged (15 000 rpm for 15 min), and the clear supernatant was recentrifuged under identical conditions. This supernatant contained the dansylcadaverine cross-linked, cytosolic proteins. Protein concentrations were determined by the Bradford method (Bradford, 1976). To detect substrates in the particulate fraction, the pellets were washed twice with TE buffer and solubilized by heating to 95 °C for 3 min in a buffer containing 62.5 mM Tris, pH 6.8, 20 mM DTT, 2% SDS (w/v), 10% glycerol, and 0.01% bromophenol blue. Any remaining particulates were removed by centrifugation, and the supernatant was used directly for electrophoretic analysis.

In experiments using radiolabeled amines ([^{14}C]cadaverine and [^{14}C]putrescine, NEN, 90.4 mCi/mmol), 2.5 μCi of label in a final concentration of 1.0 mM was used in each 500-μL reaction mix.

Preparation of Antibody E7. A homogenate of human keratinocytes was cross-linked with dansylcadaverine as described above and separated on a preparative 15% polyacrylamide gel (see below). The fluorescent 12-kDa substrate was identified by exposure to long-wave ultraviolet (Kubilus & Baden, 1982). The band was excised and the protein electroeluted (Hunkapiller et al., 1983) using an Isco apparatus. Six-week-old Balb/c mice were injected intraperitoneally with 50 μg of antigen emulsified in Freund's complete adjuvant. Booster injections of 50 μg of antigen in Freund's incomplete adjuvant were administered at 3 and 6 weeks. One month later, the mice were given three additional injections of antigen (as above) every 2 days. The spleens from ether-anesthetized mice were fused with NS-1 mouse myeloma cells by standard methods (Kohler & Milstein, 1975). Clones were screened by ELISA for reactivity with the 12-kDa dansylated substrate. E7 was the product of one such clone. Monoclonal IgG was purified with a protein A MAPS column (Bio-Rad) or by chromatography on a Waters advanced protein purification system using a protein pak DEAE column (Strickler & Gemski, 1987). The source of antibody for immunoassays was either ascites or purified IgG as specified in the figure legends.

Chemical Dansylation. Bovine serum albumin (BSA) was dansylated chemically with dansyl chloride following the procedure of Jacobsen and Jacobsen (1979). This is referred to as dansylated BSA in contrast to proteins which were enzymatically cross-linked with dansylcadaverine.

Electrophoresis and Western Blotting. Electrophoresis in polyacrylamide gels was performed using the discontinuous buffer system and sample solubilization buffer described by Laemmli (1970). Proteins were electrophoretically transferred to poly(vinylidene) difluoride (PDVF) membranes using a semidry transfer system (ISS). For autoradiography, the PDVF membranes were exposed to Kodak X-Omat film. For flourography, membranes were treated with En^{3}Hance (NEN) and exposed to film at −70 °C as described above. All incubations of western blots were done in 5% nonfat dry milk (BLOTTO; Jonson et al., 1984) and probed with E7 as noted in the figure legends. After extensive washing, the membranes were reacted with horseradish peroxidase conjugated goat-anti-mouse IgG (1:3000 dilution; Bio-Rad) and visualized with 4-chloro-1-naphthol (Bio-Rad). All incubations were done at room temperature. To enhance sensitivity, some blots (noted in the figure legends) were developed using an avidin–biotin alkaline phosphatase system (Photoblot, BRL). All procedures were done according to the respective manufacturer's instructions.

ELISA Assays. Microtiter plates (96 wells, Falcon) were coated with 50 μL of test antigen overnight at 4 °C. The plates were blocked for 2 h at room temperature in 0.5% BSA and 0.05% Tween 20 in phosphate-buffered saline (PBS). After aspiration, 100 μL of E7 diluted into PBS containing 0.5% BSA was placed in the wells and incubated at room temperature for 1 h. Wells were washed eight times in buffer (10 mM phosphate, pH 7.2, 0.15 M NaCl, 1.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.05% Tween 20, and 0.02% NaN_3), coated with 100 μL of biotinylated goat-anti-mouse IgG (BRL, 1:1000 dilution in washing buffer), and incubated for 30 min at room temperature. After washing, wells were coated with 100 μL of strepavidin–β-galactosidase (BRL, 1:1000 dilution in washing buffer) and incubated for 30 min at room temperature. The reaction was visualized by incubation with *p*-nitrophenyl β-D-galactopyranoside (BRL) according to the manufacturer's instructions. The reaction was quantitated on a Bio-Rad ELISA plate reader (Model 2550) at 405 nm.

