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# Identification of Cytoplasmic Actin as an Abundant Glutaminyl Substrate for Tissue Transglutaminase in HL-60 and U937 Cells Undergoing Apoptosis*

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**A lysine derivative, 3-[$N^{\alpha}$[$N^{\epsilon}$-[2′,4′-dinitrophenyl]-amino-*n*-hexanoyl-L-lysylamido]-propane-1-ol, a novel amine substrate of transglutaminases, was synthesized and delivered into intact HL-60 and U937 human leukemia cells to probe the function of the intracellular enzyme. The novel substrate compound was covalently incorporated into intracellular proteins in these cells expressing high levels of tissue transglutaminase and undergoing apoptosis following the induction of their differentiation with dimethyl sulfoxide and retinoic acid. Immunoaffinity purification and microsequencing of labeled proteins identified cytoplasmic actin as the main endogenous glutaminyl substrate in these cells. As shown by confocal image analysis, cells revealed distinct labeling of the microfilament meshwork structures by the novel compound as the result of the intracellular action of transglutaminase.**

Transglutaminases (TGases)[1] are $Ca^{2+}$-dependent enzymes catalyzing an acyl transfer reaction in which new γ-amide bonds are formed between a γ-carboxamide group of peptide-bound glutamine residues and various primary amines (1, 2). Under physiological conditions the amine substrates are either naturally occurring polyamines or ϵ-amino groups of peptide-bound lysyl residues. In the latter case the reaction results in the formation of γ-glutamyl-ϵ-aminolysine cross-links between proteins. The number of reactive glutaminyl substrates is highly restricted, whereas the specificity for amine substrates is modest (3, 4), although peptide-bound lysyl residues are better substrates than other diamine derivatives (1). Several members of the TGase family have been identified (2), and the specificity using different glutaminyl substrates varies depending on the type of transglutaminase.

The ubiquitous tissue transglutaminase is a monomeric globular protein, which has been implicated in a wide variety of functions resulting in intra- and/or extracellular structural alterations (2). These include modeling of the extracellular matrix (5), stimulus-secretion coupling (6), receptor-mediated endocytosis (7), cell differentiation (8), tumor growth (9), and programmed cell death (10–12). However, specific modification of defined intracellular substrate proteins by tissue transglutaminase has not yet been linked to the suspected intracellular functions mainly because of the difficulties in specific labeling of such proteins without compromising cellular integrity. Attempts have been made in the past to distinguish putative and real intracellular substrates of TGases (13–15); these trials, however, were restricted by the poor cellular uptake of the amines used. The approach presented here is based on a novel derivative of L-lysine linked with a dinitrophenol haptenic group through the α-amino group of an aminohexanoic acid spacer and bearing a hydrophilic head on its α-carboxyl group. It was found that this compound was utilized by intracellular TGase for modification of its glutaminyl substrates without any obvious violation of cellular integrity.

Several lines of evidence, including isolation of highly polymerized protein products from apoptotic cells (12, 16) and immunohistological studies (17, 18), have established tissue TGase as a useful biochemical marker of programmed cell death without elucidating what its exact role in the death process is. It is still debated whether the enzyme is involved in execution of the death program as part of the killing mechanism or whether, alternatively, it modulates the course of apoptosis by temporary stabilization of dying cells (19). Although the complexity of biochemical processes during programmed cell death can only be clarified by investigating living tissue, carefully chosen *in vitro* cell systems can also deliver relevant contributions to our understanding of the function of tissue TGase in the course of apoptosis. Leukemia-derived human cell lines, such as the promyelocytic HL-60 and the histiocytic U937, can be induced to differentiate along the granulocytic or monocytic pathways by several natural and synthetic inducers, and the differentiated cells undergo apoptosis. $Me_2SO$ and retinoic acid induce HL-60 to differentiate mostly to promyelocytes (20, 21), whereas U937 cells are maturated to monocyte/macrophage-like phenotypes. Both type of cells express high levels of transglutaminase when undergo apoptosis following differentiation (22–24). The maturated, macrophage-like phenotypes adhere to glass and contain much more nonspecific esterase activity as compared with granulocytic forms (25, 26). We examined the glutaminyl substrate proteins of tissue TGase in apoptotic cell cultures by analyzing the incorporation of the novel haptenized derivative of lysine into proteins. We show that cytoplasmic actin serves as a main substrate for cross-linking and that the incorporation of the label is structurally associated with the actin cytoskeleton.

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[1] The abbreviations used are: TGase, transglutaminase; DALP, 3-[$N^{\alpha}$[$N^{\epsilon}$-[2′,4′-dinitrophenyl]-amino-*n*-hexanoyl-L-lysylamido]-propane-1-ol; RA, retinoic acid; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; CAPS, 3-(cyclohexylamino)propanesulfonic acid; TBS-Tx, Tris-buffered saline with Triton X-100; TRITC, tetramethylrhodamine β-isothiocyanate.

