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N-Terminal and C-Terminal Segments of Actin Participate in Binding Depactin, an Actin-Depolymerizing Protein from Starfish Oocytes†

Kazuo Sutoh* and Issei Mabuchi

ABSTRACT: A one to one complex of actin monomer and depactin (an actin-depolymerizing protein isolated from starfish oocytes) was cross-linked with the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to generate a covalently cross-linked complex of actin and depactin. Cross-linking sites of depactin in the actin sequence were determined by mapping products after partial cleavages of the actin–depactin cross-linked complex. The peptide mappings have revealed that acidic residues in the N-terminal segment of actin (Asp-1, Glu-2, Asp-3, Glu-4, and Asp-11) are candidates for one of the cross-linking sites of depactin while lysine and/or acidic residues in its C-terminal segment (Lys-358, Glu-360, Asp-362, Glu-363, and Lys-372) are candidates for another cross-linking site of depactin. Since EDC can cross-link amino and carboxyl groups only when they are in direct contact, it is very likely that some of those residues in the N-terminal and C-terminal segments of actin participate in binding depactin.

Actin plays pivotal roles in muscle and nonmuscle cells. In muscles, interactions between actin and other proteins such as myosin and tropomyosin are the basis of their contraction and relaxation. In nonmuscle cells, actin is a major constituent of both their contractile systems and their cytoskeletal structures. In these nonmuscle cells, many types of "actin-binding proteins" regulate the supramolecular structure of actin [as a review, see Craig & Pollard (1982)]. The primary structure of actin is highly conserved (Elzinga et al., 1973; Elzinga & Lu, 1976; Vandekerckhove & Weber, 1978a–d), and therefore, actin from various sources can bind a large number of proteins isolated from various muscle and nonmuscle cells. It seems that the surface of the actin molecule is covered with a large number of binding sites specific for these proteins. In a sense, actin is a protein which has highly evolved so that it can bind a large number of proteins.

In echinoderm eggs, actin is a constituent of contractile and cytoskeletal structures such as microvilli or the contractile ring. Several actin-binding proteins which might function in organization and disorganization of these structures have been isolated (Kane, 1976; Ishimoda-Takagi, 1978; Mabuchi & Hosoya, 1982; Mabuchi, 1981, 1983). One approach to an understanding of the molecular mechanism by which these actin-binding proteins exert their effects on the supramolecular structure of actin would be to locate their binding sites in the primary as well as three-dimensional structures of actin.

A simple method has been developed to identify actin segments which participate in binding various proteins, and this method has been applied to locate binding sites of myosin (Sutoh, 1982a) and DNase I (Sutoh, 1984). The same method has also been employed to locate the actin–actin contact site responsible for self-association of subunits into F-actin (Sutoh, 1984). Since X-ray diffraction studies on actin–DNase I crystals are now in progress (Suck et al., 1981; Sakabe et al., 1983), it will soon become possible to locate binding sites for various proteins in the three-dimensional structure of actin once their locations in the primary sequence of actin are identified by the above method.

In this paper, we identified actin segments participating in binding depactin, an actin-depolymerizing protein isolated from starfish oocytes (Mabuchi, 1981, 1983).

Materials and Methods

Proteins and Reagents. Depactin was prepared as previously described (Mabuchi, 1983). Actin was prepared according to the method of Spudich & Watt (1971). It was further purified by chromatography on Sephadex G-150. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC)[1] was purchased from Nakarai Chemical Co. (Tokyo, Japan). BNPS-skatole was from Pierce Chemical Co. (Rockford, IL). *N*-[7-(Dimethylamino)-4-methyl-5-courmarinyl]maleimide (DACM) was from Wako Chemical Co. (Tokyo, Japan).

Cross-Linking of the Depactin–Actin Complex. G-Actin (1 mg/mL) in 2 mM Tris-HCl, 0.1 mM ATP, and 0.1 mM $CaCl_2$ (pH 8.0) was labeled with DACM at a molar ratio of 1.0:0.8 (actin monomer:DACM) for 5 min at 0 °C. Under the conditions, Cys-373 of actin was selectively labeled with the fluorescent dye (Sutoh, 1982a). The DACM-labeled actin in the above solvent was mixed with depactin in 0.1 M KCl,

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine; DACM, *N*-[7-(dimethylamino)-4-methyl-5-coumarinyl]-maleimide; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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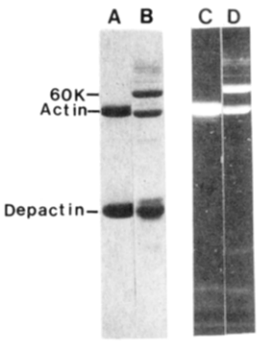

