A Calcium-binding, Asparagine-linked Oligosaccharide Is Involved in Skeleton Formation in the Sea Urchin Embryo

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Abstract. We have previously identified a 130-kD cell surface protein that is involved in calcium uptake and skeleton formation by gastrula stage embryos of the sea urchin *Strongylocentrotus purpuratus* (Carson et al., 1985. *Cell.* 41:639–648). A monoclonal antibody designated mAb 1223 specifically recognizes the 130-kD protein and inhibits Ca^{+2} uptake and growth of the $CaCO_3$ spicules produced by embryonic primary mesenchyme cells cultured in vitro. In this report, we demonstrate that the epitope recognized by mAb 1223 is located on an anionic, asparagine-linked oligosaccharide chain on the 130-kD protein. Combined enzymatic and chemical treatments indicate that the 1223 oligosaccharide contains fucose and sialic acid that is likely to be *O*-acetylated. Moreover, we show that the oligosaccharide chain containing the 1223 epitope specifically binds divalent cations, including Ca^{+2}. We propose that one function of this negatively charged oligosaccharide moiety on the surfaces of primary mesenchyme cells is to facilitate binding and sequestration of Ca^{+2} ions from the blastocoelic fluid before internalization and subsequent deposition into the growing $CaCO_3$ skeleton.

A FUNDAMENTAL problem in cellular biology is to understand the means by which certain cells in multicellular organisms differentiate to a state in which they are capable of laying down mineralized tissues. To achieve this differentiated state, precursor cells must acquire the ability to select appropriate ions from the surrounding fluid and organize these ions into a crystalline or semicrystalline form. Sea urchin embryos provide a useful system in which to study the developmental process of biomineralization. Of particular interest is the mechanism by which the fifty or so primary mesenchyme cells (PMCs)[1] of the gastrula stage embryo acquire the ability to sequester Ca^{+2} ions and build the embryonic $CaCO_3$ skeleton. Using a monoclonal antibody, mAb 1223, a 130-kD cell surface protein has been identified that plays an essential role in Ca^{+2} uptake by PMCs (Carson et al., 1985). This antibody has been shown to selectively interfere with the uptake process rather than with the subsequent phase during which ionic calcium is converted to crystalline $CaCO_3$ (Grant et al., 1985). Recent studies have shown that the 130-kD protein is specifically expressed on the surface of the PMC at the time that the cells begin to accumulate calcium (Farach et al., 1987). At this stage of development, no other cell types within the embryo synthesize or express significant amounts of this antigen.

A number of laboratories have reported the development of antibody probes that recognize PMC-specific antigens similar to the 130-kD protein recognized by mAb 1223. Leaf et al. (1987) recently identified a 130-kD protein encoded by a PMC-specific cDNA clone. Polyclonal antibodies directed against a portion of this sequence expressed as a fusion protein with β-galactosidase were prepared. This research group also has isolated a monoclonal antibody, mAb B2C2, which apparently recognizes the same 130-kD protein that is recognized both by their fusion protein polyclonal antibody and by mAb 1223 (Anstrom et al., 1987). However, neither of these antibodies is able to inhibit calcium uptake by cultured PMCs (Farach-Carson, unpublished observations). At least two other groups also have described the use of PMC-specific probes that recognize proteins that may be antigenically related to the 130-kD protein recognized by mAb 1223. We have tested the antibody designated 1G8 (Wessel et al., 1984) and found that it, like B2C2, does not inhibit calcium influx into PMCs (Farach-Carson and Wessel, unpublished observations). Shimizu et al. (1988) recently described yet another PMC-specific monoclonal called P4 that recognizes a series of proteins including one with an apparent molecular mass of 130 kD. The functional effects of this antibody on calcium uptake have not been investigated.

The unique ability of mAb 1223 to inhibit biological processes as complex as calcium uptake and embryonic skeleton formation led us to investigate the nature of the epitope on the 130-kD protein recognized by mAb 1223. In this report, we present evidence that the 130-kD protein recog-

1. *Abbreviations used in this paper*: Endo, endoglycosidase; PNGase, peptide: *N*-glycosidase; PMC, primary mesenchyme cells.

nized by mAb 1223 in *Strongylocentrotus purpuratus* embryos is a Ca^{+2} binding glycoprotein. Furthermore, we find that the epitope recognized by mAb 1223 is contained within a 3,200 M_r complex-type asparagine-linked oligosaccharide that is attached to the 130-kD glycoprotein on PMCs. The pronase-derived glycopeptide bearing the 1223 antigen exhibits a net negative charge at neutral pH. Chemical and enzymatic treatments that selectively destroy particular oligosaccharide structures indicate that substituted sialic acid residues comprise an essential part of the 1223 epitope. Furthermore, we find that the isolated pronase glycopeptide containing the 1223 epitope specifically binds divalent cations including Ca^{+2}. The unique properties of the oligosaccharide epitope recognized by mAb 1223 may account for the functional effect seen on skeleton formation in cultured spicule-forming cells.

Materials and Methods

Materials

All materials used for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). $Na^{125}I$ (carrier free), [^{3}H]leucine (45 Ci/mmol) and $^{45}CaCl_2$ (10–40 mCi/mg) were obtained from Amersham Corp. (Arlington Heights, IL). $^{35}SO_4$ (carrier free) and [6-^{3}H]glucosamine (40 Ci/mmol) were purchased from ICN Biomedicals Inc. (Irvine, CA). The Vectastain ABC (anti-mouse) kit for peroxidase staining of immunoblots was from Vector Laboratories, Inc. (Burlingame, CA). Tissue culture media and fetal calf serum were from Hazelton Biologics, Inc. (Lenexa, KS). Horse serum was purchased from Gibco Laboratories (Grand Island, NY). Tunicamycin, all detergents, and enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) with the exception of chondroitinase ABC and keratanase that were obtained from Miles Scientific Div. (Naperville, IL) and neuraminidases that were from Calbiochem-Behring Corp. (San Diego, CA). Normal mouse IgG was from Pel-Freez Biologicals (Rogers, AR). Anti-mouse IgG (goat) and the immunoglobulin subisotyping kit were purchased from HyClone Laboratories (Logan, UT). Proteins (fetuin, hemoglobin, BSA, cytochrome c) as well as all chromatography resins were purchased from Sigma Chemical Co. (St. Louis, MO). Purified calmodulin was a gift of Dr. A. Means and Endo N was generously provided by Dr. F. Troy. The monoclonal antibody B2C2 and the fusion polyclonal were provided by Dr. R. Raff. All other chemicals used were reagent grade or better.