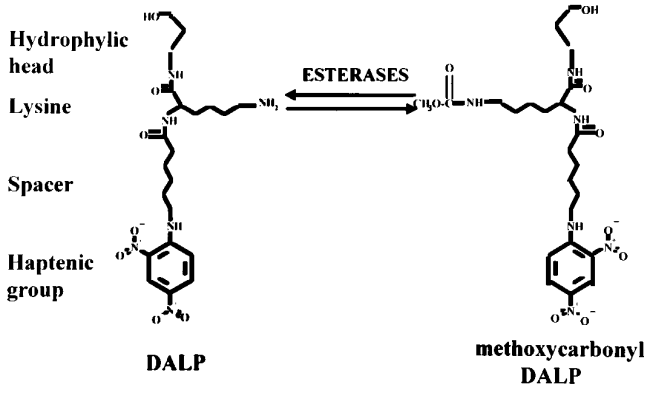


FIG. 1. **The structure of DALP, a novel transglutaminase substrate, and its membrane-permeable methyl carbamate derivative.**

## EXPERIMENTAL PROCEDURES

*Synthesis of DALP and DALP-Methyl Carbamate*—All applied reagents were obtained from Sigma, unless otherwise indicated. Platinated charcoal and dimethylformamide was obtained from Fluka AG, Buchs, Switzerland. Applied solvents were of analytical grade and were from Reanal Rt., Budapest, Hungary. The synthesis was started by the conversion of 5 g of $N^{\alpha}$-carbobenzoxy-$N^{\epsilon}$-(tertiary-) butyloxycarbonyl-L-lysine-*p*-nitrophenyl ester to 3-[$N^{\alpha}$-carbobenzoxy-$N^{\epsilon}$-(tertiary)-butyloxycarbonyl-L-lysylamido]-propane-1-ol by dissolving it in 20 ml of dimethylformamide, then adding 1 ml of 3-amino-1-propanol and stirring overnight at room temperature. Solvent was removed in vacuum, and residue was taken up in $ChCl_3$. This solution was washed with 0.01 M HCl, water, and 0.1 M $Na_2CO_3$, then crystallized from petroleum ether. The previous product (2.7 g) was dissolved in 15 ml of methanol containing 4.4% formic acid and stirred with 0.15 g of platinated charcoal for 4 h at room temperature. Catalyst was filtered, and the solution was concentrated in vacuum, taken up in 50% methanol, and passed through a 1 × 5-cm Dowex 1 column in OH form. The resulting amorphous material (3-{$N^{\epsilon}$-(tertiary)-butyloxycarbonyl-L-lysylamido]-propane-1-ol, 1.8 g) was dissolved in 12 ml of dimethylformamide, dried over $P_2O_5$, and coupled with 3 g of *N*-2,4-dinitrophenyl-ϵ-amino-*n*-caproic acid by adding 0.7 g of 1-hydroxybenzotriazole and 1.1 g of dicyclohexylcarbodiimide to the solution and stirring overnight at room temperature. After addition of 1 ml of water, the dicyclohexylurea was filtered off and the mixture was concentrated in vacuum, taken up in ethyl acetate, and washed as above. After removal of the solvent in vacuum, the residue was treated with 100% trifluoroacetic acid for 30 min on ice. Excess acid was evaporated in vacuum, and the final product was purified on an Isco preparative C18 HPLC column (Isco Inc., Lincoln, NE) using an isocratic mobile phase of 25% acetonitrile and 0.1% trifluoroacetic acid. The final yield was 1.55 g of 3-[$N^{\alpha}$-[$N^{\epsilon}$-[2′,4′-dinitrophenyl]-amino-*n*-hexanoyl]-L-lysylamido]-propane-1-ol (DALP, structure shown in Fig. 1) in trifluoroacetic acid salt form (26% of theoretical). The structure of the final product was verified by proton and $^{13}C$ NMR as well as by mass spectroscopy. The methyl carbamate form of DALP was always prepared freshly by adding 5 μl of methyl chloroformate (Aldrich) to 25 mg of DALP dissolved in 0.25 ml of triethylamine (100 mM) and by subsequent lyophilization.

*Verification of Transglutaminase Substrate Properties of DALP*—Dimethylated casein was incubated with DALP in the presence of partially purified guinea pig liver tissue transglutaminase (Sigma) and $Ca^{2+}$ for 60 min, after which unbound DALP was removed by gel filtration. Protein was exhaustively digested by proteases (27) and analyzed by reverse phase HPLC chromatography on a NovaPack 3.9 × 150-mm C18 column (Waters, Division of Millipore, Milford, MA) by using a 1 ml/min isocratic elution with 25% acetonitrile in 0.14 M sodium acetate, pH 6.35. Absorbance was detected at 365 nm. The distinct peak eluted at 22 min was identified as γ-glutamyl-DALP by its digestibility with γ-glutamyl-amine cyclotransferase (prepared from rabbit kidney according to Fink and Folk; Ref. 28), which resulted in the release of DALP (eluted at 33 min under the same conditions). The same approach was used to prove that the incorporation of DALP into proteins of intact cells was TGase-dependent; in this case the labeled proteins were eluted from SDS-polyacrylamide gel and exhaustively digested by proteases, and then the digest was analyzed by HPLC. A $K_m$ of 3 μM was determined for the incorporation of DALP into dimethylcasein at 37 °C and pH 8.5 by using a plated enzyme-linked immunosorbent assay (29) in which the originally used 5-(biotinamido)pentylamine was replaced by varying concentrations of DALP. The DALP-methyl carbamate did not exert significant inhibitory properties on this reaction at its maximal solubility concentration in water (32 μM at 37 °C).

