Marsh, J. B. (1976) *J. Lipid Res. 17*, 85–90.
Nunez, J. F., & Swaney, J. B. (1984) *J. Biol. Chem. 259*, 9141–9148.
Pownall, H. J., Massey, J. B., Kusserow, S. K., & Gotto, A. M., Jr. (1978) *Biochemistry 17*, 1183–1188.
Pownall, H. J., Pao, Q., & Massey, J. B. (1985) *J. Biol. Chem. 260*, 2146–2152.
Scanu, A. M. (1972) *Biochim. Biophys. Acta 265*, 471–508.
Schonfeld, G., & Pfleger, B. (1974) *J. Clin. Invest. 54*, 236–246.
Schumaker, V. N., & Puppione, D. L. (1986) *Methods Enzymol. 128*, 155–170.
Sundarem, G. S., Shakir, K. M., Barnes, G., & Margolis, S. (1978) *J. Biol. Chem. 253*, 7703–7710.
Surewicz, W. K., Epand, R. M., Pownall, H. J., & Hui, S.-W. (1986) *J. Biol. Chem. 261*, 16191–16197.
Swaney, J. B. (1980) *J. Biol. Chem. 255*, 8791–8797.
Swaney, J. B. (1983) *J. Biol. Chem. 258*, 1254–1259.
Swaney, J. B., & O'Brien, K. (1978) *J. Biol. Chem. 253*, 7069–7077.
Swaney, J. B., & Chang, B. C. (1980) *Biochemistry 19*, 5637–5644.
Swaney, J. B., Orishimo, M. W., & Girard, A. (1987) *J. Lipid Res. 28*, 982–992.
Tall, A. R., Small, D. M., Deckelbaum, R. J., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 4701–4711.
Trauble, H., Middelhoff, G., & Brown, V. W. (1974) *FEBS Lett. 49*, 269–275.
Valtersson, C., Filipsson, L., & Dallner, G. (1986) *J. Lipid Res. 27*, 731–741.
Verdery, R. B., III, & Nichols, A. V. (1974) *Biochem. Biophys. Res. Commun. 57*, 1271–1278.
Windler, E., & Havel, R. J. (1985) *J. Lipid Res. 26*, 556–565.

Probing the Transglutaminase-Mediated, Posttranslational Modification of Proteins during Development†

L. Cariello,‡ P. T. Velasco,§ J. Wilson,§ K. N. Parameswaran,§ F. Karush,‖ and L. Lorand*,§

Stazione Zoologica, Naples, Italy, Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208, and Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received November 7, 1989; Revised Manuscript Received February 6, 1990

ABSTRACT: *Sphaerechinus granularis* eggs were fertilized in seawater in the presence of 0.2 mM dansylcadaverine, and development was allowed to take place with this compound in the medium. γ-Glutamyldansylcadaverine, indicative of the utilization of the amine tracer by intrinsic transglutaminase, was isolated from the embryonic proteins, and identity of the product with the chemically synthesized γ-glutamyl derivative of dansylcadaverine was confirmed. Covalent labeling of proteins occurring during development was examined by means of electrophoresis in $NaDodSO_4$, followed by immunoblotting with an antibody that specifically recognized the dansyl hapten. There was an increase in the total uptake of the tracer at an essentially constant rate with each cell divison, from 2- to 8- and 64-cell stages. Moreover, multiple protein labeling was evident in all specimens. The described concept of studying posttranslational modifications in vivo by transglutaminase through detection of the haptenic or specific ligand recognizable group of an incorporated small amine substrate will undoubtedly be of general utility for probing the functions of this family of enzymes in other cell types as well.

Activation of transglutaminase (Lorand & Conrad, 1984), as measured by the incorporation of the naturally occurring polyamine, putrescine, into proteins, was shown to occur within a few minutes following fertilization of sea urchin eggs, suggesting that this enzyme might play a role in the early posttranslational modification of embryonic proteins (Cariello et al., 1984). An appreciable degree of protein cross-linking by N^{ϵ}-(γ-glutamyl)lysine side-chain bridges, yet another indicator of the physiological functioning of transglutaminases, was also demonstrated. These observations serve as the background for the present study which examines in vivo patterns of amine incorporation into proteins in the 2-, 8-, and 64-cell embryo. Because natural polyamines can undergo a variety of metabolic reactions, synthetic dansylcadaverine[1] was employed as the tracer (Lorand et al., 1968). This compound is a good general substrate for transglutaminases of all types and is known to compete effectively against the incorporation of polyamines into proteins following fertilization. The availability of a

† This work was aided by USPHS Research Career Awards (HL-03512 to L.L. and AI-14012 to F.K.) and by grants from the National Institutes of Health (HL-02212 and AI-09492).

* Address correspondence to this author.
‡ Stazione Zoologica.
§ Northwestern University.
‖ University of Pennsylvania.