Culture of Sea Urchin Embryos and Primary Mesenchyme Cells

Sea urchins were purchased from Pacific Bio Marine Laboratories Inc. (Venice, CA). Gametes were harvested and embryos cultured in artificial sea water at 14°C as described (Heifetz and Lennarz, 1979). Developmental stages were defined according to the criteria set forth by Galileo and Morrill (1985). Primary mesenchyme cells were isolated and cultured in vitro as described (Carson et al., 1985). In certain experiments, PMCs were treated with tunicamycin on the second day after plating. Tunicamycin (0.1 μg/ml) was added from a sterile stock solution prepared in DMSO. Control cultures received DMSO alone. Protein synthesis was monitored in tunicamycin-treated cells by labeling cells with [^{3}H]leucine as described previously (Farach et al., 1987). Radioactivity incorporated into macromolecular material was assessed after TCA precipitation as described (Farach et al., 1988).

Preparation of Labeled Embryo Extracts

Gastrula stage embryos were labeled with [^{3}H]leucine or [^{3}H]glucosamine (250 μCi/ml) as described (Carson et al., 1985), except that labeling with glucosamine was extended to 6 h. The embryos were then washed with artificial sea water and solubilized overnight in a Tris-buffered solution containing 2% (wt/vol) cholate. The insoluble material was removed by centrifugation for 5 min in a microfuge (Beckman Instruments Inc., Palo Alto, CA). The soluble fraction was chromatographed on a 1.5 × 26-cm Sephadex G-50 column eluted with 10 mM sodium acetate, pH 7.0, 5% (vol/vol) ethanol and 0.02% (wt/vol) NaN_3. All labeled material eluting in the void volume was combined and concentrated by lyophilization. This fraction is termed the "macromolecular fraction" throughout this text.

Monoclonal Antibody 1223

Hybridoma 1223 was cultured in Iscoves medium supplemented with 10% FCS as previously described (Carson et al., 1985). mAb 1223 was purified from conditioned medium by passage over Protein A Sepharose. Subclass identification has demonstrated that mAb 1223 is an IgG_1. Immunoaffinity beads were prepared by coupling mAb 1223 or preimmune mouse IgG to cyanogen bromide-activated Sepharose 4B (Carson et al., 1985).

Immunoblotting

Immunoblots were performed with ^{125}I-labeled second antibody as described previously (Farach et al., 1987) except that all blotting steps were performed using an Omniblot apparatus (ABN, Emeryville, CA). The blots were dried and analyzed by autoradiography. Radioactive spots were quantitated by direct scanning of the radiograms using a transmittance/reflectance scanning densitometer (GS-300; Hoefer Scientific Instruments, San Francisco, CA) equipped with a data software system (GS-350; Hoefer Scientific Instruments). For rapid detection of immunoreactive material when quantitation was unnecessary (such as screening column fractions), a peroxidase assay system was employed. In this case, samples were dot blotted onto a nitrocellulose filter using a 96-well apparatus (Bio-Rad Laboratories). The filter was blocked for 30 min with a solution containing 0.05% (vol/vol) Tween 20 in PBS, pH 7.5. At the end of the blocking period, this solution was aspirated and replaced with 5 μg/ml of mAb 1223 in a fresh Tween-PBS solution. Incubation with first antibody was continued for a minimum of 4 h, but was usually overnight. At the end of the incubation period, the filter was washed 4–5 times in PBS without Tween 20 and immediately developed upon the addition f 4-chloro-1-napthol and H_2O_2. Development was stopped by replacing this solution with water. Blots developed in this manner were immediately photographed.

Sample Preparation and Analysis

Gastrula-stage embryos were collected using a hand driven centrifuge and washed twice with freshly filtered artificial sea water. Embryo pellets were either used immediately or stored at −70°C until use. Embryos were routinely solubilized overnight at 4°C in a buffer consisting of 2% (wt/vol) Na cholate in 10 mM Tris-HCl, pH 8.0, and a battery of protease inhibitors (Carson et al., 1985). In other cases, PMCs were scraped from tissue culture plates, washed in artificial sea water and then solubilized in this same buffered detergent solution. Protease inhibitors were omitted only when pronase digestion was to be employed in subsequent steps of the protocol.

Pronase digestions using predigested pronase were carried out for 48 h at 50°C as described by Hart and Lennarz (1978). Samples were diluted approximately twofold during the entire procedure. Glycosidase digestions were performed in all cases exactly according to the manufacturer's specifications. The details are included in the figure legends where applicable. Enzyme digestions were routinely performed on samples containing 100 μg of solubilized protein. Protease inhibitors were present during all glycosidase digestions. Internal standards were included in a parallel sample to insure that the individual enzymes were active in the solubilization buffer.

Isolation of the 1223 Pronase Glycopeptide

Embryos were extracted in solubilization buffer and digested with pronase as described above. At the end of the digestion, any insoluble material was removed by low speed centrifugation and the soluble fraction was passaged over Sepharose 4B beads to which either mAb 1223 or preimmune mouse IgG was coupled as described (Carson et al., 1985). Bound material was eluted from each column with 6 M guanidine HCl and placed into dialysis to remove the chaotrope. The two fractions are henceforth referred to as "1223 glycopeptide" and "preimmune glycopeptide control."