*Induction of Differentiation and Labeling of Cells*—Mycoplasma free HL60 and U937 cell lines (ECACC, Wilts, United Kingdom (UK)) were grown in RPMI 1640 medium containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C with air/$CO_2$ (19:1). Both cell lines were seeded at 5 × $10^5$ cells/ml in RPMI 1640 with 10% fetal bovine serum, 1.25% $Me_2SO$ (Hybrimax quality, Sigma), 1 μM all-*trans*-RA, and 25 μM DALP-methyl carbamate carrying it out in darkness to prevent photoisomerization of RA. Cell viability was assessed by a negative reaction with the "*in situ* cell death detection kit (AP)" (Boehringer Manheim). Immunofluorescent staining for confocal microscopy was carried out either on cytocentrifuge preparations or glass microscopic slides placed into the culture vessels. Thin layer chromatography was used to detect the formation of DALP ($R_F$ 0.6) from its methyl carbamate ($R_F$ 0.85) after 48 h in $Me_2SO$- and RA-treated cells using cell lysates and Kieselgel 60 silica plates (Merck, Darmstadt, Germany) in ethyl acetate: pyridine:acetic acid:water (40:20:6:11) after precipitation of proteins with 5% trichloroacetic acid.

*Measurement of Transglutaminase Activity*—Transglutaminase activity in cell lysates was assayed by determining the incorporation of [$^3$H]putrescine (Amersham Corp., Bucks, UK) into dimethylcasein as described previously (30). Results shown represent the mean of five independent determinations.

*Identification of Proteins by Western Blot Analysis*—Samples were diluted with an equal volume of 2 × Laemmli sample buffer and boiled for 5 min, then subjected to PAGE in the presence of SDS using 10% slab gels and 4% stacking gel (31). Proteins were reduced with 100 mM DTT, alkylated with 2% iodoacetamide, and were detected by staining with Coomassie Brillant Blue R or after transfer onto Immobilon P (Millipore Corp., Bedford, MA). Binding of primary rabbit anti-dinitrophenyl antibodies was visualized by alkaline phosphatase coupled swine anti-rabbit antiserum (Dakopatts, Glostrup, Denmark) or, alternatively, with sheep anti-rabbit antibodies coupled to peroxidase (Dakopatts). Monoclonal anti-tissue TGase antibody (P. J. Birckbichler, Oklahoma Medical Research Foundation, Oklahoma City, OK) was detected with rabbit anti-mouse immunoglobulins coupled to alkaline phosphatase. Monoclonal anti-actin antibodies (clone AC-40 and AC-15, Sigma) were reacted with peroxidase-conjugated sheep anti-mouse IgG (Sigma). Alkaline phosphatase-coupled antibodies were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as chromogenic agents; peroxidase was detected by chemiluminescence (Supersignal, Pierce). Stripping of antibodies from membranes for reprobing was done by incubating them in 2% SDS, 1% 2-mercaptoethanol for 1 h at 55 °C.

*Isolation of DALP-labeled Proteins from Cells*—U937 cells treated for 72 h as above were collected and washed twice with centrifugation and resuspending in cold buffer A (15 mM phosphate sodium, 1 mM EDTA, pH 7.5, 140 mM NaCl) and then lysed by boiling in 2% SDS, 100 mM DTT, 5 mM EDTA, 50 mM Tris, pH 8.0. Sulfhydryl groups were alkylated by incubation with 0.1 M iodoacetamide for 30 min at room temperature. This protein solution was precipitated with cold acetone, treated with 100% trifluoroacetic acid for 30 min on ice to hydrolyze carbamate esters, lyophilized, and redissolved in 2% SDS, 125 mM Tris-Cl, pH 6.8, and 0.01% bromphenol blue. Then, the sample was applied on top of a 37 × 150-mm Bio-Rad PrepCell 491 preparative SDS-PAGE column filled with 3% gel. Column eluate harboring eluted proteins in 192 mM glycine, 25 mM Tris-HCl, pH 6.8, and 0.1% SDS was led through a 0.5 × 5-cm column filled with rabbit anti-dinitrophenyl immunoglobulins coupled hydrazide-linked beads. The latter was prepared by Affi-Gel Hz immunoaffinity kit (Bio-Rad) according to the protocol described by the supplier. In control experiments it was determined that neither DALP nor DALP-labeled proteins bind to either uncoupled beads or those coupled with non-specific rabbit antibody, and unlabeled cell homogenates were not immunoreactive with anti-dinitrophenyl antibody under the conditions described. Sample electrophoresis and eluate affinity purification was carried out for 24 h, which duration was determined to be more than double the passage time of thyroglobulin (mass of 336 kDa). Affinity-bound proteins were released from the beads by boiling in 2% SDS, 125 mM Tris, pH 6.8. This solution was subjected to SDS-PAGE and then stained with Coomassie Brilliant Blue R. In some experiments, the major lower bands running at 45 kDa were collected and electro-eluted in 10 mM CAPS, pH 11, 0.1% SDS.

*Separation and Sequencing of Peptides from Isolated Proteins*—Electro-eluted proteins were precipitated with cold acetone and fragmented

by an equal weight of cyanogen bromide (Aldrich) in 70% formic acid overnight at room temperature. Fragments were isolated on a Nova-Pack C18 (Waters) reverse phase column using a linear gradient from 0 to 40% acetonitrile with 0.1% trifluoroacetic acid (solvent B) *versus* 0.1% trifluoroacetic acid in water (solvent A). Absorbance was detected at 206 nm. Peptides giving major and solitary peaks were analyzed by a Knauer 910 protein microsequencer (Herbert Knauer GmbH, Berlin, Germany). Sequences were searched against the EMBO SwissProt data base.