FIGURE 1: Cross-linking of the depactin–actin complex with EDC. A one to one complex of depactin and actin monomer was cross-linked with EDC to generate the covalently linked product with an apparent molecular weight of 60 000. Lanes A and C, before the cross-linking reaction; lanes B and D, after the cross-linking reaction. Acrylamide gels [15% acrylamide–0.4% bis(acrylamide)] were illuminated with a UV lamp to detect fluorescent peptide bands immediately after electrophoresis (lanes C and D) and then stained with Coomassie Blue (lanes A and B). A peptide band corresponding to the covalently linked product of depactin and actin is indicated as "60K" on the gels. Cross-linking conditions were as follows: solvent, 0.1 M NaCl, 20 mM imidazole, and 1 mM $MgCl_2$ (pH 7.0); cross-linker, EDC (20 mM); temperature, 25 °C; reaction time, 2 h; proteins, 0.8 mg/mL depactin and 1.0 mg/mL actin.

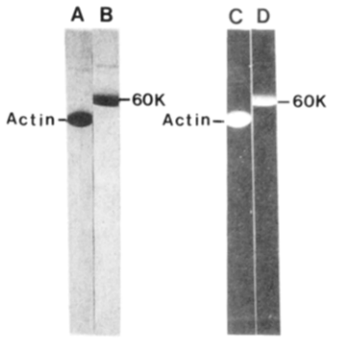

FIGURE 2: Purity of peptides isolated from acrylamide gels. Electrophoretically purified actin and the 60K product were electrophoresed in the presence of $NaDodSO_4$ on acrylamide gels [15% acrylamide–0.4% bis(acrylamide)]. Lanes A and C, actin; lanes B and D, the 60K cross-linked product. Acrylamide gels were illuminated wiht a UV lamp after electrophoresis to detect fluorescent peptides (lanes C and D) and then stained with Coomassie Blue (lanes A and B).

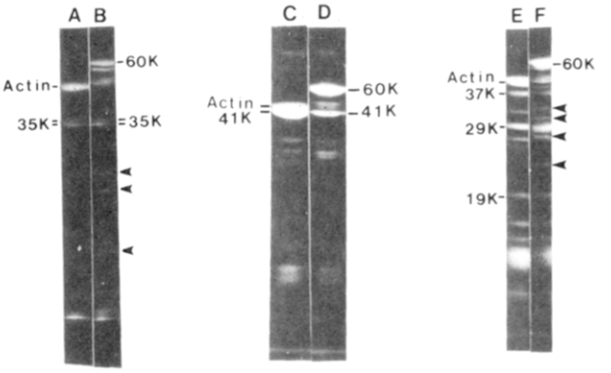

FIGURE 3: Chemical cleavages of the control actin and the 60K product. Lane A, actin was cleaved with BNPS-skatole; lane B, the 60K product was cleaved with BNPS-skatole; lane C, actin was cleaved with hydroxylamine; lane D, the 60K product was cleaved with hydroxylamine; lane E, actin was cleaved with CNBr; lane F, the 60K product was cleaved with CNBr. Arrowheads on lanes B and F indicate fluorescent fragments which were detected only in cleavage mixtures of the 60K product. Electrophoresis was carried out on acrylamide gels [15% acrylamide–0.4% bis(acrylamide)] in the presence of $NaDodSO_4$.

0.2 mM DTT, and 10 mM MOPS (pH 7.4) at a molar ratio of 2:1 (depactin:actin monomer). After the mixture was incubated for 1 h at 0 °C, it was dialyzed against 0.1 M NaCl, 20 mM imidazole, and 1 mM $MgCl_2$ (pH 7.0). To the solution was added 0.1 volume of 0.2 M EDC in 0.1 M imidazole (pH 7.0) to initiate the cross-linking reaction. After 2 h at 25 °C, 0.01 volume of 2-mercaptoethanol was added to quench the cross-linking reaction.

Chemical Cleavages of Purified Peptides. Cross-linked products were electrophoresed in the presence of $NaDodSO_4$ according to the method of Laemmli (1970). Monomeric actin and a covalently linked complex of actin and depactin were identified on acrylamide gels by illuminating them with a UV lamp (they were fluorescent because actin was labeled with DACM). Fluorescent bands corresponding to actin monomer and the actin–depactin complex were cut out, and fluorescent peptides were eluted out of gels into small dialysis tubes electrophoretically. The collected peptides were again electrophoresed on $NaDodSO_4$–acrylamide gels. Fluorescent bands were again cut out under illumination with a UV lamp. They were washed with 10% methanol and then with 50% methanol and dried in vacuo.