Immunohistochemistry. Blocks of skin frozen in OCT compound (Miles) were cut into 4-μm sections and placed on slides coated with Histostick (Accurate). Slides were stored airtight at −20 °C and used within 2 weeks. For assays, slides were thawed for 20 min at room temperature, fixed in methanol (10 min at −20 °C), rinsed three times (2 min each) in acetone at −20 °C, and air-dried. They were reacted with a 1:10 000 dilution of E7 ascites using an avidin–biotin–horseradish peroxidase detection system (Histostain, HP-Zymed Labs) according to the manufacturer's instructions. Using this detection system, a red color denotes positive immunoreactivity; hematoxylin is used as a counterstain.

Transglutaminase Assays. The ELISA assay using E7 was compared to the traditional assay for transglutaminase activity which monitors the incorporation of [^{3}H]putrescine into dimethylcasein (Lorand et al., 1972). When transglutaminase activity was measured radiometrically, the reaction mixture consisted of the following in a total volume of 100 μL: 15 μL of enzyme (purified guinea pig liver transglutaminase, 0.667 units/mL, or crude cell homogenate as stated in the figure

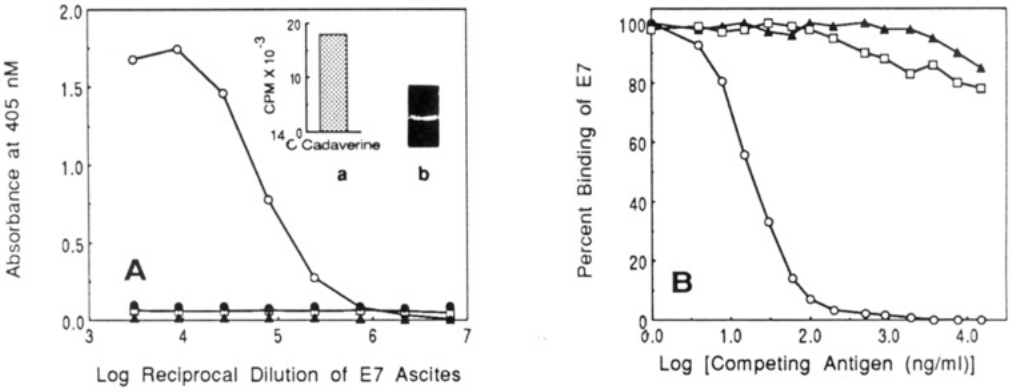

FIGURE 1: ELISA characterization of E7. (A) Titration curves of E7 binding to test antigens: (○) mouse keratinocyte proteins cross-linked with dansylcadaverine, (□) mouse proteins cross-linked with [^{14}C]cadaverine, (●) chemically dansylated BSA, and (▲) mouse keratinocyte proteins. The 96-well microtiter plates coated with 10 μg of test antigens were reacted with serial dilutions of E7 ascites. Binding was determined as described (Materials and Methods). (Inset a) cpm/mL mouse keratinocyte homogenate cross-linked with [^{14}C]cadaverine for 2 h. (Inset b) Long-wave UV photograph of dansylated BSA. (B) Competition by test antigens for binding of E7 to dansylcadaverine cross-linked proteins. Test antigens were incubated for 2 h at room temperature with 1 μg/mL E7 (final concentration); the resulting mixture was used as the primary antibody in a conventional ELISA (see Materials and Methods). Note: only antibody which has not been absorbed by the test antigen is available for binding to the coating antigen. The ELISA plate was coated with dansylcadaverine cross-linked proteins (10 μg); results are expressed as percent reactivity normalized to the mean value of wells with no competing antigen (100%). Competing antigens: (○) dansylcadaverine, (□) cadaverine, and (▲) dansylated BSA.

legends), 2.5 mg/mL *N,N*-dimethylcasein, 1 mM putrescine (1 μCi, 16.7 Ci/mmol), 10 mM $CaCl_2$, and 10 mM DTT in a buffer containing 0.15 M NaCl and 10 mM Tris, pH 7.5 (TBS). The reaction was initiated by the addition of $CaCl_2$, incubated for 30 min at 37 °C, and quenched by trichloroacetic acid (TCA) addition (10%). After chilling on ice for 30 min, the precipitate was filtered over a 0.45-μm glass fiber filter (Millipore), washed extensively with 10% TCA, and counted in a Beckman LS 3801 β-counter.

When transglutaminase activity was measured by ELISA using E7, the reaction mix contained 15 μL of enzyme, 2.5 mg/mL *N,N*–dimethylcasein, 5 μM dansylcadaverine, 10 mM $CaCl_2$, and 5 mM DTT in TBS in a total volume of 100 μL. The reaction was initiated by the addition of $CaCl_2$ and quenched by addition of 80 μL of reaction mixture to 20 μL of 0.1 M EDTA (final concentration 20 mM). This 100-μL solution was used to coat a well on a 96-well microtiter plate and reacted with E7 as described above. Transglutaminase activity was expressed as absorbance units per 100 μg of protein per 30 min. All assays were done in triplicate.