[1] Abbreviations: dansylcadaverine or Dc, *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide; γ-glutamyldansylcadaverine or γ-GluDc, N^1-L-(γ-glutamyl)-N^5-[5-(dimethylamino)-1-naphthalenesulfonyl]diaminopentane; Dns, dansyl or 5-(dimethylamino)-1-naphthalenesulfonyl; cadaverine, 1,5-diaminopentane: TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; Boc, *tert*-butyloxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; K, 10^3.

0006-2960/90/0429-5103$02.50/0

sensitive immunoblotting procedure, based on an antibody to the dansyl moiety (Lorand et al., 1986), made this compound a particularly suitable choice for purposes of such investigations.

Materials and Methods

Embryonic Cultures. Methods for the harvesting, washing, counting, and fertilizing of eggs from *Sphaerechinus granularis* (collected in the Bay of Naples) were essentially the same as described in the earlier work (Cariello et al., 1984). A 20-mL suspension containing 10^6 eggs was incubated (20 min, ca. 20 °C) in the dark with 1 mM dansylcadaverine in a filtered seawater medium enriched with 2 mM calcium chloride. The suspension was diluted 5-fold with the seawater prior to the addition of sperms. Aliquots (30 mL) were withdrawn from the developing culture at approximately 1.5, 3.5, and 7 h, corresponding to 2-, 8-, and 64-cell embryos. Following sedimentation in a hand-driven centrifuge, ca. 0.5-mL pellets were formed, which were resuspended in 10 mL of 10 mM Tris-HCl, pH 8.3, containing 10 mM EDTA, 0.1 M methylammonium hydrochloride (Sigma, St. Louis, MO) and 1 mM phenylmethanesulfonyl fluoride (Sigma). Suspensions were homogenized (1 min, Ultra-Turrex, TP 10; IKA, West Germany) and concentrated by freeze-drying. Control cultures were raised, harvested, and processed in a parallel manner, but without dansylcadaverine in the medium.

Electrophoresis and Immunoblotting. Materials were taken up in 2% $NaDodSO_4$ (Bio-Rad, Richmond, CA) and 40 mM dithiothreitol (Sigma) in 50 mM Tris-HCl, pH 7.5 (37 °C, 2 h), and insoluble residues were removed by centrifugation (Microfuge, Beckman, Fullerton, CA). Samples corresponding to approximately 100 μg of proteins were applied to 3% polyacrylamide stacking gels and were electrophoresed (protean slab gel apparatus; Bio-Rad) either in 6% or in 10% polyacrylamide resolving gels according to the method of Laemmli (1970). Molecular weight markers (MW-SDS-200 and 70L kits) were purchased from Sigma. Gels were transblotted to nitrocellulose (0.2-μm pore size, Schleicher & Schuell, BA83, Keene, NH) by using an LKB 2005 Transphor unit (Houston, TX) and employing the buffer system [25 mM Tris, 0.192 M glycine, and 20% (v/v) methanol, pH 8.3] recommended by Towbin et al. (1979). Processing of nitrocellulose sheets, staining with 0.2% amido black (Sigma) for proteins, and immunoblotting with anti-dansyl IgG were carried out as previously described (Lorand et al., 1986). Dilutions of rabbit anti-dansyl IgG (1:2000), of biotinylated anti-rabbit IgG (1:500, Vectastain, Vector, Burlingame, CA), and of the avidin–peroxidase complex (1:250) were chosen to obtain maximal color development against a clear background. Immunoblots were photographed, negatives were scanned (Zeineh, Fullerton, CA), and appropriate areas of the tracings were weighed to estimate the relative distribution of immunostained material.

Isolation of γ-Glutamyldansylcadaverine (γ-GluDc) from 64-Cell Embryos Raised in the Presence of Dansylcadaverine. The dry material (300 mg) was taken up in a solution (8.5 mL) of 25 mM Tris-HCl, 200 mM glycine, pH 8.3, 10 mM EDTA, 2% $NaDodSO_4$, and 1% 2-mercaptoethanol. A slight sediment was removed by centrifugation, the pellet was rinsed with 0.5 mL of the above, and the supernatants were combined. Passage through an Extracti-gel D (Pierce Chemical, Rockford, IL, 1.5 × 20 cm) column, using the above solution without $NaDodSO_4$ as eluant, removed the detergent. Proteins were precipitated by the addition of trichloroacetic acid (10%) at 4 °C (16 h); the precipitate was washed twice with 5 mL of each of the following: 5% trichloroacetic acid, ethanol/acetone 1:1 (v/v), and acetone. The dried proteins were taken up in 2.5 mL of 0.1 N NaOH and dialyzed against 0.1 M NH_4HCO_3, pH 8. Enzymatic digestion, following the example given by Cariello et al. (1984), was carried out with the sequential addition of 0.4 mg of subtilisin (twice), 0.4 mg of pronase CB (twice), 0.4 mg of carboxypeptidase Y, 0.15 mg of leucine aminopeptidase (twice), and 0.15 mg of prolidase. The insoluble residue was removed (Microcentrifuge, Brinkman, Westbury, NY), and the precipitate was dried and dissolved in 2 mL of 0.002 N HCl. Amino acid analysis was performed according to the procedure of Griffin et al. (1982) to estimate the total amount of protein in the sample (21.9 mg). Correction was made by subtracting amino acid contributions from the proteases used for digestion (0.9 mg). Thus, a value of 21 mg was calculated as representing sea urchin proteins.