For chemical cleavages of the purified peptides, dried gel slices were soaked in 70% formic acid containing 20 mM CNBr, in 60% acetic acid containing BNPS-skatole (1 mg/mL), or in 6 M guanidine hydrochloride containing 0.5 M hydroxylamine as previously described (Sutoh, 1981, 1982a,b, 1983, 1984). After cleavage reactions, resulting gel slices were washed with 50% methanol containing 1% 2-mercaptoethanol and then with 50% methanol. They were dried in vacuo. These dried gels were soaked in 1% $NaDodSO_4$, 10 mM Tris-HCl, and 1% 2-mercaptoethanol (pH 8.0) for 1 h at 37 °C and then loaded on a slab gel directly for $NaDodSO_4$–acrylamide gel electrophoresis (Laemmli, 1970).

Protein Concentrations. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Cys-373 of actin was covalently labeled with the fluorescent dye DACM (Sutoh, 1982a). The DACM–actin was then mixed with excess depactin to form a one to one complex of actin and depactin (Mabuchi, 1983). The resulting actin–depactin complex was treated with the zero-length cross-linker EDC to introduce cross-links between actin and depactin at their interface. As shown in Figure 1 (lane B), EDC treatment generated a cross-linked product with an apparent molecular weight of 60 000 with concomitant loss of actin and depactin polypeptides, consistent with previous observations (Mabuchi, 1983). When the fluorescence of DACM covalently linked to Cys-373 of actin was examined on the $NaDodSO_4$–acrylamide gel, it was found that the 60K product as well as monomeric actin was fluorescent (lane D in Figure 1).

The fluorescent actin and the 60K product were electrophoretically purified to homogeneity as shown in Figure 2. These purified peptides were subjected to various chemical cleavages. First, we used BNPS-skatole, which cleaves tryptophanyl bonds very selectively (Fontana, 1972). As shown in Figure 3 (lane A), digestion of the fluorescent actin with BNPS-skatole generated doublet fluorescent bands with apparent molecular weights of ~35 000 together with a low molecular weight fluorescent band on a $NaDodSO_4$–acrylamide gel. Since there are only four tryptophan residues in actin, i.e., Trp-79, Trp-86, Trp-339, and Trp-355 (Elzinga et al., 1973), the doublet bands seem to correspond to actin

fragments spanning residues 80–374 and 87–374. The low molecular weight band seems to correspond to fragments spanning residues 340–374 and 356–374. When the 60K fluorescent product was digested with BNPS-skatole, the doublet fluorescent bands were again observed (lane B in Figure 3). This result indicates that at least one of the cross-linking sites of depactin is located outside these actin segments spanning residues 80–374 and 87–374. In other words, a cross-linking site of depactin is located within an actin segment spanning residues 1–79.

Although we assumed in the above argument that the fluorescent doublet bands generated from the 60K cross-linked peptide correspond to free C-terminal fragments of actin, we have to consider another possibility. If BNPS-skatole cleaves depactin to generate a small fragment containing a binding site of actin, a complex with similar electrophoretic mobility to actin might be formed with any region of actin. Thus, the fluorescent doublet bands generated from the 60K product might be complexes of the actin fragments and a small depactin fragment. If this is the case, it is expected that the fluorescent band pattern (with respect to both mobilities and fluorescent intensities of bands) generated from the 60K product is very similar to that of actin up to the 42K actin band. As shown in Figure 3, however, the BNPS-skatole digestion of the 60K product did not release the 42K fluorescent peptide, indicating that the above possibility is very remote.

It must be noticed here that several fluorescent bands generated from the 60K product with BNPS-skatole digestion are not in alignment with those from the control actin (indicated by arrowheads in lane B in Figure 3). Since the BNPS-skatole digestion of the control actin never released these extra fluorescent fragments, they must be cross-linked complexes of fluorescent actin fragment(s) and depactin fragment(s). Fluorescent actin fragments of residues 80–374 and 87–374 cannot be constituents of these extra fragments since the apparent molecular weights of these extra fragments are smaller than those of the doublet fluorescent fragments of residues 80–374 and 87–374. Therefore, it is very likely that these extra fragments contain the actin fragment of residues 340–374 or 356–374. This argument leads us to conclude that another cross-linking site of depactin resides within an actin segment spanning residues 340–374.