Guinea pig and mouse livers were obtained from animals which were freshly sacrificed for other purposes. Whole organs were washed extensively in ice-cold Earle's balanced salt solution, homogenized in an equal volume of 0.25 M sucrose and 1 mM EDTA, and centrifuged at 65 000*g* for 60 min. The supernatant was used to monitor transglutaminase activity. Confluent cultures of human keratinocytes (NM-1 cells) were scraped from 100-mm dishes, homogenized briefly in TE buffer, and centrifuged for 15 min in a microcentrifuge. The pellet was subjected to trypsin solubilization according to Thacher and Rice (1985) to release the membrane-associated transglutaminase.

Incorporation of Dansylcadaverine by Organ Cultures of Mouse Skin. Balb/c mice (2–4 days old) were sacrificed, and their skin was cut into 5 × 20 mm strips as noted above. Human neonatal foreskins were obtained fresh from circumcisions. The strips were sterilized by incubation in half-strength bleach (30 s) and rinsed extensively in Earle's balanced salt solution. They were then incubated in Dulbecco's MEM plus 10% fetal bovine serum and 1.5 mM dansylcadaverine in a 5% CO_2 incubator (37 °C). Controls were incubated for 24 h in medium without dansylcadaverine. At successive time points, four to six strips were withdrawn, washed extensively in PBS, and processed. A 5 × 5 mm section was frozen at −20 °C in OCT compound for immunohistochemical analysis. The epidermis was separated from the remaining strips (as noted above) and homogenized in TE buffer with the addition of 10 mM EDTA to prevent transglutaminase activity during cell disruption. The particulates were homogenized and solubilized in SDS buffer as noted above.

Incorporation of [^{3}H]glycine was measured by incubation of skin strips in 10 μCi/mL [^{3}H]glycine (47.5 Ci/mmol) for 4 h after either a 1- or 20-h preincubation in tissue culture medium at 37 °C. To control for nonspecific binding to nonviable tissue, specimens were frozen in liquid nitrogen for 2 min and thawed prior to preincubation. Epidermis was separated from dermis (see above) and homogenized in 10% TCA. The sample was applied to a 0.45-μm glass fiber filter and washed extensively with 10% TCA and 1% nonradioactive glycine prior to liquid scinitillation counting.

Results

Definition of the Epitope of E7. ELISA titration and antibody absorption experiments were used to precisely define the epitope of E7. To test for binding to dansyl without the cadaverine portion of the molecule, chemically dansylated BSA was used. Free cadaverine and cadaverine enzymatically cross-linked to protein were used to test for cross-reactivity to cadaverine. Free dansylcadaverine was used to determine whether covalent binding of the molecule to protein was necessary to confer reactivity. Dansylcadaverine cross-linked proteins and unreacted proteins served as positive and negative controls, respectively. Figure 1A shows the titration curves for dansylcadaverine cross-linked proteins, mouse keratinocyte proteins, cadaverine cross-linked proteins, and chemically dansylated BSA. The only reactive species is dansylcadaverine cross-linked proteins. Inset a shows the incorporation of [^{14}C]cadaverine into the sample. This labeling reaction was done under identical conditions and in parallel with the dansylcadaverine cross-linking reaction. The fluorescent band in inset b is evidence of successful chemical dansylation of this sample. To document that a comparable number of dansyl groups were available for binding in this sample, the dansylated BSA and dansylcadaverine cross-linked proteins were compared by western analysis (data not shown). When bands of

