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Spermidine is bound to a unique protein in early sea urchin embryos

(polyamines/posttranslational modification/embryogenesis)

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ABSTRACT **Spermidine is rapidly taken up and becomes bound to protein during the very early hours of sea urchin embryogenesis. During the first 6 hr after fertilization of freshly obtained sea urchin eggs (*Strongylocentrotus purpuratus*), which are incubated in the presence of exogenous [^{3}H]-spermidine, up to 7% of the total cell-associated spermidine appears uniquely as spermidine bound in macromolecular form. This unique protein containing spermidine migrates as a single radioactive band in gel electrophoresis. It has a M_r of ≈30,000 and is readily distinguishable from the protein initiation factor eIF-4D, which has a M_r of 18,000, the only other identifiable protein known to date to be posttranslationally modified by polyamines.**

In very early sea urchin (*Strongylocentrotus purpuratus*) embryogenesis we have discovered a protein containing intact, covalently bound spermidine. Putrescine, spermidine, and spermine, collectively referred to as the parent polyamines, are essential for the life of a cell and their intracellular concentrations are elevated during rapid cell division and growth (1–3). In addition, agents known to interfere with polyamine levels and metabolism block growth and differentiation (4). Yet, until recently, it has been difficult to assign particular roles to these small aliphatic amines, which are the most abundant endogenous cellular cations. The finding of hypusine, a polyamine metabolite, as a covalent component of the eukaryotic protein translation initiation factor eIF-4D was, to our knowledge, the first example of a polyamine derivative in a specific macromolecule (5). We now find that intact spermidine is present as a posttranslational modification of a unique protein with a M_r of ≈30,000, an example of an intact polyamine bound covalently to a specific macromolecule.

The cellular uptake of [^{3}H]spermidine by freshly fertilized sea urchin eggs and its distribution between the trichloroacetic acid (CCl_3COOH)-soluble and CCl_3COOH-insoluble fraction of cells are presented in Fig. 1. Total uptake of spermidine continued rapidly, reaching about 20% of the added radioactivity at 4 hr (four-cell stage embryo) (Table 1). Although there was dilution by endogenous spermidine, the specific activity of the internal pool readily became high enough to permit us to observe the rapid appearance and accumulation of significant radioactive label in CCl_3COOH-insoluble material.

A balance sheet of the intracellular and extracellular distribution of radioactive label derived from exogenously added [^{3}H]spermidine by 4 hr after fertilization is presented in Table 1. As label accumulated intracellularly, the external medium was depleted of label. Resolution of CCl_3COOH-soluble intracellular components by HPLC (9) showed that 93% of the spermidine that had been taken up persisted intact as spermidine and the remaining 7% was metabolized to spermine. Of the total cell-associated [^{3}H]spermidine, 7.5% became CCl_3COOH-insoluble and was quantitatively recovered as spermidine.