γ-GluDc was identified by HPLC (Waters, Bedford, MA) using three solvents (Cariello et al., 1984): methanol, H_2O (Milli-Q + Organex-Q, Millipore Corp., Bedford, MA), and 40 mM potassium acetate, pH 5.5, each containing 1% (v/v) tetrahydrofuran. The Zorbax C_8 column (Du Pont, Wilmington, DE) was equilibrated at a solvent composition of 40% methanol and 30% each of H_2O and potassium acetate. Two minutes following sample (0.2 mL) injection, a linear increase of methanol commenced, with a decrease of the other solvents. With a flow rate of 1.8 mL/min, the methanol concentration was allowed to reach 100% at 22 min. Fluorescence of the effluent was monitored (λ_{exc} = 334 nm, λ_{em} = 530 nm) with a Waters Model 420 AC detector at highest sensitivity.

A 0.2-mL aliquot of the proteolyzed digest of embryonic proteins was dried and redissolved in 0.2 mL of 50 mM sodium phosphate, pH 7.5. Ten microliters of partially purified γ-glutamylamine cyclotransferase (Fink et al., 1980) containing 0.2 activity unit was added, and the sample was incubated at 37 °C for 60 min. The reaction was stopped by adding 20 μL of 0.6 N HCl, and the sample was analyzed by HPLC as described above.

Biochemical Preparation of [^3H]-γ-Glutamyldansylcadaverine ([^3H]-γ-GluDc) Standard. The compound was isolated from the proteolytic digest of *N,N'*-dimethylcasein, following the transglutaminase-mediated labeling of this protein with [^{3}H]dansylcadaverine. The 3-mL mixture in 50 mM Tris-HCl, pH 7.5, comprised 45 mg of *N,N'*-dimethylcasein (Curtis & Lorand, 1976), 9 μg of guinea pig liver transglutaminase (Connellan et al., 1971), 5 mM $CaCl_2$, 5 mM dithiothreitol (Sigma), and 2 mM [^{3}H]dansylcadaverine (Amersham, Arlington Heights, IL; diluted with the cold amine to an activity of 5520 cpm/nmol). After 21 h of incorporation at 37 °C, the sample was dialyzed against water and was taken to dryness (Speedvac, Savant, Hicksville, NY). It was dissolved in 2.5 mL of 0.1 M NH_4HCO_3, pH 8, and was hydrolyzed by the sequential addition of proteolytic enzymes as previously described (Cariello et al., 1984), using 0.6 mg of subtilisin (twice), 0.6 mg of Pronase (twice), 0.3 mg of carboxypeptidase Y, 0.225 mg of leucine aminopeptidase (twice), and 0.225 mg of prolidase. The digest was filtered (0.45 μm, Millipore Corp.) and dried. It was dissolved in 2 mL of water and was redried three times.

Separation of [^{3}H]-γ-GluDc was achieved by HPLC with a Zorbax C_8 column using a linear gradient between two solvents [A, 0.1% (v/v) triethylamine acetate, pH 6.8, and 1% (v/v) tetrahydrofuran; B, 90% (v/v) acetonitrile in solvent A]. The protein digest was taken up in 2.5 mL of solvent A and was processed in suitable aliquots through HPLC. The solvent composition at injection was 70% solvent A and 30% solvent

B. Two minutes later the percentage of solvent B was allowed to rise linearly, reaching 100% at 22 min. The flow rate was set at 1.5 mL/min, and fluorescence of the effluent was monitored (λ_{exc} = 360 nm, γ_{em} = 530 nm) with the detector set to its lowest sensitivity. The presumed [^{3}H]-γ-GluDc peak representing about 64% of the total radioactivity, and eluting at 10.3 min, was pooled from all the aliquots chromatographed and dried. The material was finally dissolved in 0.01 N HCl and stored at −20 °C. Another radioactive peak (ca. 36% of the total isotope) eluting later from the HPLC column was identified as free [^{3}H]dansylcadaverine.