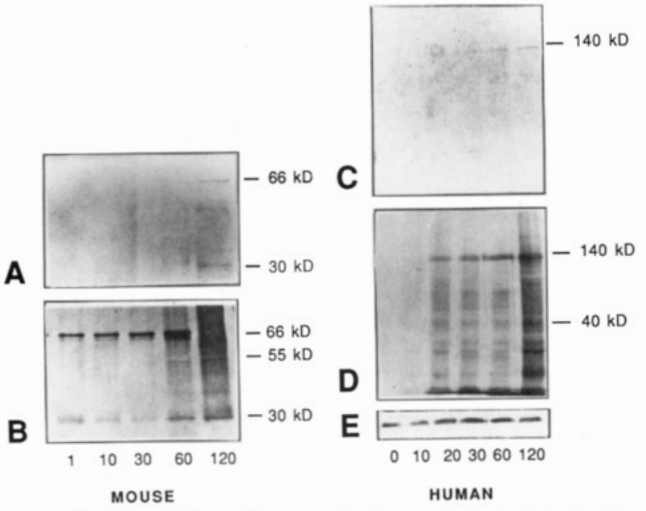

FIGURE 2: Comparison of transglutaminase substrate detection by [^{14}C]putrescine incorporation or by western analysis with E7. Panels A and B represent mouse epidermal homogenates. Panels C–E represent human cultured keratinocyte homogenates. Homogenates were incubated for the indicated times in minutes with either [^{14}C]putrescine (panels A and C) or dansylcadaverine (panels B, D, and E) as described under Materials and Methods. Cytosolic proteins from the quenched reaction mixtures were separated by SDS–PAGE and transferred to PDVF membranes. Panels A and C were developed after a 30-day exposure. Panels B and D were analyzed by western blot using E7 ascites (1:10 000). Panel E shows the samples from panel D reacted with an antibody to involucrin.

equal fluorescence were compared, enzymatically cross-linked dansylcadaverine gave a strong reaction whereas the dansylated BSA was negative.

Small molecules such as dansylcadaverine and cadaverine bind poorly to plastic. To test the potential cross-reactivity of these compounds, antibody absorption studies were used. Dansylated BSA were retested by this method also. Figure 1B shows that as the concentration of dansylcadaverine incubated with E7 increases, binding to the ELISA plate coated with dansylcadaverine cross-linked proteins decreases. Dansylcadaverine was thus able to absorb the reactivity of E7, which was not observed with cadaverine or dansylated BSA. These results demonstrate specific binding of E7 to dansylcadaverine, whether free in solution or cross-linked to protein.

Detection of Substrates by Western Analysis vs Autoradiography. E7 was used to detect transglutaminase substrates in extracts from newborn mouse epidermis and cultured human keratinocytes. When incorporation of dansylcadaverine into cytosolic murine keratinocyte proteins was examined as a function of incubation time, substrates of 66 and 30 kDa were detected within 1 min (Figure 2B). A third substrate of 55 kDa was observed at 60 min while a fourth substrate (42 kDa) was present after 2 h. By comparison, proteins labeled with [^{14}C]putrescine (Figure 2A) identified only two substrates (30 and 66 kDa) after 2 h. These experiments were repeated using human keratinocyte homogenates. The incorporation of label into involucrin, a well-characterized substrate in this system (Watt & Green, 1981; Banks Schlegel & Green, 1981; Rice & Green, 1979), served as control. Twenty minutes after dansylcadaverine addition, involucrin and other low molecular weight substrates were detected by western analysis (Figure 2D). In contrast, involucrin was the only substrate detected after 2 h by autoradiography of [^{14}C]putrescine incorporation (Figure 2C). Figure 2E identifies the 140 kDa protein as involucrin and demonstrates equal protein loading of all lanes.

The assay was further standardized with respect to the antibody dilution and the amount of protein applied per lane. Figure 3A shows that the ability of E7 to detect substrates

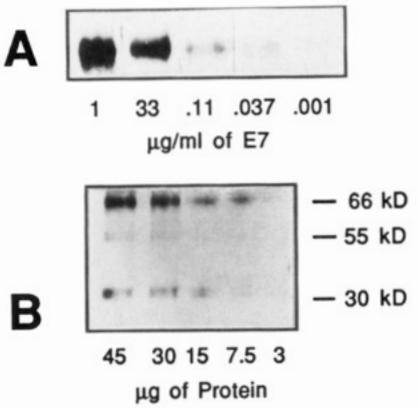

FIGURE 3: Standardization of western analysis. Mouse epidermal homogenates were incubated with dansylcadaverine for 20 min and the cytosolic proteins subjected to western analysis using E7. (A) A total of 20 μg of protein was loaded on all lanes. Strips were reacted with serial dilutions of E7. (B) A 1:10 000 dilution of ascites was reacted with protein loads as shown.

incubated for 20 min with dansylcadaverine decreases between 0.33 and 0.11 μg/mL E7 when 20 μg of protein is loaded per lane. When the amount of protein was varied (Figure 3B), optimal detection was observed with a 30-μg load (gel thickness = 1.5 mm).

Western analysis with E7 identifies major substrates after short incubation times (1–10 min) while simultaneously identifying putative substrates not detected by autoradiography. In addition to lengthy incubation times, the [^{14}C]putrescine incorporation studies require a prolonged film exposure (ca. 30 days in Figure 2).