Ion Exchange Chromatography

The column used for analysis of the behavior of all preparations during anion exchange was a 3-ml column of DE 52. The starting buffer consisted of 10 mM sodium phosphate, pH 7.2. Samples were adjusted to the running buffer by dialysis before application to the column. Samples were eluted

from the column first with 5 ml of running buffer followed by a linear gradient from 0 to 0.5 M NaCl, and then with 5 ml of 0.5 M NaCl. Aliquots (200 μl) of 1.0 ml column fractions routinely were dot blotted onto nitrocellulose and antibody-binding fractions were identified from immunobinding blots as described above. Salt concentrations were measured from conductivities determined using a CDM 83 conductivity meter (Radiometer, Copenhagen, Denmark).

Gel Permeation Chromatography

Samples were analyzed by molecular exclusion on a 1.5 × 45 cm column of Sephadex G-75 (superfine). The running buffer for this column consisted of PBS either with or without 1.0 mM EDTA depending upon the experiment. The column was calibrated with a series of protein and saccharide molecular mass markers. On some occasions, the samples were dialyzed versus 10 mM EDTA before application to this column. Aliquots (10–20% of the total volume) of the column fractions were dot blotted onto nitrocellulose, probed with mAb 1223, and the antibody-binding fractions identified.

Calcium Binding Assays

Two complementary assays were employed to test the ability of various preparations to bind Ca^{+2}.

Nitrocellulose Assay. The procedure we used was modified from that of Maruyama et al., (1984). The sample(s) to be tested was dot blotted onto a piece of nitrocellulose that was then soaked for 20 min in a solution consisting of 60 mM KCl, 5 mM $MgCl_2$ and 10 mM Bicine, pH 6.8. The solution was changed three times and incubated each time for 20 min with this buffer, following which $^{45}CaCl_2$ was added at a final concentration of 0.5 μCi/ml. After 1 h, the filter was washed three times with 50 ml of deionized water for 10 min/wash, then dried, and autoradiographed.[2]

Gel Filtration Assay. Ca^{+2} binding to soluble fractions was monitored using a modification of the gel filtration method originally described by Hummel and Dreyer (1962). A Sephadex G-10 column (1 × 27 cm) was equilibrated with various concentrations of $^{45}CaCl_2$ in a running buffer consisting of 10 mM Tris-HCl containing 0.15 M NaCl and 5 mM KCl. The effluent solution was collected in 1.0 ml fractions, and the baseline radioactivity levels were determined by counting 25-μl aliquots from each fraction in a scintillation counter. Samples to be tested for calcium binding ability were applied to the column and the shape of the elution profile determined by counting the column fractions. These profiles reflect the association–dissociation phenomenon occurring between the applied sample and the free calcium in the running buffer and give a characteristic "peak and trough" when binding occurs (Ackers and Thompson, 1965).

Other Procedures

Protein concentrations were determined using the method of Lowry et al., (1951) using BSA as a standard. SDS-PAGE was performed on 8.75% polyacrylamide gels as described previously (Carson et al., 1985). Hydrazinolysis and reacetylation were performed using the standard procedure of Takasaki et al. (1982). Fetuin glycopeptides to be used as standards for gel filtration were prepared as described by Spiro and Bhoyroo (1974).

Results

The 1223 Epitope Is An Oligosaccharide

In preliminary experiments, we were unable to immunoprecipitate the 1223 antigen-bearing protein from [^{35}S]methionine-labeled products prepared by translating RNA isolated from PMC cultures. This suggested that part of the epitope might be a product of posttranslational processing. We therefore tested the ability of a series of proteases and glycosidases of varying specificity to degrade the 1223 antigen. Preliminary experiments showed that the epitope was not destroyed by either treatment with pronase (see below) or by hydrazinolysis and subsequent reacetylation (not shown). In contrast, treatment with various glycosidases (see below) disrupted antigen–antibody binding to differing extents. In light of these preliminary results, further experiments were undertaken to characterize the oligosaccharide epitope recognized by mAb 1223.

Previous studies have shown that although the 130-kD glycoprotein is a major protein synthesized de novo in gastrula stage sea urchin embryos, it is a minor protein in total embryonic extracts (Farach et al., 1987). We therefore used Western blotting to take advantage of the high affinity of mAb 1223 for the 130-kD protein as a means of detecting the antigen in embryo extracts after various test endoglycosidase treatments. In Fig. 1 is shown the results of blotting with mAb 1223 after treatment with either Endoglycosidase F

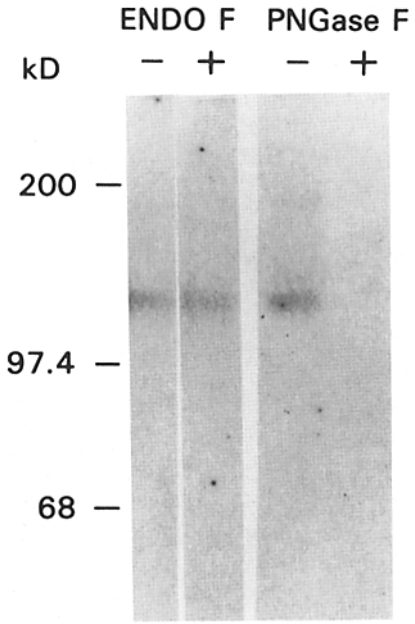

Figure 1. Effect of endoglycosidases on the 1223 antigen. Gastrula stage embryos were solubilized in a solution containing 10 mM Tris-HCl, pH 8.0, 2% sodium cholate and a battery of protease inhibitors. Insoluble material was removed by low speed centrifugation. The material remaining in the supernatant is referred to as the "gastrula extract." In this experiment, 100-μg aliquots of the gastrula extract were treated under denaturing conditions with the endoglycosidases Endo F (1.9 mU) and PNGase F (2.0 mU) before Western blot analysis with mAb 1223 and iodinated second antibody. Autoradiography was for 4 h at −70°C. The conditions for the deglycosylation reactions were as follows: total gastrula extracts were dialyzed against 10 mM Tris-HCl, pH 8.0, containing 0.01% sodium cholate and 0.02% NaN_3. For PNGase F treatment, samples were heat denatured in 0.5% (wt/vol) SDS and 1% β-mercaptoethanol and then diluted to 0.1% (wt/vol) SDS in a solution containing (final concentrations) 0.1 M Na phosphate buffer, pH 8.6, 0.02 M Na EDTA, and 0.6% (wt/vol) BIGCHAP. Incubation with PNGase was overnight at room temperature. Parallel deglycosylation reactions were performed with immunoglobulin G and ovalbumin as substrates. The protocol used for Endo F was the same except that a 0.05 M (final concentration) Na acetate buffer, pH 5.0, was used in place of the phosphate-EDTA buffer. As shown above, treatment with PNGase removes the immunoreactive portion of the 130-kD glycoprotein. No effect of PNGase F was seen under nondenaturing conditions.