*Immunofluorescent Staining and Confocal Image Microscopy*—Cells adhered or centrifuged to microscopic slides were fixed with St. Marie's fixative (2% acetic acid in ethanol) at −20 °C for 30 min and air-dried. Nonspecific binding of antibodies was blocked with 5% nonfat milk powder, 2% bovine serum albumin, 0.5% sheep serum at 37 °C in Tris-buffered saline containing 0.1% Triton X-100 (TBS-Tx). After washing in TBS-Tx actin microfilaments were visualized with phalloidin-TRITC (Sigma Chemical Co.) at 0.25 $\mu$g/ml concentration in the presence of 1% bovine serum albumin in TBS-Tx. DALP was reacted with rabbit serum containing anti-dinitrophenol antibodies and, consecutively, with biotinylated donkey anti-rabbit immunoglobulins (Amersham), then with streptavidin-fluorescein isothiocyanate (Boehringer Mannheim, Penzberg, Germany) at 10 $\mu$g/ml, all in TBS-Tx. Tissue TGase was detected using the above described monoclonal antibody and applying goat anti-mouse IgG-TRITC (Sigma) in the presence of TBS-Tx and 1% bovine serum albumin. Immunofluorescent stainings were analyzed using a confocal laser scanning invert microscope LSM 410 (Carl Zeiss, Oberkochen, Germany) equipped with external argon ion (488/514 nm) and internal helium-neon (543 nm) lasers. Plan Neoflutar ×100 objective was used. To visualize fluorescein isothiocyanate labeling, cells were exposed to laser excitation at 488 nm and emission was detected through a filter BP 510–525 nm. TRITC was excited at 543 nm and emission was detected through an LP 570 filter. The laser beam was attenuated to 10% with a neutral filter to reduce the bleaching of fluorescence. The image acquisition was performed under the following conditions. (*a*) The pinhole unit in front of the detector was opened and fitted to Airy Unit 1, (*b*) the average of four images was recorded in 1 s and 512 × 512 pixels, (*c*) the optical section thickness (*z* step) was approximately 0.5 $\mu$m, and (*d*) the scanning zoom was varied between 1.5 and 3.5. Images were digitized and stored on floppy disks.

## RESULTS

*Induction of Tissue Transglutaminase and Apoptosis in HL-60 and U937 Cells by $Me_2SO$ and All-trans-retinoic Acid*—Synergistic effect of $Me_2SO$ and RA caused growth arrest and massive cell death of both U937 and HL-60 cells. Features of cellular morphology were consistent with the apoptotic process, such as blebbing of cytoplasmic margins, nuclear shrinkage, or karyorrhexis, in agreement with previous findings (23, 24). Decrease of cell survival was maximal between 60 and 84 h of treatment (Fig. 2). Remnants of dead cells were found to undergo autolysis and swelling on prolonged incubation; thus, the time of treatment was limited to 72 h. TGase activity of cell homogenates surged from background values up to 7.4 nmol/min/mg for HL60 and 15.6 nmol/min/mg mean activity values for U937 cells by 48 h of induction and declined afterward in correlation with decreasing cell viability (Fig. 2). Induction of tissue TGase was demonstrated by immunoblotting (Fig. 2), which revealed undetectable low levels of the antigen in untreated cultures. Cultures treated with RA alone did not show expression of the enzyme, either, in agreement with previous findings (21). Detergent-insoluble, cross-linked apoptotic bodies could also be isolated, suggesting that tissue TGase is activated and polymerizes proteins in the dying cells (2, 19, 32).

*Synthesis of DALP, a Novel Transglutaminase Substrate, and Its Incorporation into Cellular Proteins*—In an attempt to identify intracellular glutaminyl substrates of TGase, a novel hapten-labeled amine substrate was designed. The compound DALP (Fig. 1) was synthesized with an acceptable 26% yield in a four-step reaction procedure. Substrate properties of DALP have been verified by incorporating it into dimethylated casein by tissue TGase and chromatographic identification of γ-glutamyl-DALP. The determined $K_m$ value of DALP (3 $\mu$M) is about 50 times lower than the best amine substrate of TGase found to date: (5-biotinamido)pentylamine (29). DALP concentrations below 1 mM were found to be non-toxic to HL-60 and U937 cells. As it is the case with other amine substrates like monodansylcadaverine (33) or biotin-cadaverine (25), the DALP itself did not show applicable uptake by HL-60 or U937 cells. However, its carbamate methyl ester derivative was found to penetrate both types of cells. This derivative, which was not toxic in the applied concentration to the examined cells