Quantiation of Transglutaminase Activity: ELISA vs [^{3}H]Putrescine Assay. We compared the standard [^{3}H]putrescine incorporation assay with an ELISA assay which utilized dansylcadaverine and E7 to measure the activity of a commerically available transglutaminase. There is no significant difference between these assays with regard to measurement of enzyme activity; as little as 4 milliunits/mL can be detected with ether method (Figure 4A). These measurements are similar to those previously reported by Velasco et al. (1988) and Jeon et al. (1989). Relative measurements of transglutaminase activity from a variety of crude tissue extracts are also similar (Figure 4B). The correlation between the mean values of 45 assays (r^2 = 0.924) demonstrates the utility of the ELISA assay in measurement of transglutaminase.

In Vivo Substrates of Epidermal Transglutaminase and Histological Localization of Enzyme Activity. An organ culture system was used to investigate the in vivo labeling of keratinocyte substrates of transglutaminase. Slices of newborn human and mouse skin were incubated with or without dansylcadaverine as described under Materials and Methods. Analysis of specimens by both immunohistochemistry and western blot was done at sequential time points. This allowed the localization of particulate transglutaminase activity along with the identification of in vivo substrates.

When enzyme activity was visualized by immunohistochemistry, the addition of 0.5 mM dansylcadaverine resulted in specific labeling of the enzyme with low background (Figure 5, A panels, controls). Four hours after incubation, enzyme activity was noted in the granular keratinocytes of both murine and human neonatal skin. A small amount of E7 reactivity was also noted in stratum corneum and is presumably due to cross-linked proteins which have moved into the stratum corneum. There was also faint labeling of some fibrillar material in the papillary dermis, which is likely due to

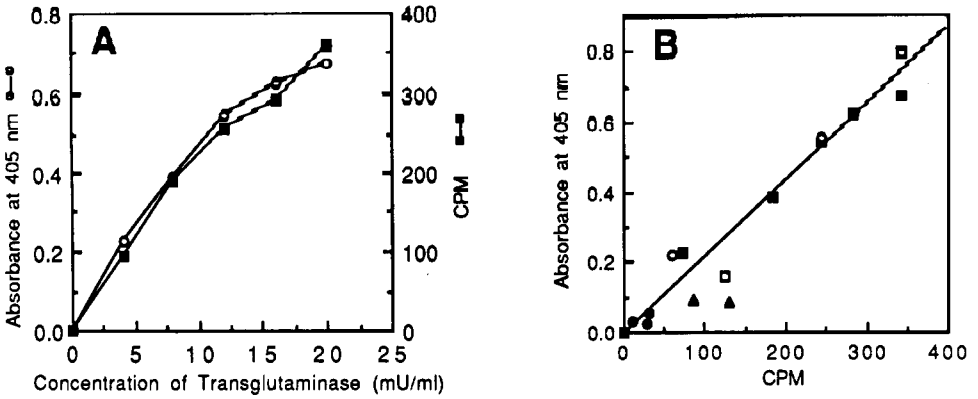

FIGURE 4: Comparison of transglutaminase activity: ELISA vs [^{3}H]putrescine incorporation. Panel A compares the two assays by measuring the activity of varied amounts of purified guinea pig transglutaminase. Data points represent the means of five different assays. Panel B compares the values for the two assays using enzyme from a number of sources: (■) purified guinea pig liver transglutaminase, (□) mouse liver homogenate, (○) guinea pig liver homogenate, (●) human keratinocyte cytosol, and (▲) human keratinocyte particulate. Each data point is the mean of at least three assays.

cross-linking of dansylcadaverine into dermal proteins by tissue transglutaminase(s).

For visualization of in vivo substrates by western blot, pilot studies showed optimal substrate detection with 1.5 mM dansylcadaverine (data not shown). In the cytosolic fraction of human epidermal extracts, a substrate of 140 kDa labeled at 4 h after dansylcadaverine addition; the label intensity of this band increased with time (Figure 5, B panels, bottom). When the same blot was probed with an antibody to involucrin (inset i, Figure 5, B panels), the 140-kDa protein was also visualized, confirming that the substrate was involucrin. Densitometric comparison of staining with E7 and the involucrin antibody showed that approximately 14% of the involucrin in the sample was labeled at 4 h with approximately 87% at 24 h. This type of kinetic analysis with a known transglutaminase substrate demonstrates further the potential use of this system for examining the dynamics of cross-linking in vivo. It also provides evidence of cellular uptake and cross-linking of dansylcadaverine in this system. In the particulate fraction, substrates of 200, 60, and 42 kDa were identified. These substrates are currently being further characterized. The 66-kDa band seen in all human particulate lanes is an electrophoretic artifact.

In murine epidermal extracts, a cytosolic substrate of 55 kDa and a particulate substrate of 45 kDa were visible at 4 h (Figure 5, B panels, top). By 24 h, cytosolic substrates of 66, 55, 42, and 30 kDa were visible, while only one particulate substrate (45 kDa) was detected.