As the next step for mappings of cross-linking sites of depactin in the actin sequence, we employed hydroxylamine, which cleaves an Asn–Gly bond in actin (Asn-12–Gly-13) (Elzinga et al., 1973; Bornstein & Balian, 1977; Sutoh, 1982a). When fluorescent actin was subjected to hydroxylamine cleavage, the Asn–Gly bond in actin was cleaved to release a fluorescent fragment with an apparent molecular weight of 41 000 as revealed by $NaDodSO_4$–acrylamide gel electrophoresis (lane C in Figure 3), consistent with the previous result (Sutoh, 1982a). The fluorescent 41K fragment spans residues 13–374. Hydroxylamine treatment of the 60K product released the 41K fluorescent fragment as shown in lane D (Figure 3), indicating that at least one of the cross-linking sites of depactin is located outside the 41K fragment. Thus, it seems that a cross-linking site of depactin resides within an actin segment spanning residues 1–12. On the basis of the same argument described for the BNPS-skatole cleavage, we can exclude the possibility that the 41K fluorescent fragment released from the 60K product is not a free C-terminal fragment of actin but a cross-linked complex of the fragment and a small depactin fragment spanning only several residues.

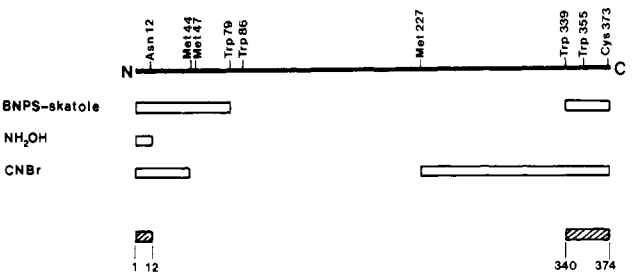

FIGURE 4: Locations of cross-linking sites of depactin in the actin sequence. In the upper part of the figure, locations of several residues cited in the text are depicted along the actin polypeptide (shown as a horizontal line). Horizontal open bars indicate peptide segments identified as participating in cross-linking with depactin. The shortest actin segments participating in cross-linking with depactin are deduced from these results and shown in the lower part of the figure as hatched bars. Numbering of residues follows that by Elzinga et al. (1973), though actin actually contains 375 residues.

Partial CNBr digestion of the control actin generated a series of fluorescent fragments (lane E in Figure 3). All of these fragments have been assigned to segments in the actin sequence (Sutoh, 1982a, 1984). When the 60K product was subjected to partial CNBr cleavage, a similar fluorescent band pattern was observed on a $NaDodSO_4$–acrylamide gel (lane F in Figure 3). Up to the 19K band, fluorescent bands released from the 60K product are completely in alignment with those of actin. In the 19–37K region, however, several fluorescent bands released from the 60K product are not in alignment with those of actin (indicated by arrowheads in lane F of Figure 3). Those extra fragments must be cross-linked complexes of fluorescent actin fragment(s) and depactin fragment(s). Therefore, it seems that at least one of the cross-linking sites of depactin is located within the C-terminal 19K segment of actin, which spans residues 228–374 (Sutoh, 1982a, 1984). Furthermore, it was found that CNBr cleavage of the 60K product generated fluorescent fragments whose mobilities on the gel were the same as those of the 29K and 37K fluorescent fragments of the control actin. Although we cannot exclude the possibility that these 29K and 37K fluorescent fragments in lane F (Figure 3) are cross-linked products as other extra fluorescent fragments, we tentatively identify them as free C-terminal fragments of actin by taking the results of the BNPS-skatole and hydroxylamine cleavages into account. Thus, it seems likely that one of the cross-linking sites of depactin is located outside the C-terminal 37K segment of actin, i.e., within the N-terminal segment spanning residues 1–44 (Sutoh, 1982a, 1984).

The results of peptide maps are summarized in Figure 4. Taking all of those results into account, we conclude that at least two sites in actin are involved in cross-linking with depactin, one in its N-terminal segment spanning residues 1–12 and the other in its C-terminal segment spanning residues 340–374. Since EDC cross-links amino and carboxyl groups very selectively, Asp-1, Glu-2, Asp-3, Glu-4, and Asp-11 in the N-terminal segment and Lys-358, Glu-360, Asp-362, Glu-363, and Lys-372 in the C-terminal segment (Elzinga et al., 1973) are candidates for cross-linking sites of depactin.