Synthesis of N^1-L-(γ-*glutamyl*)-N^5-[5-(*dimethylamino*)-1-*naphthalenesulfonyl*]*diaminopentane* was necessary to confirm the identity of the γ-GluDc obtained by the biochemical procedures described above. To a stirred and cooled (−15 °C) solution of N^α-Boc-glutamic acid α-benzyl ester (1.0 g, 2.96 mmol; Chemical Dynamics, South Plainfield, NJ) in 10 mL of dry tetrahydrofuran was added *N*-methylmorpholine (0.34 mL, 3.0 mmol), followed by isobutyl chloroformate (0.38 mL, 2.9 mmol). After 15 min, a precooled (0 °C) solution of dansylcadaverine fumarate (1.28 g, 2.83 mmol; Sigma product converted to the fumarate salt) in 10 mL of dry dimethylformamide containing *N*-methylmorpholine (0.34 mL, 3.0 mmol) was added. The reaction mixture was stirred at −10 °C for 30 min and then at 0 °C for 3 h, followed by 17 h at room temperature. The solvents were removed by evaporation in vacuo, and the residue was taken up in ethyl acetate (150 mL). The ethyl acetate extract was washed successively with cold 1 N HCl (1 × 25 mL), 5% $NaHCO_3$, and saturating NaCl. It was dried (Na_2SO_4), filtered, and concentrated to a 10-mL volume under reduced pressure. This was diluted with petroleum ether and cooled (0 °C), and the precipitate was triturated repeatedly with additional cold (0 °C) petroleum ether, filtered, and dried in vacuo to give 1.74 g (94%) of a light yellow solid: mp (uncorrected) 49–52 °C; TLC homogeneous (fluorescent under UV light of 365 nm, but negative with 0.25% ninhydrin stain in 1-butanol); R_f 0.84 in solvent I [chloroform/methanol/acetic acid 10:2:1 (v/v)] and 0.86 in solvent II [ether/ethanol/NH_4OH 14:4:2 (v/v)]. The ^{1}H NMR ($CDCl_3$, 90 MHz) spectrum was in agreement with the structure for the blocked intermediate, N^α-Boc-α-benzyl-γ-glutamyldansylcadaverine. A solution of this material (1.64 g, 2.5 mmol) in 125 mL of ethanol was hydrogenated in the presence of 0.4 g of 5% Pd on a carbon catalyst for 4 h at atmospheric pressure. The catalyst was filtered off, and the filtrate was concentrated. Upon addition of 60 mL each of anhydrous ether and petroleum ether, followed by trituration of the precipitate with petroleum ether, a light yellow solid was obtained which was filtered off and dried in a vacuum desiccator to give 1.26 g (89%): mp 83–88 °C; TLC homogeneous (fluorescent, but ninhydrin negative); R_f 0.79 in solvent I and 0.15, 0.78, and 0.23 in solvents II, III [95% ethanol/10% NH_4OH 10:2 (v/v)], and IV [chloroform/methanol 10:2 (v/v)], respectively. To deblock the Boc group, a solution of this intermediate (0.3 g, 0.53 mmol) in 2.0 mL of 50% trifluoroacetic acid in dichloromethane was allowed to stand at room temperature for 1 h. Excess trifluoroacetic acid was removed by adding more dichloromethane and evaporating under vacuum and triturating the residue with cold anhydrous ether (2 × 25 mL). The precipitate was filtered off and recrystallized twice from a mixture of anhydrous methanol and anhydrous ether with a yield of 0.185 g (60%) of L-γ-glutamyldansylcadaverine: mp (uncorrected) 171–173 °C; TLC homogeneous (fluorescent and ninhydrin positive); R_f 0.1 in solvent I, 0.05 in solvent II, and 0.65 in solvent III;

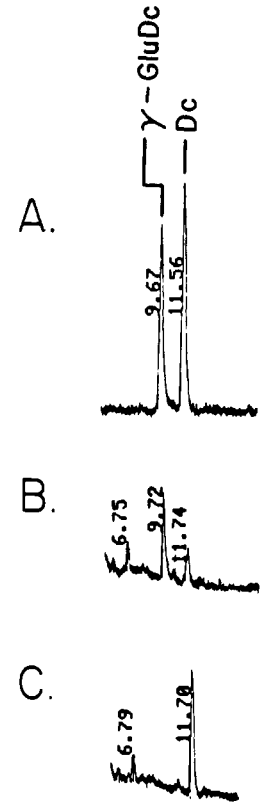

FIGURE 1: Identification of γ-glutamyldansylcadaverine (γ-GluDc) in the hydrolysate of proteins from the 64-cell embryo, raised in the presence of dansylcadaverine (Dc). Panel A shows the HPLC profile for a standard mixture containing [^{3}H]-γ-GluDc (100 pmol) and Dc (100 pmol), as monitored by fluorescence (see Materials and Methods). Panel B is the pattern for the hydrolysate of embryonic proteins (1.8 mg) which, upon further treatment with γ-glutamylamine cyclotransferase, was converted to the pattern shown in panel C.

^{1}H NMR (Me_2SO-d_6) δ (expressed as parts per million and using tetramethylsilane as internal standard) 1.1–1.3 (6 H, m, $(CH_2)_3$ of diaminopentyl moiety), 1.8–1.9 (2 H, br m, βCH_2 Glu), 2.2 (2 H, app t, γCH_2 Glu), 2.75–2.88 [10 H, m of $NHCH_2$(4H) + S of $N(CH_3)_2$(6 H) centered at 2.85], 3.2 (1 H, app t, αCH Glu), 3.35 (3 H, br s, $-N^+H_3$), 7.95 (2 H, br t, SO_2NH + CONH), 7.25, 8.1, 8.3, 8.45 (each 1 H, d, naphthyl of Dns), 7.6 (2 H, m, naphthyl of Dns); FAB MS, *m/z* 465.2 (M^+ + 1). Anal. Calcd for $C_{22}H_{32}N_4O_5S$: C, 56.87; H, 6.94; N, 12.05. Found: C, 56.19; H, 6.89; N, 11.74.