2. Since the completion of the majority of this work, we have found that Zeta-Probe (Bio-Rad Laboratories) provides a much improved matrix on which to perform overlays with $^{45}Ca^{+2}$. Not only does the negatively charged 1223 glycopeptide bind more efficiently to this cationized nylon membrane, but also the background is consistently reduced with shorter wash times.

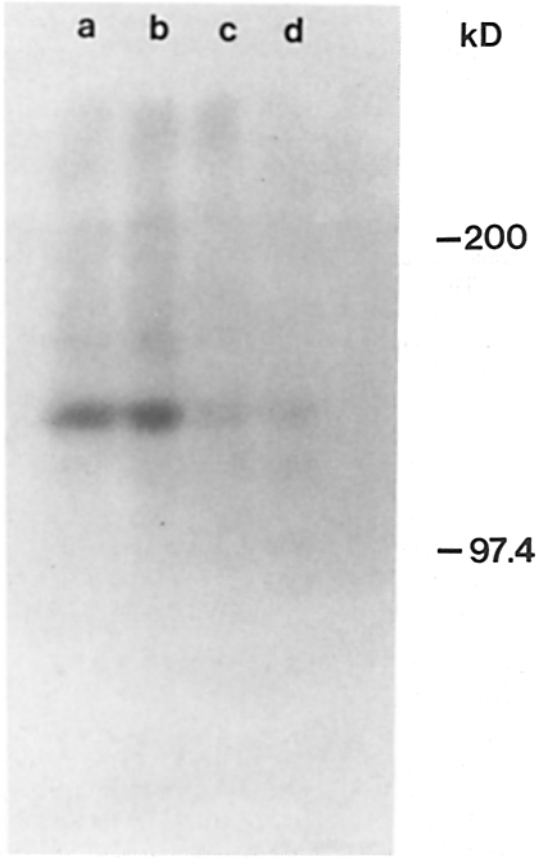

Figure 2. Tunicamycin inhibits the assembly of the 1223 epitope on the 130-kD glycoprotein. Primary mesenchyme cells were cultured as described in Materials and Methods. On the second day after plating, tunicamycin (0.1 μg/ml) was added from a sterile stock solution prepared in DMSO. Control cultures received DMSO alone. On day 3, PMCs were scraped from the plates, solubilized as described in Fig. 1 and analyzed by Western blotting using mAb 1223 and iodinated second antibody. Autoradiography was carried out overnight. Lanes *a* and *b*, duplicate control cultures; lanes *c* and *d*, duplicate cultures receiving 0.1 μg/ml tunicamycin.

(Endo F) or peptide: *N*-glycosidase (PNGase F). As seen in the autoradiograms, treatment with PNGase but not Endo F selectively removed the portion of the molecule containing the mAb 1223 binding site. Additional experiments also demonstrated that treatment with Endo H had no effect on the ability of mAb 1223 to recognize the 130-kD glycoprotein (data not shown). Consistent with these results, we found that PNGase digestion of the 130-kD glycoprotein metabolically labeled with [^{3}H]leucine destroyed the ability of the 130-kD polypeptide to bind to an immunoaffinity column prepared by coupling mAb 1223 to Sepharose 4B.

The release of the 1223 epitope by PNGase F led us to investigate whether tunicamycin treatment, which interferes selectively with assembly of N-linked chains on core proteins, would decrease the immunoreactivity of the 130-kD glycoprotein. Cultures of PMCs were prepared from dissociated embryos. Previous studies (Farach et al., 1987) have shown that on the second day after plating the synthesis of the 1223 epitope-bearing glycoprotein by PMCs increases dramatically. Consequently, we examined the effect of tunicamycin on assembly of the 1223 glycoprotein by PMCs during day 2 in culture. As shown in Fig. 2, cultures receiving 0.1 μg/ml of tunicamycin contained considerably less immunoreactive 130-kD glycoprotein than did control cultures. Quantitative densitometry showed that mAb 1223 binding in the experiment shown in Fig. 2 was reduced by 64%. To assess the effects of tunicamycin treatment on overall protein synthesis by PMCs, parallel cultures were incubated with 20 μCi/ml [^{3}H]leucine throughout the experimental course described in the legend to Fig. 2. Radioactivity incorporated into macromolecular material was then determined in TCA precipitates. Little if any effect of tunicamycin was observed at 0.1 μg/ml, although higher doses (0.5–1.0 μg/ml) did inhibit leucine incorporation by 20–30%. These findings, coupled with the sensitivity to PNGase F, provide strong supporting evidence that the oligosaccharide chain containing the epitope recognized by mAb 1223 is part of a complex-type oligosaccharide chain that is linked to the core protein via an asparagine residue.