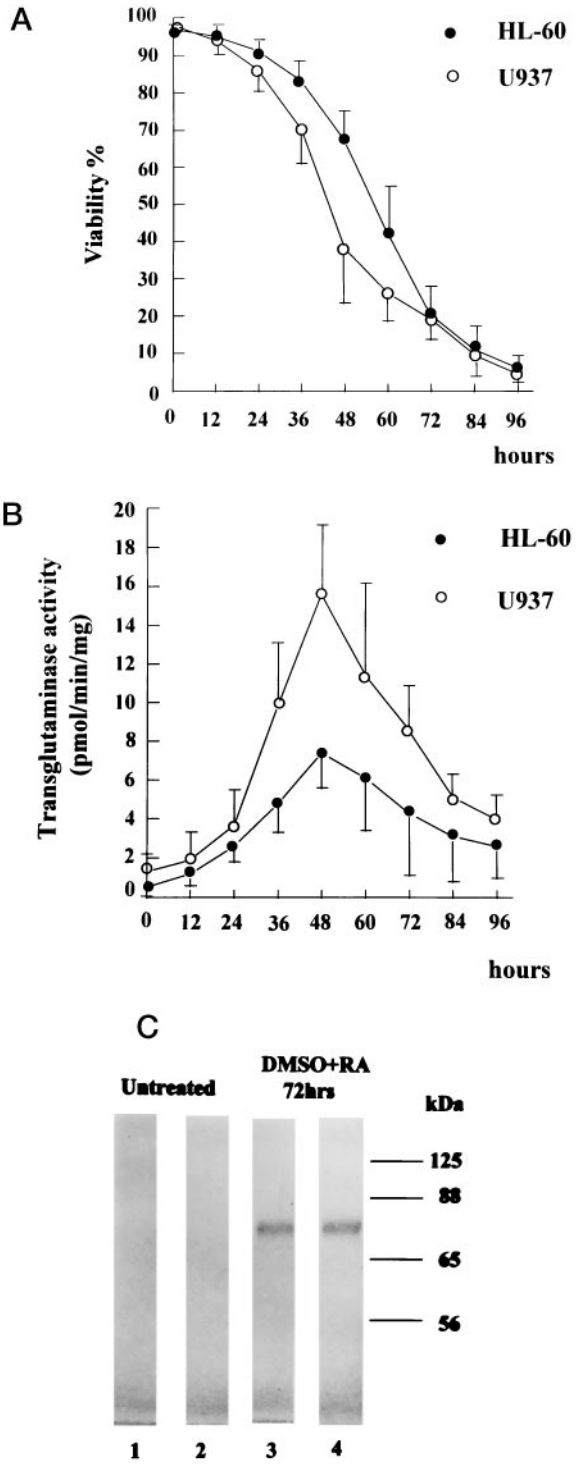


FIG. 2. **Induction of apoptosis (*A*) and tissue transglutaminase (*B*) in HL-60 (*1*) and U937 cells (*2*) following the combined treatment of $Me_2SO$ and all-*trans*-retinoic acid.** Immunoblot analysis (*C*) of tissue TGase shows the presence of the enzyme protein 72 h after the addition of $Me_2SO$ and RA to the culture.

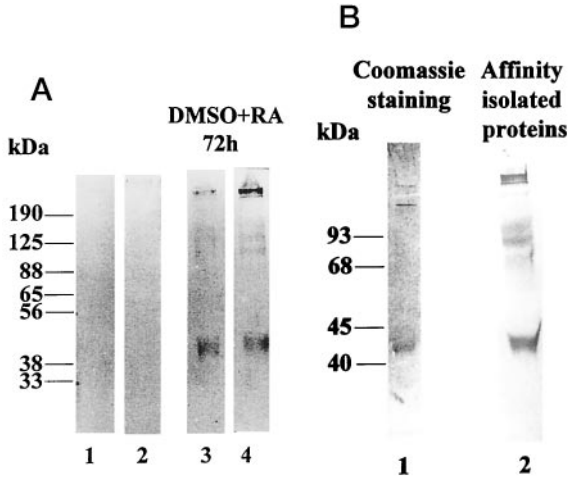


FIG. 3. **Detection of DALP-labeled proteins in cell lysates.** *A*, Western blot analysis of DALP-labeled proteins from HL-60 (*1* and *3*) and U937 (*2* and *4*) cells to detect the dinitrophenol haptenic group. DALP-labeled proteins could not be found in cells not treated with $Me_2SO$ and RA (*1* and *2*). *B*, analysis of DALP-labeled proteins by PAGE and Coomassie staining (*1*) and by Western blotting using anti-dinitrophenol antibody (*2*) from U937 cells following their purification by affinity chromatography utilizing anti-dinitrophenol antibodies.

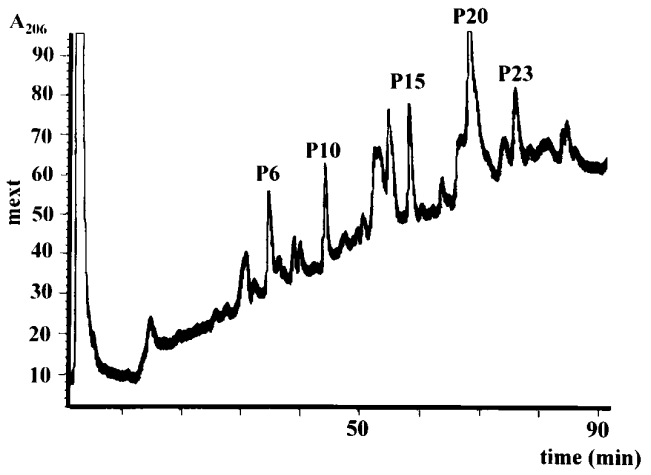


FIG. 4. **Separation of CNBr peptides from the 43-kDa DALP-labeled and affinity-purified protein from U937 cells by reverse phase HPLC.**

and did not influence either their differentiation or the rate of apoptosis, became hydrolyzed inside cells, as demonstrated by thin layer chromatography of non-protein fraction of cells cultured for 48 h in the presence of $Me_2SO$ and RA (data not shown). In cells incubated in the presence of DALP-methyl ester and treated with $Me_2SO$ and RA, the incorporation of DALP into intracellular proteins was detectable after 72 h with immunoblot analysis using anti-dinitrophenyl antibodies (Fig. 3). The covalent incorporation of DALP into cellular proteins was a specific result of cellular Tgase activity as the formation of DALP derivatives have not been observed unless tissue TGase and apoptosis were induced by the combined treatment of $Me_2SO$ and retinoic acid and the incorporated DALP could be recovered as γ-glutamyl-DALP from proteolytic digests of cellular proteins (data not shown). Intracellular DALP labeling of living cells revealed transglutamination of very few cellular proteins contrasted to nonspecific transglutaminase-mediated labeling of cell lysates resulting in a continuous spread of labeled proteins on Western blots (data not shown).

