Vol. 80, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1978 Pages 833-840

GLYCOPROTEIN SYNTHESIS IN DEVELOPING SEA URCHIN EMBRYOS[1]

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Received January 4, 1978

Summary Surface membrane glycoproteins have been postulated in many mammalian cells to be involved in external surface membrane functions such as cell adhesion, cell-cell recognition, and cell movement. In developing echinoderm embryos, cell adhesion, recognition, and movement of individual cell types have been attributed to differences in the external surface membranes of these cells. Results reported here suggest that the three cell types of 16-cell sea urchin embryos have a mechanism that could establish differences in the carbohydrate portion of glycoproteins located in the external surface membrane. The results demonstrate 1) that glycoproteins are synthesized during early sea urchin development and 2) that slightly different rates of glycoprotein synthesis exist for the three types of blastomeres from 16-cell sea urchin embryos.

Introduction The location of glycoproteins on the external cell surface membrane has been established for many cells. The carbohydrate portion of glycoproteins and other complex glycoconjugates may provide part of the high degree of molecular specificity necessary for complex cell functions such as cellular adhesion, cell-cell recognition, and embryonic cell movement. In echinoderm development it has been suggested that certain movements of the embryonic cell may be explained by differences in the external surface membranes (Gustafson & Wolpert, 1967; Karp & Solursch, 1974; Roberson & Oppenheimer, 1975). During gastrulation, groups of cells often appear to move independently of each other in such a manner as could be explained by differences in their external surface membranes (Gustafson & Wolpert, 1967). In sea urchin embryos three cell types are recognizable as early as the 16-cell stage. Differences in the adhesiveness of these 16-cell blastomeres have led to the speculation that the surfaces of these early embryonic cells may be different (Hynes and Gross, 1970). In addition, evidence has been presented suggesting differences in the external membrane surface carbohydrates of the individual blastomere types that appear to

[1]Supported in part by USPHS grants NS 12461, CA 13220, and GM 00032 . H.B.B. is a Scholar of the Leukemia Society of America.

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0006-291X/78/0804-0833$01.00/0

correlate with individual cell movement and the general adhesive properties of the blastomere types (Karp & Solursch, 1974; Roberson & Oppenheimer, 1975; Neri et al., 1975).

Since the possibility clearly existed that external surface membrane glycoproteins of the individual sea urchin blastomeres were different and that these differences might contribute to such important developmental phenomena as embryonic cell movement, the aim of the research described here was to determine whether glycoproteins are synthesized during early sea urchin development and, if so, to determine the patterns of synthesis of these macromolecules in the three types of sea urchin blastomeres at the 16-cell stage. The importance of this work is supported by the fact that in a recent abstract (Chamberlain, 1977), slight differences in proteins synthesized by the three cell types of 16-cell embryos were described.

The incorporation of radiolabeled glucosamine into acid precipitable sea urchin macromolecules was used to measure glycoprotein synthesis. Glucosamine incorporation into acid precipitable macromolecules has been used in many mammalian cell systems as a measure of glycoprotein synthesis. It has been demonstrated that glucosamine is a precursor for N-acetylglucosamine, N-acetylgalactosamine, and sialic acid residues of glycoproteins (Kornfeld & Ginsburg, 1966; Hayden, Crowley & Jamieson, 1970; Cook & Stoddard, 1973).

Methods

Handling of sea urchins, gametes, and embryos. Arbacia punctulata were purchased from Mr. Glendle Noble, Panama City, Florida. Lytechinus pictus were purchased from Pacific BioMarine, Inc., Venice, California. Although most of the experiments reported here have been performed with both species and have yielded similar results, we have not specifically tested the hypothesis that species differences exist. Thus the present data pertain to the A. punctulata from the west coast of Florida.

The procedures used for maintenance of sea urchins and the collection and fertilization of gametes are similar to those described by Harvey (1956). The artificial sea water used was made daily according to the method of Spiegel & Rubinstein (1972). The embryos were incubated at a concentration of 10^3 to 10^4 embryos per ml in a Bellco hanging bar magnetic stirrer (Bellco Biological Glassware and Equipment, Vineland, New Jersey), with the stirrer operating just fast enough to keep the embryos suspended. In order to obtain the most synchronous development, A. punctulata were incubated at 18°C. The embryos divide within 5 min of each other; this degree of synchronization can be observed in A. punctulata through the 16-cell stage. Only batches of embryos that showed more than 99% fertilization and normal synchronous divisions were used in these investigations.