Results

Pretreating of *Sphaerechinus* eggs for 20 min with 1 mM dansylcadaverine and lowering the concentration of the amine to 0.2 mM just prior to the introduction of sperms were compatible with a good fertilization rate (>90%) and an apparently normal mode of development for the duration of the experiments. Embryos were harvested at the 2-, 8-, and 64-cell stages at approximately 1.5, 3.5, and 7 h after fertilization, homogenized, and processed as described under Materials and Methods.

First, the identity between a biochemically isolated γ-GluDc and the chemically synthesized N^1-L-(γ-glutamyl)-N^5-[5-(dimethylamino)-1-naphthalenesulfonyl]diaminopentane had to be demonstrated. Therefore, γ-GluDc was obtained from the proteolytic digest of *N,N'*-dimethylcasein into which [^{3}H]dansylcadaverine was previously incorporated through the action of liver transglutaminase. By employing the HPLC protocol for Figure 1, the biochemically prepared and the synthetic product coeluted at about 9.7 min. In addition, treatment of either compound with γ-glutamylamine cyclo-

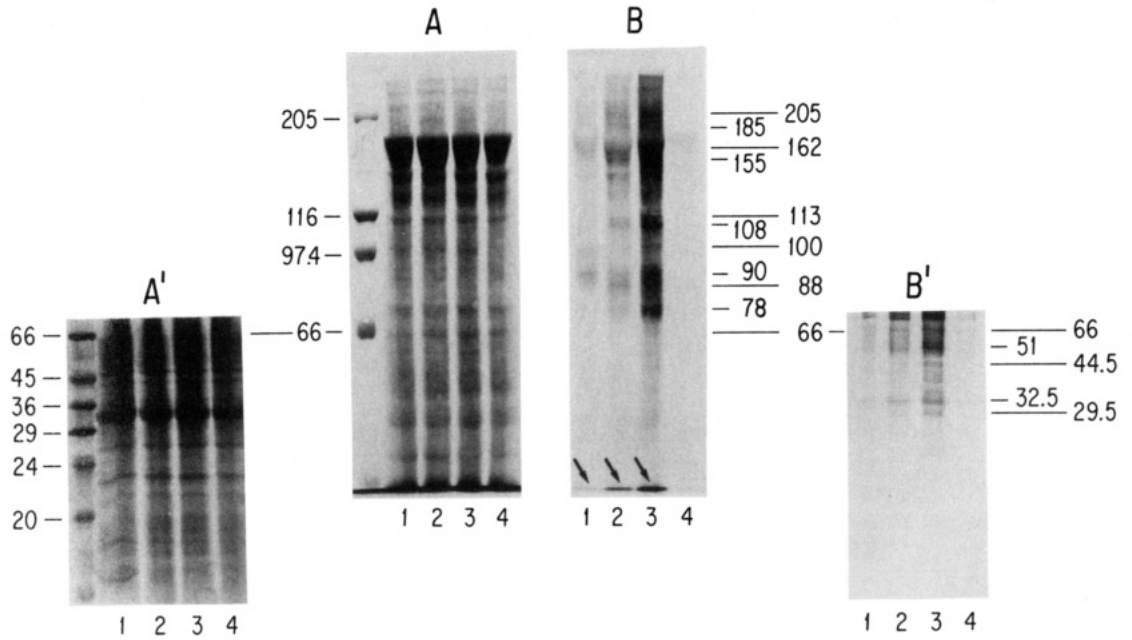

FIGURE 2: Electrophoretic separation and immunoblotting of embryonic proteins with anti-dansyl antibody. Panels A and A′ show the protein profiles obtained n 6% and 10% polyacrylamide gels, respectively, by the staining of nitrocellulose transblots with amido black, whereas panels B and B′ repesent the corresponding immunoblots with the anti-dansyl antibody (see Materials and Methods). Lanes 1, 2, and 3 in each panel refer to samples from the 2-, 8-, and 64-cell embryos grown in the presence of dansylcadaverine; lane 4 is always the control 64-cell specimen which was raised in the absence of dansylcadaverine. Molecular weight markers are included in panels A and A′, and calculated relative molecular weights for prominent bands in the immunoblots are given for panels B and B′. The arrows in panel B mark the position of ≤50K proteins.

transferase resulted in the loss of the 9.7-min peak but gave rise to a similarly fluorescent material which was eluted around 11.6–11.8 min. This second peak was identified as dansylcadaverine (Dc).

Panel A in Figure 1 illustrates the resolution for a reference mixture of 100 pmol each of γ-GluDc and Dc standards, and panel B shows the HPLC fluorescence profile for the proteolytic digest of the 64-cell embryonic proteins. The analysis revealed the presence of γ-GluDc at a frequency of about 2.4 mol/10^8 g of protein and of free Dc at about 0.9 mol/10^8 g of protein. When the digest in panel B was exposed to γ-glutamylamine cyclotransferase, as presented in panel C, there was a complete loss of γ-GluDc; however, free Dc accumulated to the extent of about 3.1 mol/10^8 g of protein.