Assay of Glycopeptides

To further characterize the structural and functional properties of the oligosaccharide chain containing the 1223 antigen, we first prepared pronase glycopeptides from gastrula stage embryos or cultured PMCs. These glycopeptides then were dot blotted onto a piece of nitrocellulose and assayed for binding with mAb 1223 as described in Materials and Methods. Although there are limitations to this assay system (see below), it nonetheless enabled us to characterize a quantitatively minor antigen that could only be followed by binding of our monoclonal antibody. As indicated above, the epitope recognized by 1223 was resistant to treatment with pronase, although the efficiency of binding of mAb 1223 was only 10–15% control after protease digestion. Control experiments were required to determine whether the observed loss in binding efficiency was because of a loss in antigenicity or to a loss in efficiency of binding of the 1223 antigen to nitrocellulose after pronase treatment. To test this, gastrula stage embryos were labeled with either [^{3}H]leucine or [^{3}H]glucosamine and the efficiency of binding to nitrocellulose measured for these two preparations either before or after pronase digestion. As shown in Table I, we found that the efficiency of binding of the total pool of glucosamine-labeled pronase glycopeptides was only 10%, which correlated with the signal reduction we saw with mAb 1223 binding. From this, we concluded that if mAb 1223 recognized a typical pronase glycopeptide from gastrula stage embryos, the 10–15% binding activity that was retained after pronase digestion was about the maximum expected.

Table I. Binding of Various Metabolically Labeled Macromolecular Fractions to Nitrocellulose*

Radiolabel	Treatment‡	% Bound§
[^{3}H]Leucine	None	78 ± 12
[^{3}H]Leucine	Pronase	2 ± 0.5
[^{3}H]Glucosamine	None	53 ± 8
[^{3}H]Glucosamine	Pronase	10 ± 3

* Gastrula stage embryos were labeled for 2 h (leu) or 6 h (gln) with ^{3}H precursors and the macromolecular fraction isolated as described in Materials and Methods. 20,000 cpm of labeled material was used per well in all assays.
‡ Macromolecular fractions were either treated with pronase as described in Materials and Methods or incubated in parallel in the absence of enzyme.
§ Nitrocellulose was 0.45 μm pore size. Wash steps using Tween-20 in PBS were as described in Materials and Methods.

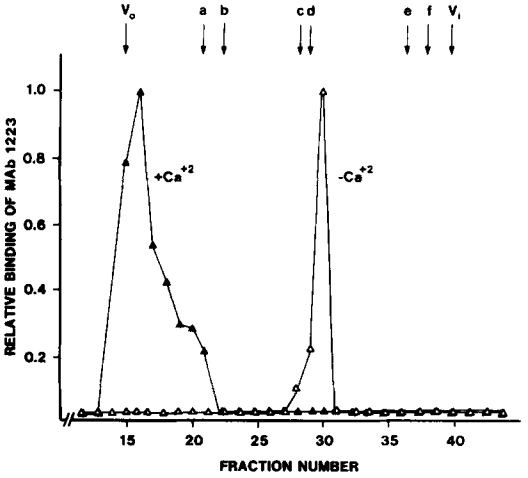

Figure 3. Behavior of the 1223 antigen-bearing pronase glycopeptide on Sephadex G-75. The 1223 glycopeptide was prepared as described in Materials and Methods. The behavior of the glycopeptide during gel permeation chromatography was assessed using a Sephadex G-75 column as described in the text. The elution position of the glycopeptide bearing the 1223 epitope was monitored using a nitrocellulose assay and an immunobinding dot blot protocol as described in Materials and Methods. Quantitation of dot intensity was performed using a densitometer. The elution position of the 1223 glycopeptide was found to be calcium dependent. Samples prepared in the presence of calcium (which is used during the pronase digestion) and run directly on the column using PBS as the running buffer elute in a broad peak beginning at the void volume of the column (▲−▲). Dialysis of the sample versus EDTA before application to the column results in conversion to a lower molecular mass form (△−△). The molecular mass markers are as follows: (*a*) hemoglobin; (*b*) 9 kD dextran; (*c*) 3.5 kD fetuin glycopeptide; (*d*) cytochrome c; (*e*) 1.5 kD fetuin glycopeptide; and (*f*) oxytocin. The V_o and V_i markers were blue dextran and potassium dichromate, respectively.

Sizing of the 1223 Epitope-bearing Pronase Glycopeptide

The 1223 pronase glycopeptide was prepared as described in Materials and Methods. The size of the glycopeptide was assessed by comparison of its elution position on Sephadex G-75 compared to a series of polypeptide and glycopeptide standards. As shown in Fig. 3 and Table II, the elution position of the 1223 epitope-bearing pronase glycopeptide was found to be dependent upon the presence of divalent cations. In the presence of divalent cations the 1223 glycopeptide eluted as a broad peak beginning at the void volume of the column. When the sample was treated with EDTA before application to the column or when EDTA was added to the running buffer, the 1223 glycopeptide was converted to a lower molecular mass form. Comparison of the elution position to that of a series of standards indicates a molecular mass of ~3,200 D for the 1223 epitope-bearing pronase glycopeptide. This result led us to investigate the "aggregation–disaggregation" of the 1223 glycopeptides in the presence and absence of various ions.

In Table II, it can be seen that the aggregation of the 1223 glycopeptide takes place in the presence of any of the divalent cations (Ca^{+2}, Mg^{+2}, or Ba^{+2}) tested. In contrast, monovalent cations, even at concentrations of 0.15 M, did not induce aggregation. Partial disaggregation could be achieved by the inclusion of 4 M urea in the sample. These results suggested that the isolated oligosaccharide was capable of binding divalent cations. One phenomenon repeatedly observed was that the intensity of the signal obtained in dot blots was dependent on the presence or absence of "aggregates" of the pronase glycopeptide. As illustrated in Fig. 3, the signal obtained by integrating the area under the peak for the lower molecular mass form was considerably reduced compared to the signal obtained when the sample was aggregated and eluted in the void volume. This cannot be explained by sample loss, because the full intensity of the signal is recovered when calcium is added (not shown). These observations suggest that mAb 1223 binds better to the aggregated form of the antigen.

The apparent size of the oligosaccharide released by PNGase treatment of the gastrula extract (as assessed by passage over G-75 and immunoblotting as above) was approximately the same as that of the pronase glycopeptide. This means that the size estimated for the pronase glycopeptide containing the 1223 antigen is close to the size of the oligosaccharide chain and is not because of large amounts of a protease resistant core protein associated with the oligosaccharide chain.