To ascertain possible deleterious effects of dansylcadaverine incubation on cellular metabolism under the conditions described above, [^{3}H]glycine uptake was measured. Strips incubated in tissue culture medium for 1 or 20 h in the presence or absence of 1.5 mM dansylcadaverine showed no difference in the uptake of [^{3}H]glycine during a 4-h pulse (Table I). To control for nonspecific binding of label to dead tissue, strips were freeze/thawed and preincubated for 20 h; under these conditions, there was no detectable uptake of the amino acid. Further evidence of viability was obtained by examination of histologic sections from the control strips of the dansylcadaverine incorporation experiments. As shown in Figure 5 (A panels, controls), these specimens showed no histologic evidence of cell death.

Table I: [^{3}H]Glycine Uptake by Strips of Newborn Mouse Skin after a 4-h Pulse with 10 μCi/mL[a]

	time of preincubation	
	1 h	20 h
+DC[b]	5354[c]	7378
−DC	5600	5851
control		100

[a] Control samples were frozen in liquid nitrogen for 2 min and thawed before preincubation. [b] DC = dansylcadaverine added at 1.5 mM. [c] Units are cpm/cm^2 of epidermal surface area.

DISCUSSION

In this report, we have characterized a mouse monoclonal antibody (E7) and described its utility in measuring transglutaminase activity and labeling of enzyme substrates. We have shown that substrate detection by western analysis using E7 is dramatically more sensitive than autoradiography. We have also coupled the detection of enzyme activity in tissue with the detection of in vivo substrates.

The data in Figure 1 clearly demonstrate that the epitope for E7 is dansylcadaverine. Neither chemically dansylated BSA nor cadaverine reacts with E7 in direct binding or competitive ELISA studies. It appears that the cross-linking of dansylcadaverine to protein is not necessary for binding of E7 to the epitope, as evidenced by the ability of dansylcadaverine to compete with E7 for binding of dansylcadaverine cross-linked proteins (Figure 1B). The poor binding of E7 to dansylated BSA contrasts to the strong binding to dansylcadaverine cross-linked proteins, suggesting that the five-carbon aliphatic linkage between dansyl and the protein may be important for binding.

Examination of labeling of involucrin, a well-characterized transglutaminase substrate from cultured human keratinocytes (Rice & Green, 1979), was used to establish the reliability of the system for in vitro substrate detection. Panels C and D of Figure 2 reproduce published data on transglutaminase substrates in human cultured keratinocytes. Rice and Green (1979) used [^{14}C]putrescine to label the 140-kDa protein (involucrin) as shown in Figure 2C. Kubilus and Baden (1982) noted substrates of 140 and 12 kDa after a 2-h incubation when dansylcadaverine was measured by fluorescence. These correspond in molecular weight to the prominent substrates seen in Figure 2D.

The use of E7 to detect transglutaminase substrates in vitro is advantageous from two perspectives. First, the time required for visualization of labeled proteins is short, circumventing the problems associated with incubation of samples at 37 °C for prolonged times which more traditional techniques cannot address. Second, this technique identifies putative substrates at sequential time points. Figure 2B illustrates labeling of proteins of 66, 30, 55, and 42 kDa at different times, which