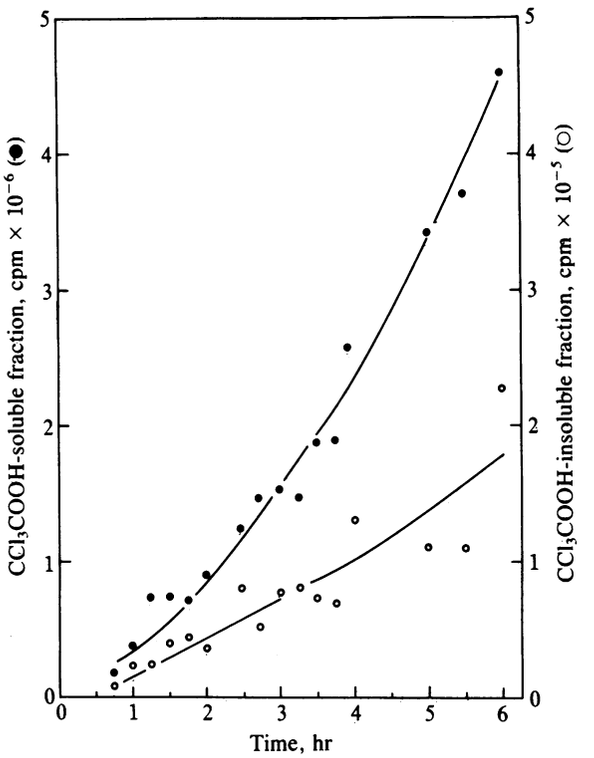

FIG. 1. Incorporation of spermidine into sea urchin embryos. Sea urchin eggs were obtained, fertilized, and cultured at 15°C as described (6). Fertilized eggs were suspended at a concentration of 0.5×10^5 eggs per ml and 30 min after fertilization spermidine was added at 10 μCi/ml (0.67 μM) ([1,8-*methylene*-^{3}H]spermidine; New England Nuclear; 15.5 Ci/mmol; 1 Ci = 37 GBq). Samples equivalent to 7.5×10^3 eggs, taken at various times after fertilization, were diluted with a large excess of sea water and were harvested by centrifugation at 1000 × *g* for 1 min at 4°C. The pelleted embryos were lysed (7) and aliquots of the lysate were used for measurement of CCl_3COOH-soluble (●) and CCl_3COOH-insoluble (○) radioactivity. CCl_3COOH-insoluble fractions were collected on glass fiber filters and assayed for radioactivity in a xylene-based liquid scintillation mixture; CCl_3COOH-soluble radioactivity was determined in tritosol (8). Reported values correspond to 2.5×10^5 cells to facilitate comparison with Table 1 and Fig. 2.

Pronase digestion (11) of the radioactively labeled material from 2.5×10^5 eggs fertilized 4 hr previously yielded five distinct low molecular weight species containing spermidine, as determined by HPLC (data not shown). Since the specificity of Pronase for the peptide bond leaves the isopeptide

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Abbreviation: CCl_3COOH, trichloroacetic acid.

Table 1. Distribution of radioactivity from spermidine during sea urchin embryogenesis (4 hr after fertilization)

Fraction	Total cpm $\times 10^{-3}$	%
Extracellular medium*	16,467	
Cell-associated cpm as % of total cpm in incubation		21.1
CCl_3COOH-soluble†	4,200	
Spermidine		93
Spermine		7
CCl_3COOH-insoluble†	330	
CCl_3COOH-insoluble cpm as % of total cell-associated cpm		7.5
Spermidine		100

All data refer to 2.5×10^5 eggs; fertilized eggs, at a concentration of 1.15×10^5 eggs per ml, were exposed to [1,8-^{3}H]spermidine (10 μCi/ml), and samples were taken as for Fig. 1.

*Radioactive spermidine in the extracellular medium remains nonmetabolized, unchanged as added (analysis by HPLC, using our standard method for resolution of the polyamines and their metabolites (9) (data not shown).

†The CCl_3COOH-soluble and CCl_3COOH-insoluble samples were analyzed as follows (9). Samples of lysate obtained as described in the legend to Fig. 1, corresponding to 2.5×10^5 fertilized eggs [containing 10.0 mg of protein (10) and 330,000 cpm], were precipitated with 5% CCl_3COOH. CCl_3COOH-soluble samples were analyzed by HPLC. The CCl_3COOH-insoluble samples were washed with 5% CCl_3COOH until the washes were free of radioactivity, and, after acid hydrolysis (6 M HCl 110°C 16 hr *in vacuo*), they were analyzed by HPLC.

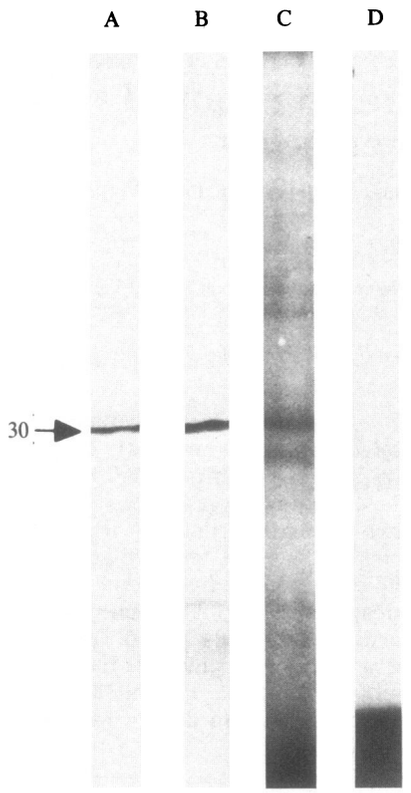

FIG. 2. Gel electrophoretic analysis of macromolecular material after incorporation of spermidine into sea urchin embryos. Samples of embryo lysates corresponding to 2.0×10^4 eggs, from the experiment described in Table 1, were used; in each case, the CCl_3COOH-insoluble material was subjected to 13% NaDodSO$_4$/0.43% bisacrylamide gel electrophoretic analysis, according to the method of Laemmli (16). Lanes: A, 4-hr embryos; B, 6-hr embryos; C, 4-hr embryos after storage of lysate at −20°C for 2 weeks; D, 4-hr embryos after treatment with proteinase K (17). After fluorography, the gels were exposed to Kodak XAR film. Lanes A and B were exposed for 5 days; lanes C and D were exposed for 20 days. The molecular weight is shown as $M_r \times 10^{-3}$.

linkage intact, these small CCl_3COOH-soluble spermidine-containing peptides or isopeptides may reflect transglutaminase-catalyzed covalent attachment of spermidine to protein.