Ionic Character of the 1223 Epitope-Bearing Pronase Glycopeptide

Ion exchange on DE 52 was chosen as the means of characterizing the charge characteristics of the 1223 glycopeptide. As shown in Fig. 4, the pronase glycopeptide bearing the 1223 epitope bound to DE 52 at neutral pH and was eluted with a salt concentration between 0.2 and 0.3 M NaCl. This negative charge character is consistent with its ability to aggregate in the presence of divalent cations. A small amount of the antibody-binding activity consistently eluted at the start of the salt gradient; in the experiment shown in Fig. 4, this represented 15% of the total.

The 1223 Glycopeptide Binds Calcium

Two separate assays were devised to detect direct binding of calcium to the pronase glycopeptide containing the 1223 epitope. Such binding would account for the tendency of the 3,200 M_r glycopeptide to aggregate in the presence of divalent cations (see above). In preliminary experiments, we

Table II. Aggregation–Disaggregation of the 1223 Glycopeptide Induced by Divalent Cations

Treatment conditions	Elution position on G-75
Pronase digest	V_o
Pronase digest dialyzed versus 10 mM EDTA	M_r = 3,200
Add either Ca^{+2}, Mg^{+2} or Ba^{+2} to sample 2*	V_o
Add Na^+ or K^+ to sample 2‡	M_r = 3,200
Pronase digest plus 4 M urea	75% V_o 25% M_r = 3,200

* Divalent cations were added back to the pooled, dialyzed, EDTA-treated pronase glycopeptide eluting from the G-75 column at a position indicating a molecular mass of 3.2 kD. The concentration of divalent cation was 1.0 mM in all cases. The samples were then rerun over the G-75 column as described in Materials and Methods.

‡ Monovalent ions (Na^+ and K^+) were present at concentrations of 0.150 M and 5 mM, respectively.

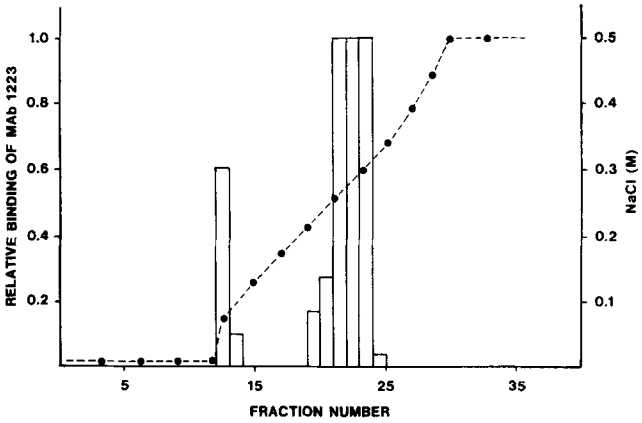

Figure 4. Behavior of the 1223-antigen bearing pronase glycopeptide during anion exchange. The pronase glycopeptide containing the 1223 epitope was prepared from gastrula stage embryos as described in the text. All samples were adjusted to the running buffer by dialysis before application to the column. The column used was a 3-ml column of DE 52 with a running buffer of 10 mM sodium phosphate, pH 7.2. Samples were eluted as described in Materials and Methods. Aliquots of the fractions were dot blotted onto nitrocellulose at the end of the run and antibody binding fractions identified and quantitated from immunobinding blots. The bar graph represents the intensity of the signal obtained densitometrically across the column fractions. Conductivities were read using a conductivity meter (●—●).

found that we could not isolate sufficient amounts of purified material to perform Scatchard analysis from equilibrium dialysis or centrifugation assays. Therefore, the assay systems used to measure $^{45}Ca^{+2}$ binding were chosen because they could detect calcium binding even with the extremely small amount of the 1223 glycopeptide that we were able to isolate. As seen in Fig. 5 *A*, the 1223 glycopeptide, but not a preimmune glycopeptide control (see Materials and Methods), binds $^{45}Ca^{+2}$ in a nitrocellulose overlay assay. Although not shown in this figure, we found that the bound calcium could not be displaced from the 1223 glycopeptide bound to nitrocellulose by overnight incubation in PBS containing 5 mM K^+ and 0.15 Na^+, although it could be readily removed by the addition of 10 mM EDTA to the wash solution. From this, we suspected that the binding affinity of Ca^{+2} to the 1223 glycopeptide was relatively high. This was tested more directly using a gel filtration assay on Sephadex G-10 as described in Materials and Methods. As shown in Fig. 5 *B*, when the column was equilibrated with $^{45}Ca^{+2}$ before application of the 1223 glycopeptide preparation, a peak of radioactivity was observed in the void volume. This peak resulted from the association of free ionic calcium in the running buffer with the much larger 1223 glycopeptide. The lowest concentration of calcium at which binding could be detected using this assay was 10^{-7} M. Neither the preimmune glycopeptide control nor the ~3.5 kD pronase glycopeptide of fetuin bound calcium during passage through the column. Calmodulin, (25 μg/run) which was used as a positive control in this assay, also began to bind calcium in this assay at 10^{-7} M. This behavior is consistent with its reported binding constant (2×10^5 M^{-1}) for calcium (Ogawa and Tanokura, 1984). Because of the similar calcium-binding behavior they displayed in this assay, it appeared that the association constant of the 1223 glycopeptide for calcium is similar to that of calmodulin. More precise analysis of the binding constant must await isolation of larger amounts of material than is presently available.

Immunoblotting of the 1223 Glycopeptide after Chemical Treatment or Treatment with Various Glycosidases

To identify structural features of the oligosaccharide bearing the 1223 epitope, we selectively modified various saccharide moieties and then assayed for retention or loss of reactivity with mAb 1223. As shown in Table III, most treatments, including various glycosidases and phosphatases, had no effect on immunoreactivity of the 1223 antigen with mAb 1223. Interestingly, α-L-fucosidase digestion increased binding from 3–10-fold in duplicate experiments (also see below, Fig. 8). In contrast, treatment with neuraminidase from several sources reduced binding by only 10–40%. The epitope was found to be quite stable to base, but was easily destroyed by mild acid hydrolysis.