Glucosamine incorporation into sea urchin eggs or embryos. In general, the embryos from one female sea urchin were cultured at a concentration of 10^3 to 10^4 embryos per ml, as described above. At the particular developmental stage desired, the embryos were harvested and concentrated to 10^5 embryos per ml. Aliquots of this suspension were incubated with D-[U-^{14}C]-

glucosamine (222 Ci/mol from New England Nuclear, or 318 Ci/mol from Amersham Searle). The final egg or embryo concentration was usually 5 x 10^4 eggs or embryos/ml, and the final D-[U-^{14}C]glucosamine concentration was 5 x 10^{-5} M. For incubations requiring different concentrations of radiolabeled precursor the total volume and egg or embryo concentration were held constant. Incubations were performed at 25°C. Under these conditions, and with gentle shaking of the incubation tubes by hand every 5 min, normal embryo development, cleaving at the same time as controls, was observed for at least 1 hr.

Reactions with the radiolabeled glucosamine were terminated by the addition of 5 ml of ice-cold 1% (by vol) phosphotungstic acid in 0.5 N HCl. Precipitates were centrifuged at 2000 x g for 5 min and washed twice with 10% (by vol) trichloroacetic acid and once with ethanol:ether (2:1, v/v) (Bosmann, Hagopian & Eylar, 1969). Precipitates were then dissolved in 1 ml of NCS (Amersham Searle) overnight in the dark, neutralized with 40 µl of glacial acetic acid, and added to 15 ml of Aquasol (New England Nuclear) scintillation cocktail for radioactive determination.

Discontinuous sodium dodecyl sulfate gel electrophoresis of D-[U-^{14}C]-glucosamine-labeled sea urchin macromolecules. Sea urchin embryos were incubated with D-[U-^{14}C]glucosamine (222 Ci/mol) for 30 min, as already described. The incubation was terminated and excess D-[U-^{14}C]glucosamine was removed with three ice-cold sea water washes (200 x g for 2 min). The SDS gel electrophoresis system used was as described by Neville (1971) and Glossman & Neville (1971). The separating gel was 11.1% polyacrylamide, and the stacking gel was 3.2% polyacrylamide. The staining procedure for localization of glycoproteins was a periodic acid-Schiff reaction that has been modified by Glossman & Neville (1971) to completely remove interfering SDS from both the gels and the proteins. Optical density of the gels after staining was monitored at 500 nm. Fractions 0.5 cm thick were sectioned, completely oxidized in 50% hydrogen peroxide, and dissolved in Aquasol (New England Nuclear). The radioactivity in each fraction was then determined.

Recovery of D-[U-^{14}C]glucosamine from A. punctulata macromolecules. In order to determine the identity of the radioactivity in the sea urchin macromolecules labeled with D-[U-^{14}C]glucosamine (222 Ci/mol), monosaccharides were released from precipitated macromolecules by acid hydrolysis and identified by paper chromatography according to Spiro (1966) and Bernacki (1973).

16-Cell sea urchin blastomere dissociation and separation. For separation of 16-cell A. punctulata blastomeres, the embryos were demembranated with papain (E.C. 3.4.4.10) (Tyler & Spiegel, 1956; Spiegel & Rubinstein, 1972), cultured to the 16-cell stage, dissociated, and then separated on a 5-15% Ficoll gradient (Hynes & Gross, 1970). Demembranated embryos were cultured by standard techniques. The demembranated embryos were collected, cooled to 4°C, and dissociated by gentle shaking in calcium- and magnesium-free sea water plus 2 mM ethylenediaminetetraacetic acid (EDTA). Two to five ml of these dissociated blastomeres were layered onto 40-ml gradients of 5-15% Ficoll in Ca^{2+}- and Mg^{2+}-free sea water at 4°C and centrifuged in a swinging bucket-type rotor at 250 x g for 1 min. The individual cell types were then removed with a Pasteur pipet and washed free of Ficoll with Ca^{2+}- and Mg^{2+}-free sea water.

Protein determination. Protein determinations were made using the procedure of Lowry et al. (1951).

Materials. Radiochemicals were obtained from New England Nuclear, Boston, Mass., D-[U-^{14}C]glucosamine hydrochloride, 222 Ci/mol, and from Amersham-Searle, Arlington Heights, Illinois, D-[U-^{14}C]glucosamine hydrochloride, 318 Ci/mol.

Chemicals used in making sea water for experimental work were obtained from Baker Chemical Company, Phillipsburg, New Jersey.

Acrylamide and N,N'-methylenebisacrylamide were obtained from Eastman Organics, Inc., Rochester, New York.