The most intensively labeled protein in apoptotic cells had a molecular mass of 43 kDa. Further bands were seen at the approximate position of 110 and 150 kDa. In addition, high molecular mass materials retarded at the top of the resolving gel (>250 kDa), as well as the stacking gel (>1 MDa) have been observed. The amount of these high molecular mass protein masses is presumably even more than the figure suggests, as proteins of these sizes are transferred to the membranes with poor efficiency. The pattern of protein labeling was the same in case of both cell lines (Fig. 3), although HL-60 showed consequently weaker labeling intensity. This is in agreement with the weaker and more limited induction of nonspecific esterase in these cells (25) as compared with U937 cells (34).

*Isolation and Identification of the Major TGase Substrate*—By labeling U937 cells with DALP in bulk quantities, endogenous substrate proteins of tissue TGase could be isolated from the total mass of cellular proteins applying a carefully chosen procedure detailed under "Experimental Procedures." After the non-incorporated label was removed by precipitation and electrophoretic techniques, the proteins bearing the haptenic group were isolated by an immunoaffinity column utilizing the phenomenon that the interaction between the applied antibodies and the labeled proteins was not affected by 0.1% SDS at room temperature. Immunoblot analysis of the isolated proteins gave a labeling pattern very similar to that of the crude cell homogenate (Fig. 4). The majority of the labeled proteins was in the 43-kDa band. The additional two upper bands of the immunoblot were at least 1 order of magnitude less in relative quantity. A direct N-terminal sequencing of the labeled proteins was not possible because of N-terminal blockage. The 43-kDa protein was then isolated by electroblotting following PAGE and fragmented with CNBr. Peptide fragments were separated by reverse-phase HPLC chromatography (Fig. 4), and amino acid sequences of the isolated peptides were analyzed by microsequencing. The obtained sequences were searched against the Swiss-Prot protein sequence data base for identical sequences. In the case of each read sequence, only actins gave full matching in the search and only non-muscle (cytoplasmic) actins contained all the identified sequences (Table I). The identity of the isolated protein with cytoplasmic actin was further confirmed using monoclonal anti-actin antibodies (Fig. 5); the 43-kDa band of the hapten-labeled proteins reacted with both anti-dinitrophenol and anti-actin antibodies. Actin immunoreactivity was also seen with the unresolved high molecular mass proteins, suggesting that actin is an ingredient of the cross-linked protein polymer formed by tissue TGase in the dying cells. The two labeled proteins of approximate molecular masses of 110 and 150 kDawere not recognized by the anti-actin antibodies. These proteins were identified as retinoblastoma protein derivatives by specific antibodies; these results will be discussed in conjunction with a separate set of data (35).

*Subcellular Localization of the Incorporated Label and Tissue Transglutaminase*—U937 cells induced to differentiate by $Me_2SO$ and RA in the presence of DALP-methyl carbamate were cytocentrifuged or adhered to glass slides. Following fixation and washing (to remove non-incorporated DALP), the number of cells undergoing apoptosis was determined and the subcellular localization of TGase and incorporated DALP was visualized by immunofluorescence and investigated by confocal image microscopy. DALP fluorescence was not detectable in cells obtained in the early phase of the culture (before 60–72 h) or in those not treated with $Me_2SO$ and RA. It was observed in triplicate experiments with double fluorescence staining for TGase and DALP, or DALP and apoptotic nuclear fragmentation, or apoptosis and TGase (Table II) that at 72 h of treatment, i) DALP incorporation occurred in a large number of cells and only those containing TGase, ii) the majority of apoptotic

TABLE I
*Identification of CNBr peptides obtained from the purified 43-kDa DALP-labeled protein by matching their sequences to known protein sequences of human origin*

| Peptide | Sequence read | Identical sequence found in: |
|---|---|---|
| P6 | MXXEITALAPS | Actin, muscle, and cytoplasmic isoforms |
| P10 | MXXTFNP | Actin, muscle, and cytoplasmic isoforms |
| P15 | MXKAGFAGDD | Actin, cytoplasmic isoforms |
| P20 | MXIKIIAPPER | Actin, muscle, and cytoplasmic isoforms |
| P23 | MATAASSSSL | Actin, muscle, and cytoplasmic isoforms |

cells had DALP labeling, and iii) almost all apoptotic cells expressed TGase. Since the difference between neither of the aforesaid three double positive population was significant (Table II), one may conclude that most of the double positive cells must necessarily be positive for all the three parameters, *i.e.* apoptosis, transglutaminase expression, and covalent cross-linking of cellular proteins very much coincide.

The pattern of DALP labeling revealed a distinct and mainly filamentous distribution by confocal slicing (Fig. 6*A*), whereas the distribution of cytoplasmic actin did not resolve any sort of structure (data not shown), since it is mainly monomer G actin that is detected by monoclonal anti-actin antibodies. On the other hand, by staining with phalloidin-TRITC, which reacts only with fibrillary actin, the stress fibers of adherent cells could be selectively stained and this staining gave a very close overlap with the pattern of DALP staining (Fig. 6, *A–C*). It is noteworthy that the incorporation pattern of DALP was mostly, but not entirely, restricted to the actin cytoskeleton (Fig. 6*C*, *green color*). The distribution of tissue TGase was diffuse throughout the cytoplasm and did not co-localize with the incorporated label (Fig. 6, *C–E*). Disrupted cells showing either necrotic or late apoptotic features lost their arranged stress fibers, and the staining pattern was diffuse similarly to that of non-adherent cells (data not shown).