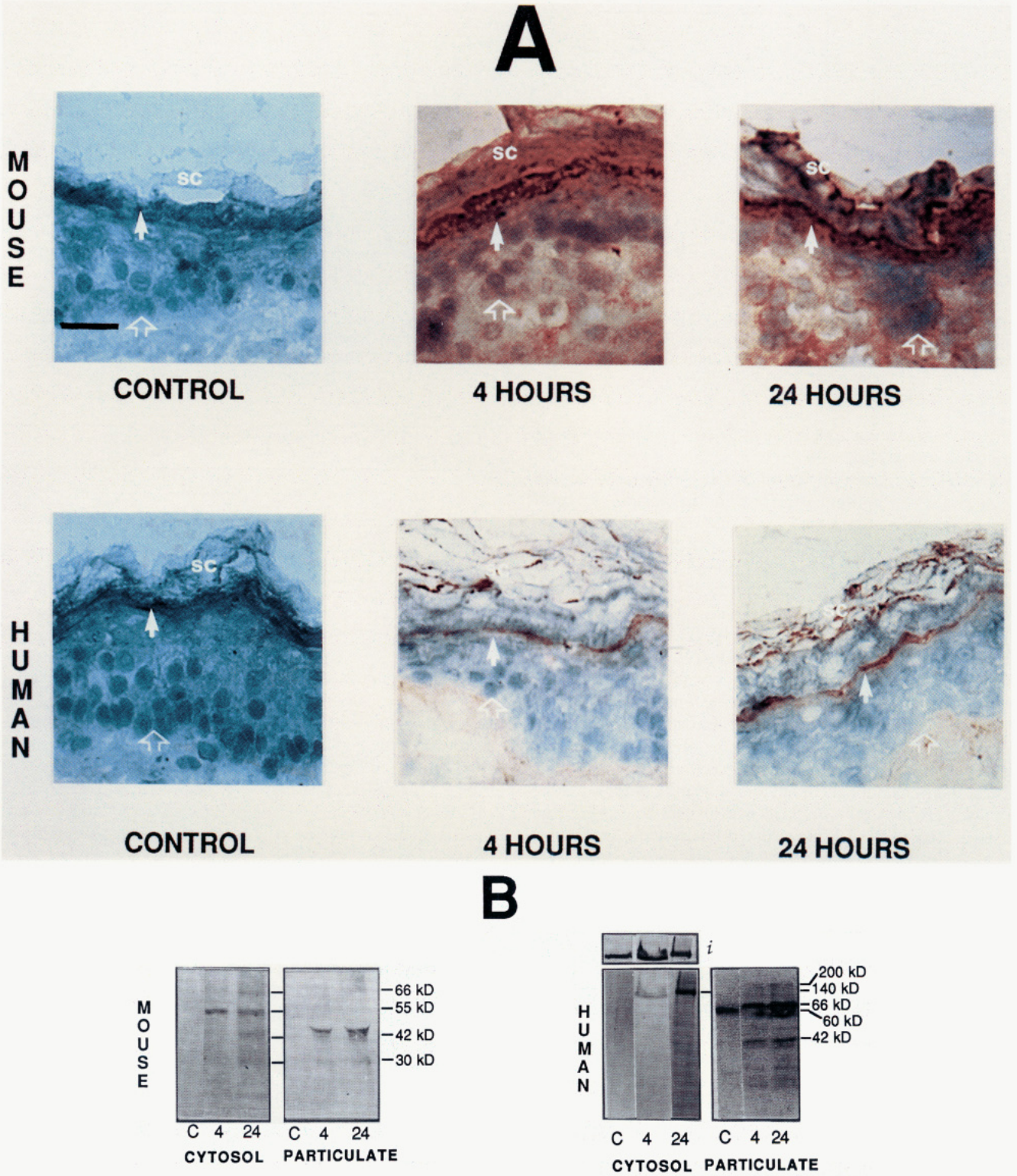

FIGURE 5: In vivo labeling of transglutaminase activity and substrates in organ culture. Slices of neonatal skin were incubated in DMEM plus 10% FBS plus dansylcadaverine at 1.5 or 0.5 mM (see discussion in text). Samples were removed at various time points and analyzed either by immunohistochemistry of the fresh frozen tissue (A panels) or by western analysis (B panels). Control samples were incubated in the absence of dansylcadaverine and stained with hematolylin, the counterstain for the immunoreactive panels. Open arrows mark the dermal–epidermal junction, solid arrows mark the granular cell layer, and sc marks the striatum corneum. Bar = 25 μm. Gel patterns are labeled C for control or by the number of hours of incubation. Inset i above the human cytosolic patterns shows staining of the blot below using the antibody to involucrin. The panel of human particulate samples was developed using the Photoblot (BRL, see Materials and Methods) detection system.

may be the result of differential binding affinities. After long incubation times (2 h), western blots with E7 often show a smear of reactive proteins (e.g., Figure 2B,D, 120 min). One possible interpretation of this result is that high-affinity substrates bind to the enzyme and label early; specific labeled proteins can be detected as early as 1 minute (Figure 2B). As these substrates become quantitatively cross-linked, proteins with less affinity for the enzyme may bind to its active site and label. At some point, the enzyme may begin to label proteins which bind weakly to its active site, resulting in a

smear of reactive proteins after 2 h. Alternatively, proteolysis, natural cross-linking, or an alteration in electrophoretic mobility due to dansylcadaverine binding may account for the smearing observed.

In addition to providing an improved method for examination of transglutaminase substrates in vitro, the high specificity and sensitivity of this antibody also permits detection of cross-linking in vivo (Figure 5). Transglutaminase activity has previously been localized in epidermis (Buxman & Wuepper, 1978; Michel & Demarchez, 1988); however, this paper presents the first system in which this information can be coupled with the detection of in vivo substrates. This ability to simultaneously localize enzyme activity and substrates makes this system unique. Thus, the organ culture system described in these experiments allows for a more definitive examination of the kinetics of cross-linking in renewing cell populations such as epidermis, as well as the content and assembly of the keratinocyte cornified envelope.