Various studies suggest that, of the naturally occurring polyamines, spermidine may be a preferred endogenous substrate for transglutaminase (12). Addition of dansylcadaverine (1 mM), the transglutaminase inhibitor (13, 14), 30 min after the addition of [^{3}H]spermidine, resulted in ≈50% inhibition of the incorporation of label into the CCl_3COOH-insoluble fraction obtained 4 hr after fertilization (data not shown). This inhibition is consistent with the known activity of dansylcadaverine as an inhibitor of transglutaminase. The possibility that dansylcadaverine interferes with cell uptake of spermidine might be considered. In this regard, however, it is worth noting that recent studies of sea urchin (*Arbacia*) embryogenesis proved conclusively that posttranslational modification of protein by the polyamine putrescine is mediated by transglutaminase (15).

The initial autoradiograph obtained after analytical gel electrophoresis (16) of samples after 4 hr and 6 hr of embryogenesis (Fig. 2, lanes A and B) revealed a major radioactive band with M_r of ≈30,000. Minor components with both higher and lower molecular weights were also evident. Since these became more clearly apparent after storage at −20°C, we suggest that the M_r 30,000 protein is particularly labile and that the secondary bands represent both aggregation and degradation of the primary product (Fig. 2, lane C). Susceptibility to degradation by the nonspecific proteolytic enzyme proteinase K may be also demonstrated (Fig. 2, lane D) (17). This confirms the protein nature of M_r 30,000.

The strikingly high level of incorporation of spermidine into the cellular protein M_r 30,000 is better appreciated when we examine some of its characteristics. For our calculations we used values for embryo protein and polyamines recorded in the literature of 4.0 mg of protein per 10^5 eggs, which remains constant after fertilization during the period of our studies (10), and 0.63 nmol of spermidine per mg of protein in the four-cell embryo (18). We also assumed that complete intracellular equilibration of all noncovalently bound spermidine occurred. We estimated that at 4 hr after fertilization there were about 52 nmol of bound spermidine per g of protein. Comparable studies in sea urchin embryos reported incorporation of 18.2 pmol of putrescine equivalents per g of protein (15). If we assume that the protein M_r 30,000 has 1 mol of spermidine per mol of protein, our calculations of 0.52 nmol of covalently bound spermidine per 2.5×10^5 embryos represent 0.016 mg of protein or 0.16% of total embryo protein. Thus, we estimate that incorporation of spermidine into the protein M_r 30,000 occurs at a rate of ≈16 pg of protein per embryo per hr, which might be compared to total protein synthesis of 250 pg per embryo per hr recorded in embryos of the same age (19).

Evidence for the presence of spermidine and spermine as posttranslational modifiers of macromolecules has been obtained recently. Significant amounts of spermidine are found in rodent seminal plasma proteins (20), small amounts are present in several small proteins in activated human lymphocytes (20) (M_r <20,000), in human amniotic fluid (21) (M_r 10,000–30,000), in conjugated form in human plasma (22, 23), in nuclear and nucleolar proteins from calf liver (24), in HTC (15), and in Friend erythroleukemia cells (25), and spermine is evident in human seminal plasma proteins (unpublished studies). However, a specific protein with bound spermidine has not been identified in any of these examples. It might be noted that spermidine recently has been found covalently linked as a unique glutathione–spermidine conjugate and in

the corresponding bis derivative N^1,N^8-bis(L-γ-glutamyl-L-hemicystinylglycyl)spermidine (26, 27).

Recent elegant studies have proven spermidine to be the proximal precursor of hypusine (5, 28) in the protein eIF-4D with M_r 18,000. The protein species we have identified in these early embryos has a M_r 30,000 with no label in hypusine. We therefore have described a protein distinct from eIF-4D, the only other specific protein known to date to be associated with polyamines. Our findings of persistent high accumulation of intact spermidine label in this unique protein of M_r 30,000 may point to a specificity by the embryonic cell for the synthesis of a unique protein. Other proteins of M_r 30,000 have been observed in sea urchin embryos. One, a major protein characteristic of the early stages of development, disappears as embryogenesis proceeds (29), and another, a glycosylated protein, seems to be associated with gastrulation (30). It is tempting to hazard a guess that our protein containing spermidine may be related to other embryonic proteins or may be a protein precursor to some other posttranslationally modified protein. Studies are necessary to evaluate the precursor hypothesis and to determine whether the M_r 30,000 protein is a marker of early embryogenesis.

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