## DISCUSSION

Tissue transglutaminase is often highly expressed at sites of programmed cell death (17–19, 32), and its induction has been observed in association with apoptosis of several cultured cell lines (23, 32, 36). However, defining the precise role of this enzyme in the cell death pathway requires the identification of its substrates cross-linked in the dying cells. Apart from numerous abundant extracellular proteins, several intracellular substrates have been shown to be *in vitro* TGase substrates including β-crystallins (37), lipocortin (38), fructose-1,6-bisphosphate aldolase (14), and actin itself (39). Although the variety of putative enzyme substrates would make it necessary to determine whether the substrate properties of particular proteins really have physiological significance or not, these results either were obtained in test tube experiments or were greatly compromised by the insufficient permeability of the applied amine substrates because of the strong dissociation of the amino groups at physiologic pH values and the potential barrier of cytoplasmic membrane for ionic charge. In our approach, the charge barrier could be overcome by the use of the methyl carbamate derivative of the amine substrate DALP, which is hydrolyzed by esterases inside cells and then specifically incorporated into cellular proteins.

HL-60 and U937 cells differentiate upon treatment with $Me_2SO$ and retinoic acid. Maturated phenotypes express high levels of tissue transglutaminase and die by apoptosis. DALP labeling of TGase substrates and studying the main labeled protein by DALP-specific purification and microsequencing identified cytoplasmic actin as one of the major targets of the enzyme in cells. Subcellular distribution of the incorporated molecules showed a strongly overlapping pattern with actin

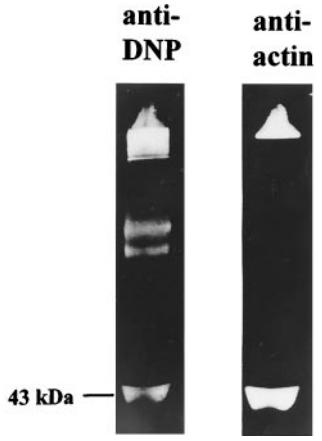


FIG. 5. **Western blot analysis of the affinity-isolated endogenous TGase substrate proteins from U937 cells with anti-DNP and anti-actin antibodies visualizing reactive bands with the chemiluminescence reaction.**

microfilaments. Actin was found to be a substrate for tissue transglutaminase in test tube experiments, both in its globular and polymeric form (39), and our present results clearly demonstrate that actin serves as the major substrate of tissue TGase in dying cells. The lack of DALP labeling of other abundant proteins of the cellular machinery (such as tubulin) also points to the specificity of actin labeling. It was also demonstrated that Gln-41 is the main amine acceptor site for the transglutaminase reaction (39, 40), and the enzyme can also form trace amounts of covalently cross-linked actin oligomers from G actin, which may act as "seeds" to allow actin to polymerize faster. Furthermore, since Gln-41 is accessible in F actin as well (39) and according to our present results the high molecular weight polymers formed by TGase are also labeled by DALP, it is very likely that additional proteins are attached covalently, through their accessible Lys residues, to the microfibrillar meshwork by the enzyme. Actin from human blood platelets or rabbit skeletal muscle has also been shown to serve as substrate for factor XIIIa (41), a transglutaminase that is different in both structure and reactivity toward glutamine containing peptide substrates (2, 3). The highly cross-linked matrix of platelets formed by the transglutaminase reaction also contains actin (42).

Changes of microfibrillar architecture have been proposed to determine the morphological events of apoptosis (5). Hallmarks of apoptosis like cytoplasmic blebbing and fragmentation of the nucleus can be inhibited by cytochalasin leading to disruption of actin filaments (43, 44). Cells unable to maintain their proper shape and cytoskeletal arrangement because of the loss of contact with the extracellular matrix die by apoptosis (45, 46). On the other hand, apoptosis results in increasing disruption of actin fibers in certain cells (47). The process of apoptosis may be regulated by proteolytic processing of actin or by other components of the microfilament system (48–51). The modification of actins and their filaments by the transglutaminase-dependent cross-linking reaction is another possible way of regulating actin-dependent morphologic and biochemical events of apoptosis and thereby executing cell death. Under the conditions of our experiments, it is not possible to decide what switches on the activity of the enzyme and when. Our data suggest that transglutaminase activation is a late event of apoptosis, since the covalent incorporation of DALP into actin filaments occurs in a significant amount after about 60 h following treatment with $Me_2SO$ and retinoic acid, *i.e.* when most cells showed molecular signs of apoptosis as assessed by the detection of DNA breaks in the nucleus. It should be noted that

TABLE II

*Immunofluorescent staining of U937 cells for the presence of TGase, DALP incorporation, and nuclear fragmentation 72 h following RA and $Me_2SO$ treatment*

Table shows results (mean ± S.E.) of triplicate experiments counting at least 100 cells in each case.

| Staining | Immunopositive cells (%) | | |
|---|---|---|---|
| | By 1st stain | By both stains | Double positives as % of 1st stain positives/2nd stain positives |
| TGase (1st stain) + DALP (2nd stain) | 78.5 ± 1.94 | 75.2 ± 3.85[a] | 95.8/100 |
| DALP (1st stain) + NF (2nd stain) | 75.4 ± 3.63 | 74.2 ± 2.54[a] | 98.4/91.8 |
| NF (1st stain) + TGase (2nd stain) | 80.8 ± 3.74 | 74.0 ± 2.82[a] | 92.6/94.2 |

[a] The difference between neither of these populations is significant by Student's test ($0.8 < p < 0.9$ for all three cases).