Having demonstrated that the bulk of the dansylcadaverine which became incorporated into embryonic proteins could be recovered from the digest in the form of γ-GluDc, a structure compatible with transglutaminase-mediated posttranslational modifications, the pattern of protein alterations during embryonic development was examined. Proteins were extracted from the 2-, 8-, and 64-cell embryos by $NaDodSO_4$ in dithiothreitol and were electophoresed in polyacrylamide, followed by transfer to nitrocellulose under electric current. Since free dansylcadaverine would not be transferred by this procedure, it could be assumed that probing the nitrocellulose with an anti-dansyl antibody would yield positive results only with proteins which were covalently modified through the incorporation of dansylcadaverine at relevant positions.

Electrophoresis was performed in 6% as well as in 10% polyacrylamide resolving gels to achieve separation of polypeptides of higher molecular mass (panels A and B of Figure 2) and lower than 66K (panels A′ and B′). Panels A and A′ show the protein profiles obtained by staining the nitrocellulose sheets with amido black, whereas panels B and B′ represent the immunoblots with anti-dansyl antibody. Lanes 1, 2, and 3 correspond to samples from the 2-, 8-, and 64-cell embryos grown in the presence of dansylcadaverine, while lane 4 in each panel refers to a control 64-cell stage specimen that was raised in the absence of dansylcadaverine. Since staining intensities with amido black were essentially the same throughout (lanes 1–4 for panel A or A′), it may be concluded that similar gel loadings were achieved.

Negative immunoblotting in lanes 4 of panels B and B′ was in accordance with the specificity of the anti-dansyl antibody because it did not react with any component in this control material. However, all the experimental specimens grown in the presence of dansylcadaverine gave positive immunostaining. Overall intensity increased with embryonic age, from lanes 1 to 3 in panels B and B′. Furthermore, in all instances, including the 2-cell samples, multiple labeling of bands was evident. Even the lower molecular weight (≤50K) material moving as a sharp band at the front of 6% gels (marked by arrows on the transblots in panel B) could be resolved into several components by electrophoresis in the 10% polyacrylamide gel (panel B′).

Densitometric scans of the immunoblots (corresponding to panels B and B′ in Figure 2) are shown in Figures 3 and 4, respectively, demonstrating the progressive labeling of embryonic constituents in a more quantitative manner. The combined areas under each tracing in Figure 3 reflect the extent of dansylcadaverine incorporation. When these were compared for the 2-, 8-, and 64-cell preparations (marked I, II, and III, respectively), the approximate ratio of 1:2.6:12.7 was obtained. Considering the fact that the 2- and 8-cell stages are separated by two cell divisions and the 8- and 64-cell stages by three cell divisions, the data indicate a fairly constant or just a slightly increasing rate (1.3–1.6-fold) of labeling of embryonic proteins. The relative distribution of labeling for the different molecular weight groups of proteins in the 64-cell blastomere was as follows: about 20% of the total dansyl label was in ≳182K bands, 32% in 182–123K, 17% in 123–98K, 23% in 98–68K, 5% in 64–51K and only 3% in ≲51K species. As shown in Figure 4, the small molecular weight material could be further resolved into several components, and labeled bands could be recognized with M_rs of 51K, 44.5K, 32.5K, and 29.5K. However, in quantitative terms, their individual

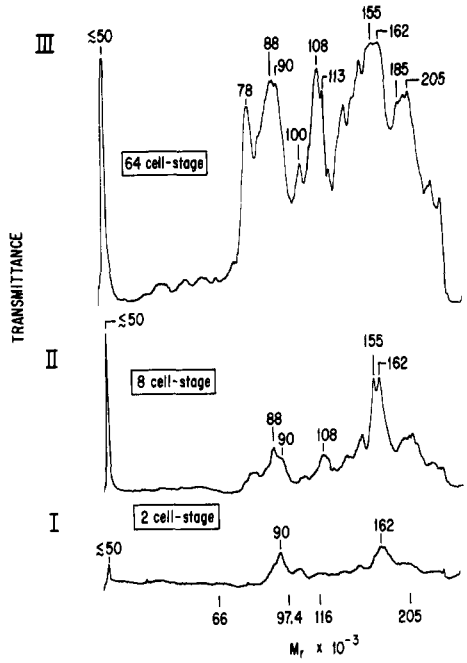

FIGURE 3: Profile of dansylcadaverine-labeled large (≥70K) embryonic proteins. Densitometric analysis was carried out for the immunoblots shown in lanes 1–3 of panel B in Figure 2.