Papain (EC 3.4.4.10; 2 x crystallized, specific activity approximately 10 U/mg), lactoperoxidase (EC 1.11.1.-) and RNase (EC 2.7.7.16) were obtained from Worthington Biochemicals, Freehold, New Jersey.

All materials not mentioned above or in the text were obtained from either Sigma Chemical Company, St. Louis, Missouri, or Fisher Scientific Company, Rochester, New York.

Results

Characterization of D-[U-^{14}C]glucosamine incorporation into 16-cell sea urchin embryos. Figure 1 shows a time course for the incorporation of D-[U-^{14}C]glucosamine (50 μM final concentration) into 16-cell sea urchin acid precipitable macromolecules. After an initial lag, the incorporation increases at a constant rate for the length of time observed--60 min.

Following incubation of 16-cell sea urchin embryos with D-[U-^{14}C]glucosamine, the acid precipitated material was hydrolyzed to liberate monosaccharides from the precipitated glycoproteins and separated by paper chromatography to identify the radioactive monosaccharides. It was determined (results not shown) that 80% of the radioactivity in the acid precipitable sea urchin macromolecules was liberated by acid hydrolysis under conditions specific for the release of neutral and amino monosaccharides. It was also found that 70% of the acid-liberated radioactive material co-chromatographed with a D-[U-^{14}C]glucosamine standard.

Association of incorporated D-[U-^{14}C]glucosamine with glycoprotein. Figure 2 shows discontinuous SDS gel electrophoresis of 16-cell sea urchin macromolecules that had been incubated with 50 μM D-[U-^{14}C]glucosamine (222 Ci/mol) for 30 min at 25°C. These results demonstrate that the radioactive material is associated with several prominent sea urchin glycoproteins. Molecular weights of these glycoproteins were estimated by comparison with known protein standards [β-galactosidase (EC 3.2.1.23), lactoperoxidase (EC 1.11.1-), ovalbumin, and RNase (EC 2.7.7.16)]. From these comparisons the radioactivity is associated with a high molecular weight glycoprotein band, ∿150,000; an intermediate molecular weight glycoprotein band, ∿45,000; and a low molecular weight glycoprotein band, ∿10,000.

Glucosamine incorporation into macromolecules by fertilized sea urchin eggs, and 2- and 16-cell sea urchin embryos. The results in Table 1 show

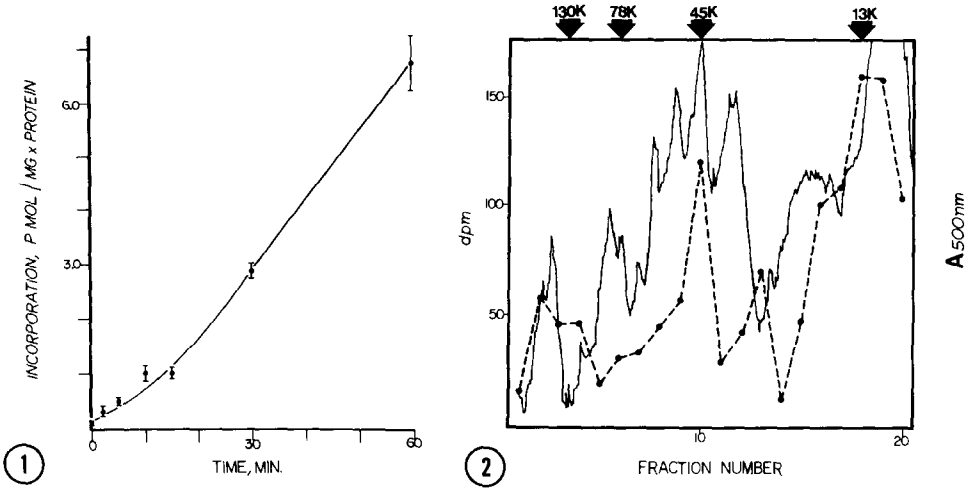

Fig. 1. Time course of D-[U-^{14}C]glucosamine incorporation into 16-cell sea urchin embryos. Sixteen-cell Arbacia punctulata embryos were incubated with 50 μM D-[U-^{14}C]glucosamine (222 Ci/mol) for various times at 25°C. Radioactivity is that present in phosphotungstic acid, 2 x trichloroacetic acid, ethanol-ether (2:1, v/v) insoluble material. Data points are means ± S.E.M. These results are from duplicate determinations performed on 2 to 4 batches of embryos (i.e., the fertilized eggs from 2 to 4 sea urchins).