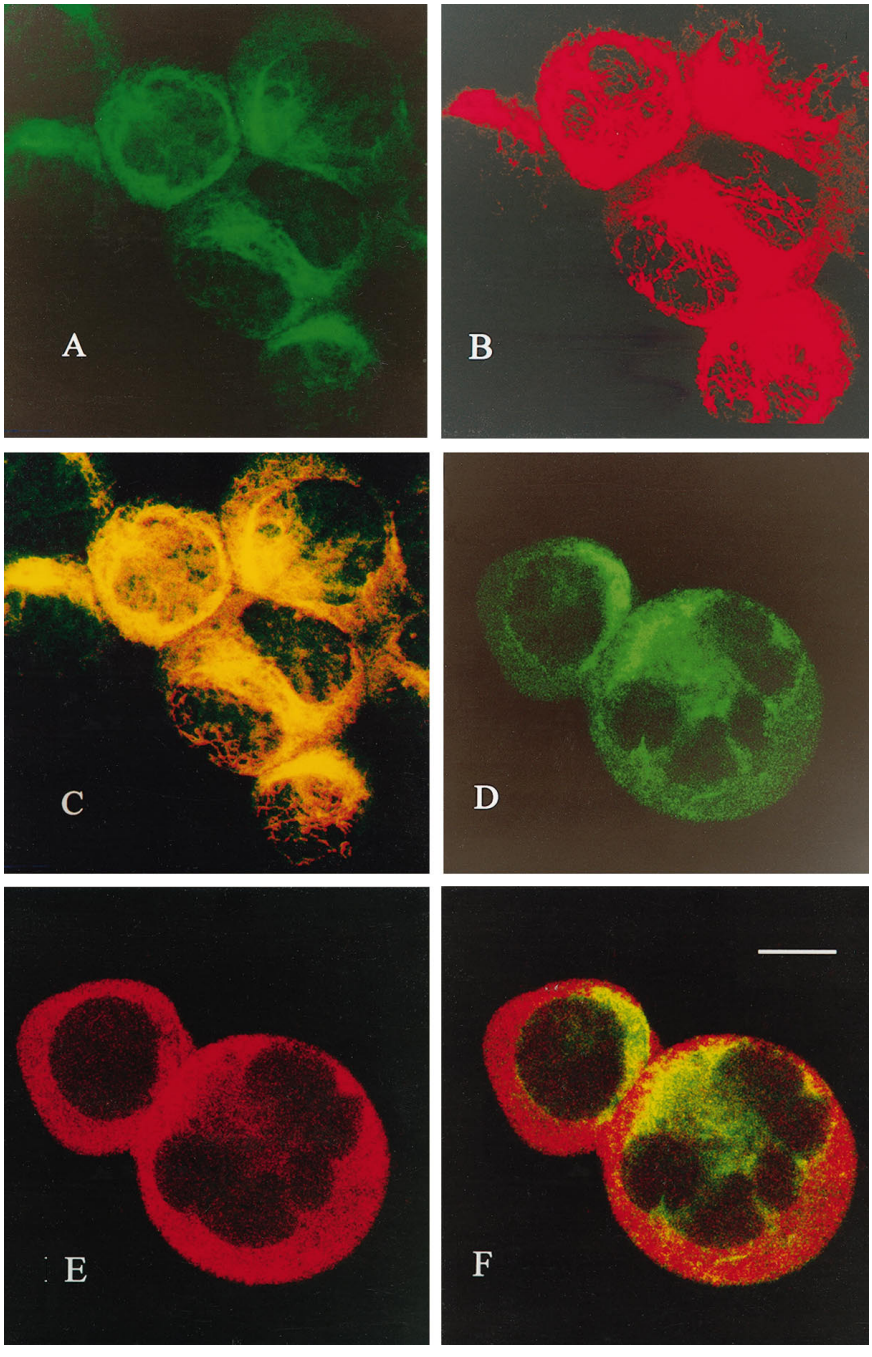


FIG. 6. **Confocal immunofluorescent images showing intracellular distribution of DALP-labeled TGase substrate proteins (*A* and *D*), actin microfilaments (*B*), tissue TGase (*E*), and superimposed images of *A* and *B* (*C*) or *D* and *E* (*F*) of U937 cells.** On superimposed images co-localization appears in *yellow*, whereas the *red* or *green color* indicates non-overlapping reactions. Intracellular distribution of DALP incorporation corresponds to actin labeling by phalloidin-TRITC (compare *A* and *B* to *C*) but not to the localization of tissue TGase (compare *D* and *E* to *F*). *Bar* = 10 μm.

these cells are not permeable to vital stains such as trypan blue.

Apoptosis, in contrast to necrosis, is a "silent" form of cell death without the release of aggressive enzymes, hidden antigens, or agents inducing inflammation (52). Even if the execution of the apoptotic process does not need novel gene expression (53), the events of this program are more ordered than necrosis in terms of both morphology and biochemistry. Order, among others, means maintained compartmentalization and restricted movements of subcellular organelles in which a crucial role of statics and dynamics of the cytoskeletal can be assumed. It may be hypothesized that cross-linking of various

intracellular proteins to actin is involved in such processes stabilizing cellular morphology, counteracting the release of cytoplasmic material from cells (19), and exerting a morphogenic role in programmed cell death (54).

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