The immunohistochemical reactions in Figure 5 provide evidence that dansylcadaverine can be taken up by keratinocytes and cross-linked by transglutaminase. This has also been demonstrated by Michel and Demarchez (1988). Other cell and organ systems have also been used to demonstrate cellular uptake and processing of dansylcadaverine (Julian et al., 1983; Cariello et al., 1984). The mechanism by which dansylcadaverine is transported across cell membranes is not known.

The optimal concentrations of dansylcadaverine used in labeling substrates and enzyme activity in vivo differ. In the latter case, the lowest concentration resulting in detectable labeling was chosen to avoid increased background. In the case of substrate detection, the optimal concentration chosen was one that could effectively inhibit the natural cross-linking activity of the enzyme by saturating amine donor sites with dansylcadaverine. Under these conditions, substrates accumulate over time in the cytosol rather than becoming cross-linked and thereby insoluble (i.e., undetectable by western analysis). The comparison of staining of involucrin by E7 and the involucrin antibody in Figure 5, B panels and inset i, illustrates this point. The labeling of involucrin (87% after 24 h) was kinetic. This is direct evidence of cellular uptake of dansylcadaverine and ongoing cross-linking of this protein to dansylcadaverine during the experiment.

Several lines of evidence suggest the skin strips were not adversely affected by a 24-h incubation in culture medium with or without dansylcadaverine. The histology of the control sections (Figure 5, A panels) shows no evidence of tissue death. The comparison of involucrin staining with E7 and the involucrin antibody (Figure 5, B panels and inset i) demonstrates increased accumulation of labeled substrate at 24 h, suggesting continuous enzyme activity. The uptake of [^{3}H]glycine by cultures suggests that the tissue slices incubated overnight at 37 °C are viable (Table I).

The localization of transglutaminase activity to the granular keratinocytes correlates with data which have shown an identical distribution of the type I enzyme (transglutaminase K) in rodent (Parenteau et al., 1986) and human (Michel & Demarchez, 1988) skin using antibodies directed to the enzyme. This enzyme is responsible for the cross-linking of the cornified envelope (Thacher & Rice, 1985; Simon & Green, 1985).

Labeling of transglutaminase activity with E7 was observed over the entire section of epidermis in all sections studied, suggesting that cornified envelope assembly takes place simultaneously as cells reach the granular layer. The dramatic changes in cell size and shape as well as protein (Dale et al., 1978, 1983; Kubilus et al., 1985) and gene expression (Rothnagel & Steinert, 1990; Mehrel et al., 1990) during the transition between the upper spinous and granular phase of kertainocyte differentiation suggest that this is a highly ordered process. Studies to determine the exact time course of envelope assembly using this model are underway.

The correlation of enzyme activity in the granular cell layer with substrate identification suggests that these substrates are cornified envelope precursors. In the more commonly studied human system, involucrin was the predominant substrate labeled in the cytosolic fraction, compatible with data which have shown this protein to be a precursor of the cornified envelope (Rice & Green, 1979; Simon & Green, 1985).

Unlike the western blots in other figures, the control lane of human particulate samples (Figure 5, B panels) shows a nonspecific band at 66 kDa. This reaction is seen when highly sensitive detection techniques such as the Photoblot system from BRL are used. An electrophoretic artifact has been described (molecular mass 66 kDa) that nonspecifically binds antibody due to the presence of reducing agent in the samples (Bath, 1988). This artifact could be reproduced using this detection method when the primary antibody was left out of the system. Initial analysis of the human particulate samples using the HRP-conjugated goat-anti-mouse IgG revealed immunoreactive bands only when the membrane was viewed under ultraviolet light (Domingo & Marco, 1989). Photographic reproducibility of these data was poor, so a more sensitive detection system was employed. The same bands visible under ultraviolet light are seen in Figure 5 with the addition of the nonspecifically labeled band at 66 kDa.

Increased labeling of a number of substrates in mouse epidermis was observed over time. One explanation for this finding is that dansylcadaverine completely inhibits natural cross-linking at 1.5 mM. Under these conditions, substrates which would normally become part of large insoluble polymers would remain soluble, allowing for detection by western analysis. Other explanations include proteolytic processing and cross-linking of fewer substrates.

In our comparison of in vivo and in vitro substrates, we see proteins of identical mobility labeling in either assay. This is particularly evident in the case of mouse cytosolic proteins (Figures 2 and 5). These data confirm the utility of the in vitro assay for identifying substrates. The earliest substrate seen in vitro is the 66-kDa substrate while in the in vivo assay, the 55-kDa protein is labeled first. This observation suggests that there may be different enzyme–substrate relationships in the two situations.

The enhanced sensitivity in identification of transglutaminase substrates with this technology has led to several areas of investigation which are in progress. This technology will be used to study the process of cornified envelope assembly which has remained a mystery in large part due to the lack of sensitivity of present assays.

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