Fig. 2. Discontinuous SDS gel electrophoresis of D-[U-^{14}C]glucosamine labeled sea urchin macromolecules. Sixteen-cell Arbacia punctulata embryos were incubated with 50 μM D-[U-^{14}C]glucosamine (222 Ci/mol) for 30 min at 25°C. After three ice-cold sea water washes the embryos were solubilized in the upper gel reservoir buffer plus]% SDS and 1% β-mercaptoethanol and heated at 95°C for 2 min. The SDS-solubilized samples were electrophoresed on an SDS acrylamide gel in a discontinuous buffer system (Neville, 1971). Stacking gel, 3.2 x 6.25; separating gel 11.1 x 0.09; upper reservoir buffer, pH 8.64, 0.04 M boric acid, 0.014 M Tris, 0.1% SDS; upper gel buffer, pH 6.1, 0.0267 M H_2SO_4, 0.0541 M Tris; lower gel buffer, pH 9.18, 0.0308 N HCl, 0.1244 M Tris; and lower reservoir buffer, same as lower gel buffer. The gels were stained with Schiff's reagent (Neville, 1971), and the optical density at 500 nm was monitored. The gels were then fractionated, and the radioactivity in each sample was determined. Data points for the radioactivity determinations are the averages, at each point, of two identical samples run on two separate gels. The solid line is the absorbancy tracing at 500 nm, and the dotted line is the radioactivity present in each fraction. The standards are, from left to right, β-galactosidase (130,000 daltons); lactoperoxidase (78,000 daltons), ovalbumin (45,000 daltons), and ribonuclease (13,000 daltons).

dramatically that fertilized eggs incorporate glucosamine at a 50% higher rate than do 2-cell embryos. Also, 16-cell embryos incorporate glucosamine at about a third the rate of the fertilized eggs when the results are expressed on a per mg basis. Thus as the embryo increases in age (differentiates?) the rate of glucosamine incorporation into macromolecules decreases

Table 1. D-[U-^{14}C]glucosamine incorporation into macromolecules by fertilized sea urchin eggs, 2-cell sea urchin embryos, and 16-cell sea urchin embryos

Eggs and embryos, prepared according to the text, were incubated with 50 μM D-[U-^{14}C]glucosamine (318 Ci/mol) for 30 min at 25°C. Radioactivity is that present in phosphotungstic acid, 2 x trichloroacetic acid, ethanol:ether (2:1, v/v) insoluble material. Results are means ± S.E.M. These results are from duplicate determinations performed on 4 batches of embryos (i.e., the fertilized eggs from 2 urchins).

	pmol/mg protein/0.5 hr
Eggs (fertilized)	6.87 ± 0.22
2-cell embryos	4.34 ± 0.37*
16-cell embryos	2.54 ± 0.15*

* Significantly different from one another; $P < 0.005$ by Student's t test.

greatly on a per mg protein basis. The rapid rate of synthesis in the just-fertilized egg may represent a requirement for extensive membrane synthesis.

Glycoprotein synthesis in separated 16-cell sea urchin blastomeres. D-[U-^{14}C]Glucosamine incorporation by the separated blastomeres is shown in Table 2. A significant difference ($P < 0.05$) in the rate of D-[U-^{14}C]-glucosamine incorporation was found when the macromeres were compared with either the micromeres or the mesomeres. No significant differences were found for any of the other blastomere cell types.

Discussion The observation reported here that glucosamine is incorporated into heterogeneous high molecular weight glycoconjugates is consistent with observations in a number of mammalian systems in which incorporation of radioactively labeled glucosamine has been used for studying the synthesis and location of the cell's hexosamine-containing glycoconjugates. Kornfeld and Ginsburg (1966) have shown that over 90% of the [^{14}C]glucosamine taken up by HeLa cells first appears in the cell's uridine diphosphate-N-acetylhexosamine pool and that this radioactivity is slowly accumulated by the macromolecular components of the cell over the next 12 hours. Hayden, Crowley & Jamieson (1970) reported that following incubation of human lymphocytes with [^{14}C]glucosamine, 84% of the radioactivity was present as uridine diphosphate-N-acetylglucosamine which was then incorporated into membrane

Table 2. D-[U-^{14}C]glucosamine incorporation after blastomere separation

Sixteen-cell whole sea urchin embryos or separated blastomeres (Hynes & Gross, 1970) were incubated with 50 μM D-[U-^{14}C]-glucosamine for 30 min at 25°C. Radioactivity is that present in the phosphotungstic acid, 2 x trichloroacetic acid, ethanol: ether insoluble material. Results are means ± S.E.M. The experiments were repeated 4 times. Each time the dissociated 16-cell blastomeres from several female urchins were pooled prior to cell separation.

	pmol/mg protein/0.5 hr	
Embryos	2.54	0.15
Micromeres	2.12	0.27
Mesomeres	2.22	0.10
Macromeres	3.02	0.30*

* Significantly different from micromeres and mesomeres; $P < 0.05$ by Student's t test.

macromolecules; in addition, radioactive glycopeptide fragments could be released from the lymphocyte cell surface with proteolytic enzyme treatment.