As mentioned above, the signal on immunoblots corresponding to the 1223 epitope was reduced, but not destroyed,

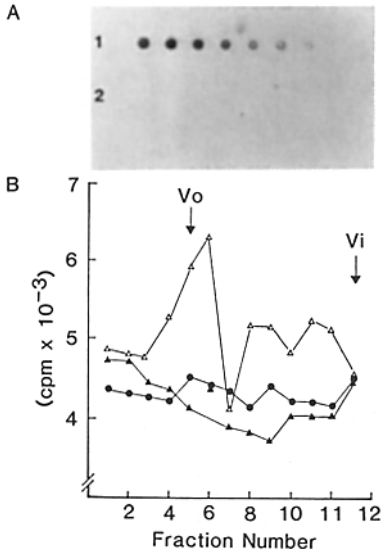

Figure 5. The 1223 antigen-bearing pronase glycopeptide binds calcium. *A* shows the autoradiogram obtained from a $^{45}Ca^{+2}$ overlay of the immunoaffinity-purified 1223 glycopeptide blotted onto nitrocellulose. $^{45}Ca^{+2}$ selectively binds to the 1223 glycopeptide but not to a control prepared using immunoaffinity beads coupled to preimmune mouse IgG. The isolated samples used in these experiments were prepared from 2 ml of packed sea urchin embryos that had been solubilized and digested with pronase as described in the text. The first well contains 10% of the 1223 glycopeptide preparation and the succeeding wells represent serial dilutions. The amount of 1223 glycopeptide used was below the level of detection using either a microhexose or microLowry assay, but could readily be detected using an immunoassay with mAb 1223. *B* shows a gel permeation assay for calcium binding that was performed as described in the text. The column was equilibrated with 10^{-7} M $^{45}Ca^{+2}$ and then loaded with the sample being tested for calcium binding. The resin (Sephadex G-10) was chosen such that the 1223 glycopeptide would elute in the void volume. (△—△) 1223 glycopeptide; (▲—▲) preimmune control; (●—●) 3.5 kD fetuin glycopeptide prepared as described in Materials and Methods. Only the 1223 glycopeptide gives the peak and trough pattern indicating calcium binding has taken place.

Table III. Effects of Various Chemical and Enzymatic Treatments on Immunobinding and Calcium Binding

Treatment*	Immunobinding	Ca^{+2} Binding
Keratanase‡ (2 U/ml) (*Pseudomonas*)	Retained	ND‖
Chondroitinase ABC‡ (1 U/ml) (*Proteus vulgaris*)	Retained	ND
Alkaline phosphatase‡ (300 U/ml) (*Escherichia coli*)	Retained	Retained
Acid phosphatase‡ (18 U/ml) (wheat germ)	Retained	ND
Neuraminidase‡ (0.1 U/ml) (*Clostridium perfringens*) (*Arthrobacter ureafaciens*) (*Vibrio cholera*)	MAb 1223 binding reduced by 10–40%	Retained
α-L-Fucosidase‡ (0.002 U/ml) (bovine epididymus)	MAb 1223 binding increased 3–10-fold	Retained
Mild acid hydrolysis§ (0.1 N TFA, 80°, 1 h)	MAb 1223 binding destroyed	Retained
β-Elimination§ (0.1 N NaOH, 37°, 48 h)	Retained	Retained
Mild base hydrolysis followed by neuraminidase (see text for details)	MAb 1223 binding destroyed	Retained

* All treatments were performed according to the manufacturer's protocols. Parallel controls were always conducted to verify enzymic activity.
‡ Samples were analyzed by quantitation of autoradiographs from Western blots as described in Materials and Methods.
§ Samples were analyzed by quantitation of immunodot blots following neutralization of the samples.
‖ *ND.*

after digestion with neuraminidase. To assess whether this partial susceptibility to neuraminidase might be because of some unusual characteristics of a sialic acid–containing oligosaccharide bearing the 1223 epitope, we tested other agents that modify sialic acid structures. As shown in Fig. 6, Endo N, which specifically cleaves α-2,8-polysialic acid chains

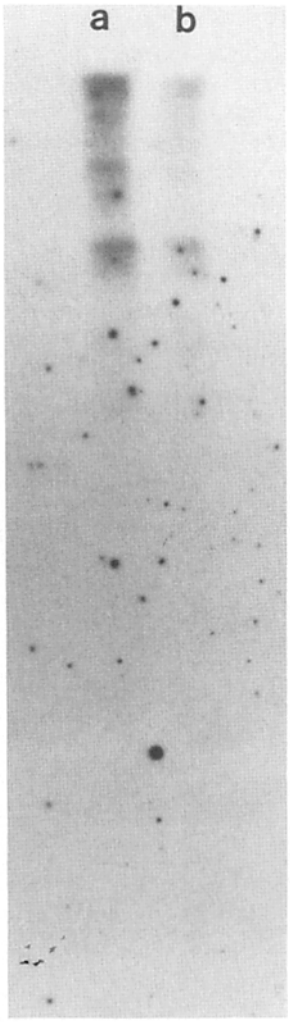

Figure 6. Digestion with Endo N reduces immunobinding of mAb 1223 to the 130-kD protein. Gastrula stage embryos were solubilized as described in Materials and Methods. Insoluble material was removed and the soluble fraction was treated with Endo N, which selectively degrades α-2,8-polysialic acid linkages. 100-Mg aliquots of total gastrula protein extracts received either buffer alone (lane *a*) or 0.5 unit of Endo N (lane *b*). After incubation overnight at 25°C, the samples were separated on 8.75% SDS-polyacrylamide gels and analyzed for immunobinding to mAb 1223 on Western blots using ^{125}I second antibody. The reduction of immunobinding (estimated by densitometry) in this experiment was 46%. Also note the aggregation of the 130-kD glycoprotein seen in this experiment. This characteristic of the protein is discussed in the text.