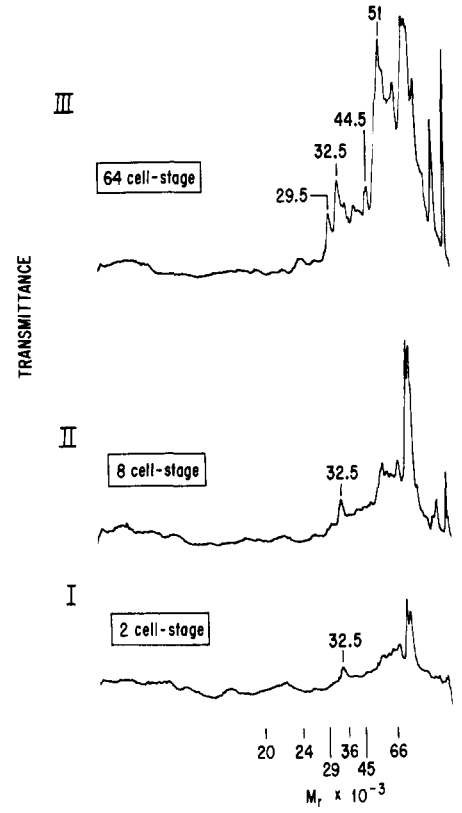

FIGURE 4: Distribution of dansylcadaverine-labeled small (≤70K) embryonic proteins. The densitometric profiles were obtained from the immunoblots shown in lanes 1–3 of panel B′ in Figure 2.

contributions to the total uptake of dansylcadaverine appeared to be rather minimal.

Discussion

The presence of transglutaminase in sea urchin eggs has been known for some time, and it has also been suggested that the subcellular distribution of the enzyme may change during embryogenesis (Campbell-Wilkes, 1973). Additional interest was focused on transglutaminase by showing that, soon after fertilization, the enzyme underwent a remarkable degree of activation (Cariello et al., 1984). Since emetine, as inhibitor of protein synthesis, did not block the increase in activity up to the first cell division, it was concluded that fertilization caused a conversion of a preexisting form of transglutaminase to an enzymatically active form. A similar pattern of activation was found when human epidermal carcinoma cells (A431) were exposed to epidermal growth factor (Dadabay & Pike, 1987). Thus, in addition to gene activation, the conversion of transglutaminase from a latent to an enzymatically functioning form may be a common response to different agonists, such as sperm or growth factors, in a variety of cell types. Altogether, the transglutaminase-mediated posttranslational modification of proteins may be of general biological importance.

By nondenaturing electrophoresis (Lorand et al., 1979a) three forms of transglutaminase could be identified in extracts of *Strongylocentrotus purpuratus* eggs (Takeuchi and Lorand, unpublished observation). Of these differently charged proteins, one was obtained in a highly purified form; the enzyme was shown to require Ca^{2+} for expression of activity and was inhibited by iodoacetamide, an indication that it functioned through a SH catalytic center.

A major aim of the present study was to define the in vivo pattern of posttranslational modification of protein substrates after fertilization, caused by the activation of transglutaminase. To highlight the difficulties inherent in this effort, it may be instructive to compare the transglutaminase-catalyzed reaction with the paradigm of protein phosphorylation by tyrosine kinases in biological systems [see Hunter and Cooper (1985)]. In that example there is but one product, a phosphotyrosyl residue, and the phosphoryl group which is transferred is always derived from the singular γ-position of the intracellular ATP pool. Importantly, also, the latter can be readily labeled to high levels of radioactivity under physiological conditions. By contrast, transglutaminase-mediated reactions, operating on protein-bound (**P**) glutaminyl residues, may generate a variety of products depending on the partitioning of the acyl-enzyme intermediate by different nucleophiles [such as water, alcohols, small primary amines (e.g., putrescine, spermidine, spermine), or the ε-lysine side chain of another protein; see Lorand and Conrad (1984)]. In every case, NH_3 is a common byproduct, generated in the formation of the acyl-enzyme intermediate, but the derivatization of the protein-bound glutamyl will differ:

$$\mathbf{P}\sim CONH_2 + H_2O \rightarrow \mathbf{P}\sim COOH \quad (1)$$

$$\mathbf{P}\sim CONH_2 + HOR \rightarrow \mathbf{P}\sim COOR \quad (2)$$

$$\mathbf{P}\sim CONH_2 + H_2NR \rightarrow \mathbf{P}\sim CONHR \quad (3)$$

$$\mathbf{P}\sim CONH_2 + H_2N\sim\sim\mathbf{P}' \rightarrow \mathbf{P}\sim CONH\sim\sim\mathbf{P}' \quad (4)$$

It would be quite a formidable analytical task to search for all of these products after cell activation. Rather, we introduced an exogenous amine tracer, dansylcadaverine, and allowed it to compete, in a manner represented by (3), against the other reaction possibilities. However, even though dansylcadaverine is one of the best among the known amine substrates for transglutaminase (Lorand et al., 1979b), it is obvious that the in vivo labeling of proteins by the compound may represent only a fraction of the totality of posttranslational modifications by this enzyme.