There is earlier evidence suggesting that glycoproteins are synthesized in sea urchin embryos. Perlmann, Bostrom & Vestermark (1959) reported that the total sialic acid content of the embryo increases during early sea urchin development. Kean and Bruner (1971) demonstrated that sea urchin eggs contain cytidine 5'-monophosphosialic acid (CMP-sialic acid) synthetase activity. This suggests that the nucleotide monosaccharide, CMP-sialic acid, necessary for the synthesis of sialoglycoconjugates is present in sea urchin embryos.

The rates of [^{14}C]glucosamine incorporation for the three blastomere types indicate a slightly higher rate of relative glycoprotein synthesis for the macromeres than for either the mesomeres or the micromeres. Whether or not this represents a higher absolute rate of glycoprotein synthesis for the macromeres cannot be concluded without information on the relative pool sizes of glucosamine for the three cell types. If the pool sizes and thus intracellular specific activity of [^{14}C]glucosamine in the three blastomere types were assumed to be the same, then the incorporation data could be interpreted as indicating a higher rate of glycoprotein synthesis in the macromeres than in either the mesomeres or the micromeres from 16-cell sea urchin embryos. If the actual rates of glycoprotein synthesis for micro-

meres, mesomeres, and macromeres are different, then possibly there are quantitative or qualitative differences in the glycoproteins of these individual cell types. The data presented here indicate a potential for differences in the glycoproteins of the 16-cell blastomeres. One may further speculate from the observed different relative patterns of glycoprotein synthesis that there may be differences in the external cell surface glycoproteins of the 16-cell blastomere types. Certainly this is speculative, but this research does indicate that at the 16-cell stage the individual blastomere types have the ability to synthesize different amounts and kind of glycoproteins to be located on the external cell surface membrane.

We thank Dr. P. Gross of this university for helpful advice and constructive criticism.

References

Bernacki, R. J. (1973). Glycoprotein Metabolism Association with Mammalian Membrane Systems. Ph.D. dissertation, University of Rochester, Rochester, New York.

Bosmann, H. B., Hagopian, A. & Eylar, E. H. (1969). Arch. Biochem. Biophys. 130, 573-583.

Chamberlain, J. P. (1977). J. Cell Biol. 75: 32a.

Cook, G. M. W. & Stoddard, R. W. (1973). Surface Carbohydrates of the Eukaryotic Cell. New York, Academic Press.

Glossmann, H. & Neville, D. M., Jr. (1971). J. Biol. Chem. 246, 6339-6346.

Gustafson, T. & Wolpert, L. (1967). Biol. Rev. Cambridge Phil. Soc. 42, 442-498.

Harvey, E. B. (1956). The American Arbacia and Other Sea Urchins. Princeton, N.J., Princeton University Press.

Hayden, G. A., Crowley, G. M. & Jamieson, G. A. (1970). J. Biol. Chem. 245, 5827-5832.

Hynes, R. O. & Gross, P. R. (1970). Dev. Biol. 21, 383-402.

Karp, G. C. & Solursch, M. (1974). Dev. Biol. 41, 110-123.

Kean, E. L. & Bruner, W. E. (1971). Exp. Cell Res. 69, 384-392.

Kornfeld, S. & Ginsburg, V. (1966). Exp. Cell Res. 41, 592-600.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). J. Biol. Chem., 193, 265-275.

Neri, A. Roberson, M., Connolly, D. T. & Oppenheimer, S. B. (1975). Nature 258, 342-344.

Neville, D. M., Jr. (1971). J. Biol. Chem. 246, 6328-6334.

Perlman, P., Bostrom, H. & Vestermark, A. (1959). Exp. Cell Res. 17, 434-446.

Roberson, M. & Oppenheimer, S. B. (1975). Exp. Cell Res. 17, 434-446.

Spiegel, M. & Rubinstein, N. A. (1972). Exp. Cell Res. 70: 423-430.

Spiro, R. G. (1966). Methods Enzymol. 8, 3-24.

Tyler, A. and Spiegel, M. (1956). Biol. Bull. 110, 196-200.