Discussion

Depactin, an actin-binding protein isolated from starfish oocytes, rapidly depolymerizes F-actin in a stoichiometric manner under physiological conditions (Mabuchi, 1983). Depolymerized actin subunits form one to one complexes with depactin [association constant of $(2–3) \times 10^6\ M^{-1}$] (Mabuchi, 1983). It has been speculated that this protein acts along the length of F-actin and actively promotes depolymerization by

binding and sequestering monomers.

Cross-linking of the one to one complex of actin and depactin with the zero-length cross-linker EDC has revealed that acidic residues in the N-terminal segment of actin (Asp-1, Glu-2, Asp-3, Glu-4, and Asp-11) as well as lysine and acidic residues in its C-terminal segment (Lys-358, Glu-360, Asp-362, Glu-363, and Lys-372) are candidates for cross-linking sites of depactin. Since EDC can cross-link amino and carboxyl groups only when they are in direct contact, some of these residues in the N-terminal and C-terminal segments of actin must be in direct contact with depactin. In other words, some of those residues must be in a region (or regions) of the actin–depactin contact. Furthermore, ionic interactions are expected between residues involved in EDC cross-linking. The ionic interactions might stabilize the one to one complex of actin and depactin. This notion is consistent with the previous observation that the actin–depactin complex is dissociated in a high ionic strength solvent (Mabuchi, 1983).

Although the N-terminal and C-terminal segments of actin were identified as binding sites of depactin, it does not necessarily mean that there are two binding sites which are spatially separated on actin. Considering the fact that depactin is a small globular protein (M_r 17000) (Mabuchi, 1983), it is quite probable that the contact surface area between actin and depactin is rather limited and that the N-terminal and C-terminal segments of actin are in proximity with each other to form a single binding site for depactin.

It has been previously shown that the N-terminal and C-terminal segments of actin also participate in binding myosin (Sutoh, 1982a,b, 1983). Acidic residues in the N-terminal segment of actin, i.e., Asp-1, Glu-2, Asp-3, Glu-4, and Asp-11, are candidates for the binding site of the myosin heavy chain, while acidic residues in its C-terminal segment, i.e., Glu-360, Asp-362, and Glu-363, are candidates for the binding site of the myosin light chain (alkaline light chain 1). Thus, in actin, the binding site of depactin overlaps with that of myosin at least partially. This is consistent with the observation that depactin cannot depolymerize F-actin when it is complexed with myosin prior to the addition of depactin (Mabuchi, 1983). Although depactin and myosin share the same contact surface on actin, their effects on actin are very different; depactin depolymerizes F-actin while myosin accelerates polymerization of actin.

It is well-known that the N-terminal segment of actin shows a disproportionately high rate of amino acid exchange (Vandekerckhove & Weber, 1978a–d). This implies that this segment actively participates in various functions of actin. This notion is consistent with our results that two functionally different proteins, myosin and depactin, use the N-terminal segment as their binding sites. In this context, it must also be noticed that the N-terminal segment of actin, especially the first 12 residues, seems to participate in binding divalent cations (Jacobson & Rosenbusch, 1976; Barden & dos Remedios, 1983).

It would be noteworthy that the C-terminal segment of actin identified as one of the cross-linking sites of depactin is actually close to a subunit contact site in F-actin; Cys-373 in an actin subunit is only 8 Å from one of the lysine residues in the CB 17 segment of a neighboring subunit (Sutoh, 1984). Thus, on the surface of actin, the actin–depactin contact site (at least a part of it) is very close to the actin–actin contact site. Therefore, it is tempting to speculate that a part of the depactin molecule in the actin–depactin complex extends to the actin–actin contact site to block self-association of actin subunits by steric hindrance.

Bovine pancreatic DNase I is another type of actin-depolymerizing protein (Lazarides & Lindberg, 1974; Hitchcock et al., 1976; Mannhertz et al., 1980). Recently it has been shown that an actin segment spanning residues 48–82 participates in binding DNase I (Sutoh, 1984). Thus, it seems that the binding site of DNase I is different from that of depactin, at least in the primary sequence of actin. The observation that the actin–depactin complex can bind to a DNase I–Sepharose column (Mabuchi, 1983) indicates that the actin–DNase I contact site is actually far from the actin–depactin contact site in the three-dimensional structure of actin, consistent with the above result. The fact that DNase I and depactin use different binding sites on actin may reflect the difference in their mode of action on F-actin.