First, it was necessary to chemically synthesize N^1-L-(γ-glutamyl)-N^5-[5-(dimethylamino)-1-naphthalenesulfonyl]diaminopentane and to show that the relevant derivative, obtained from the total proteolytic digest of a protein, was,

indeed, the same compound. For this purpose *N,N'*-dimethylcasein was prelabeled with dansylcadaverine by transglutaminase and digested. Armed with this information, it was possible to demonstrate that, apart from some free dansylcadaverine, the bulk of the tracer was present in the form of γ-GluDc when the embryos were raised in the presence of the exogenous amine (Figure 1B,C).

Analysis of embryonic proteins by NaDodSO$_4$–PAGE, coupled with immunoblotting with an anti-dansyl antibody, revealed an increasing degree of labeling with dansylcadaverine through the development period of 2-, 8-, and 64-cell stages. The increase in the covalent incorporation of the tracer occurred at a fairly constant rate (1.3–1.6-fold) with each cell division. In all instances (see Figures 3 and 4) a multiple pattern of protein labeling was found. Although for the earliest 2-cell specimen (see panel I in Figure 3) a case could be made for the preferential labeling of 90K and 162K polypeptides, it is clear that our results do not support the claim for the existence of a "unique" transglutaminase-sensitive protein of about 30K in size. That conclusion was reached by Canellakis et al. (1985), who, following our work with [^{14}C]putrescine (Cariello et al., 1984), employed [^{3}H]spermidine to identify posttranslational products of transglutaminase in fertilized sea urchin eggs without, however, proving that the polyamine was actually incorporated into γ-glutamyl residues in proteins.

The biological functions of proteins identified as transglutaminase substrates in vivo remain to be elucidated. It has been known for some time that both the fertilization envelope and hyalin may serve as amine-incorporating substrates. In fact, these embryonic structures could be isolated in a fluorescent form by exposure of the developing culture to dansylcadaverine (Campbell-Wilkes, 1973). We have recently confirmed that, following the in vivo uptake of dansylcadaverine into the fertilization envelope, γ-GluDc could be isolated, and from the fertilization envelope of control cultures, N^{ϵ}-(γ-glutamyl)lysine was obtained at the relatively high frequency of about 1 mol/330000 g of protein (Cariello, Wilson, and Lorand, unpublished results). The idea that the transglutaminase-mediated cross-linking of proteins may be important in the assembly of the fertilization envelope received impetus also from the studies of Battaglia and Shapiro (1988), who suggested that such cross-linking may actually precede the hardening of the envelope by formation of dityrosine bridges. We assume that some of the labeled proteins seen in Figures 3 and 4 may represent component polypeptides of the fertilization envelope and of the hyalin layer. Different forms of transglutaminase may be responsible for reactions at different regions of the embryo, some involved in intracellular developmental events, others in extracellular morphogenesis. The conceptual and methodological approaches outlined in the present paper should be useful for identifying specific protein substrates which are affected by posttranslational modifications through the functioning of such transamidating enzymes not only in the sea urchin embryo but also in other biological systems. Whether all the proteins that were labeled with the dansylcadaverine probe would participate in physiological cross-linking reactions in the absence of the amine remains to be answered.

References

Battaglia, D. E., & Shapiro, B. M. (1988) *J. Cell Biol. 105*, 254a, Abstract 1445.

Campbell-Wilkes, L. K. (1973) Ph.D. Dissertation, Northwestern University, Evanston, IL (Xerox University Microfilms No. 73-30,763).

Canellakis, Z. N., Bondy, P. K., & Infante, A. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7613–7615.

Cariello, L., Wilson, J., & Lorand, L. (1984) *Biochemistry 23*, 6843–6850.

Connellan, J. M., Chung, S. I., Whetzel, N. K., Bradley, L. M., & Folk, J. E. (1971) *J. Biol. Chem. 246*, 1093–1098.

Curtis, C. G., & Lorand, L. (1976) *Methods Enzymol. 45*, 177–191.

Dadabay, C. Y., & Pike, L. J. (1987) *Biochemistry 26*, 6587–6591.

Fink, M. L., Chung, S. I., & Folk, J. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4564–4568.

Griffin, M., Price, S. J., & Palmer, T. (1982) *Clin. Chim. Acta 125*, 89–95.

Hunter, T., & Cooper, J. A. (1985) *Annu. Rev. Biochem. 54*, 897–930.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lorand, L., & Conrad, S. M. (1984) *Mol. Cell. Biochem. 58*, 9–35.

Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Oner, N., & Bruner-Lorand, J. (1968) *Biochemistry 7*, 1214–1223.

Lorand, L., Siefring, G. E., Jr., Tong, Y. S., Bruner-Lorand, J., & Gray, A. J., Jr. (1979a) *Anal. Biochem. 93*, 453–458.

Lorand, L., Parameswaran, K. N., Stenberg, P., Tong, Y. S., Velasco, P. T., Jonsson, N. A., Mikiver, L., & Moses, P. (1979b) *Biochemistry 18*, 1756–1765.

Lorand, L., Murthy, S. N. P., Velasco, P. T., & Karush, F. (1986) *Biochem. Biophys. Res. Commun. 134*, 685–689.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.