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Embryonal Histone H1 Subtypes of the Sea Urchin *Strongylocentrotus purpuratus*: Purification, Characterization, and Immunological Comparison with H1 Subtypes of the Adult†

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ABSTRACT: The four H1 subtypes utilized during embryonic development of *Strongylocentrotus purpuratus* were purified. Their amino acid compositions and immunological reactivities show that they have different primary structures. Antisera against them were used to identify the H1 subtypes present in adult tissues. These experiments revealed that adult tissues do not contain detectable levels of the two H1 subtypes expressed in the earliest embryonic stages (H1cs and H1α) but do contain the two subtypes expressed from the blastula stage on (H1β and H1γ). In addition, all adult tissues examined contain H1λ, a subtype of unusually low apparent molecular weight, which is not found in the embryo prior to the feeding larval stage and is not closely related immunologically to any of the embryonal H1 subtypes.

Histone H1 exhibits the greatest variation in primary structure of the five major histone classes (Kinkade & Cole, 1966; Kinkade, 1969; Rall & Cole, 1971). Most organisms contain more than one H1 protein (termed H1 subtypes), and in mammalian cells, the subtypes present and their relative proportions depend on the cell type (Bustin & Cole, 1968; Kinkade, 1969; Seyedin & Kistler, 1980). Since H1 is involved in higher order chromatin structure (Finch & Klug, 1976; Thoma & Koller, 1977; Thoma et al., 1979; Butler & Thomas, 1980), it seems possible that different H1 subtypes may give rise to different higher order structures and in so doing affect some coarse regulation of genetic expression. This notion is supported by the observations that the proportions and kinds of H1 subtypes synthesized can be changed by hormonal stimulation (Hohmann & Cole, 1971) and by inhibition of cell division (Panyim & Chalkley, 1969a; Pehrson & Cole, 1980, 1982; Lennox & Cohen, 1983) and that the different subtypes are phosphorylated to different degrees and on different schedules during the cell cycle (Ajiro et al., 1981; Lennox et al., 1982).

The sea urchin embryo undergoes dramatic changes in the expression of H1 subtypes during its development (Hill et al., 1971; Ruderman & Gross, 1974; Newrock et al., 1978). H1cs, the H1 subtype that is stored in the egg (Newrock et al., 1978; Salik et al., 1981), is the predominant subtype present during the first five cell divisions; these cell divisions occur about once an hour. Next, H1α appears and by the blastula stage is the major H1 subtype; during this time, the rate of cell division slows significantly. Two other H1 subtypes, H1β and H1γ, appear in the chromatin during the blastula stage, and by gastrulation, they are the only H1 subtypes being made by the embryo; during this period, cell proliferation continues to slow, and many noncycling cells appear for the first time (L. H. Cohen and G. McFadden, unpublished results). A fifth H1 subtype, H1λ, first appears in the late larva and is an abundant subtype in adult tissues (L. H. Cohen, K. Newrock, and R. Hinegardner, unpublished results). A gene coding for *Strongylocentrotus purpuratus* H1α has been cloned and sequenced (Levy et al., 1982), and three *Parechinus angulosus* H1 subtypes resembling H1α, H1β, and H1γ in the timing of their expression during development have been purified and partially sequenced (De Groot et al., 1983). In this paper, we report the purification of the four *S. purpuratus* embryonal H1 subtypes and some chemical and immunological comparisons of these proteins. We have succeeded in preparing antibodies that are monospecific against H1cs and H1α and have used these together with unabsorbed antisera to investigate whether these subtypes are present in the tissues of the adult.

Materials and Methods

Nuclear Isolation and Histone Extraction. Cultivation of embryos and isolation of nuclei from embryos were carried out as described previously (Cohen et al., 1975). Adult tissues were obtained from male urchins to avoid contamination by eggs or oocytes containing H1cs. Intestines were dissected from the animal and washed several times with ice-cold seawater to remove digestive juices and food. To obtain tube feet, urchins were rapidly removed from glass beakers, leaving many tube feet attached to the beaker. Coelomocytes were obtained by first injecting the urchin with 2 mL of 10% ethylenediaminetetraacetic acid (EDTA),[1] pH 7.0, to inhibit clotting. Aristotle's lantern was then cut away, and the coelomic fluid (usually ~10 mL per urchin) was drained into a chilled beaker containing 2 mL of EDTA per urchin. The coelomic fluid was filtered through cheesecloth, and an equal volume of 0.8 M dextrose was added. Coelomocytes were spun down at 1000*g* for 10 min. The following procedures were all performed at 0-4 °C. The tissues were homogenized with a Dounce hom-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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