(Rutishauser et al., 1985), also reduced but did not abolish immunoreactivity with mAb 1223. A 50% signal reduction after digestion with Endo N also was seen on dot blots of column fractions of control and Endo N-digested 1223 glycopeptide passed over DE 52 (not shown). The elution position of the remaining immunoreactive material was not shifted, however, and eluted between 0.2 and 0.3 M NaCl as did the control. To obtain more information regarding the nature of the 1223 oligosaccharide, we turned to nonenzymatic treatments that might alter immunoreactivity more quantitatively.

The 1223 epitope was found to be stable to mild periodate treatment (2 mM, 30 min, 0°C) (see Fig. 7 *a*), although harsher treatment (2 mM, 1 h, 25°C) partially abolished reactivity of the 130-kD glycoprotein with mAb 1223 (Brooks-Scott and Lennarz, unpublished observations). The 1223 epitope also was stable to base (0.1 N NaOH, 37°C, 30 min to 24 h, in the presence or absence of 0.25 M $NaBH_4$) (Fig. 7, *b* and *c*). Interestingly, however, the mAb 1223 epitope was completely lost when the 1223 glycopeptide was treated with mild base (0.1 M NaOH, 37°C, 30 min) and then digested with neuraminidase from *Clostridium perfringens* (see Fig. 7 *d*). This observation, coupled with the resistance to periodate, is consistent with the notion that sialic acid residues on the 1223 glycopeptide might be substituted with *O*-acetyl groups (Varki and Diaz, 1984). These substitutions would confer resistance to neuraminidase treatment unless these *O*-acetyl groups were removed by prior exposure to mild base (Schauer, 1987). The samples shown in Fig. 7, *e* and *f* were both treated with acid (0.1 N HCl, 30 min, 100°C) before further analysis and were found to completely lose immunoreactivity merely as a consequence of the acid hydrolysis. From these experiments, we concluded that the epitope recognized by mAb 1223 contains sialic acid which contains *O*-acetyl side chain substitutions. Since de-*O*-acetylation does not affect antibody binding, it must be concluded that the base-labile substituent is not part of the epitope.

As mentioned above, long-term (overnight, 25°C or 4 h, 37°C) treatment of the 1223 glycoprotein with α-L-fucosidase increased the apparent binding of mAb 1223 to the glycoprotein by 3–10-fold. To demonstrate that this was a result of the enzymatic activity of the fucosidase, we tested the time-dependence of the reaction. As shown in Fig. 8, fucosidase digestion produced an essentially linear increase in im-

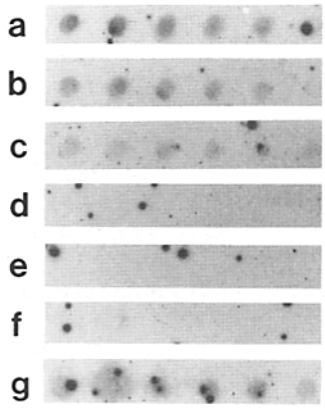

Figure 7. Immunoreactivity of the 1223 glycopeptide following various treatments. Pronase glycopeptides were prepared from gastrula stage embryos as described in the text. A series of combined chemical and enzymatic treatments were then performed to determine which types of residues were likely to constitute the 1223 epitope. After the initial treatment, samples were neutralized and sonicated to disperse the precipitates that formed during acid or base hydrolysis and/or heating. Enzymes were added where indicated to the resuspended, neutral samples. The treated samples were then dot blotted onto nitrocellulose and analyzed for immunoreactivity with mAb 1223. The irregular black spots on the filter represent ^{125}I second antibody that was not washed from the filter, a technical problem that was particularly bad in this experiment because of the particulate nature of several of the samples after treatment. The treatments were as follows: (row *a*) 2 mM periodate, 30 min, 0°C; (row *b*) 0.1 N NaOH, 24 h, 37°C; (row *c*) 0.1 N NaOH, 30 min, 37°C; (row *d*) same as row *c*, then neutralized and treated with neuraminidase (*Clostridium perfringens*), 0.1 U/ml, 4 h, 37°C; (row *e*) 0.1 N HCl, 30 min, 100°C; (row *f*) same as row *e*, then neutralized and treated with alkaline phosphatase; (row *g*) untreated pronase glycopeptides from gastrula stage embryos.

munoreactivity over a 3-h period. This finding suggests that the 1223 epitope-bearing oligosaccharide is heterogeneously fucosylated and that removal of the terminal fucose residues unmasks additional 1223 binding sites. Heterogeneous fucosylation does not explain the partial susceptibility previously seen to neuraminidase, since fucosidase treatment followed by neuraminidase digestion does not lead to complete loss of recognition by mAb 1223 (not shown).

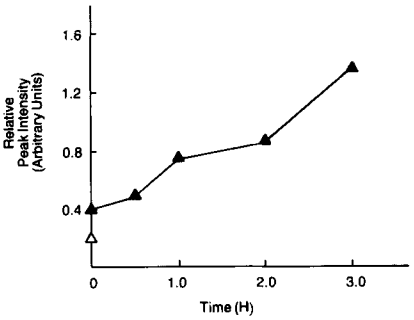

Figure 8. Time-dependent increase in immunoreactivity of the 1223 glycoprotein during digestion with α-L-fucosidase. A 3 mg/ml cholate extract of gastrula stage embryos was prepared as described in Materials and Methods. At time zero, 0.002 U/ml of α-L-fucosidase was added and 40 μl aliquots removed from the mixture at various time intervals. Proteins in the aliquots were separated by SDS-PAGE, and the immunoreactivity with mAb 1223 assayed by Western blotting with an iodinated second antibody as described in the text. The intensity of the band in the autorad at 130-kD was measured densitometrically and plotted. (▲—▲) Fucosidase-treated samples; the open triangle represents baseline immunoreactivity of an aliquot removed before the addition of fucosidase.

The Epitope Recognized by mAb 1223 and the Calcium Binding Site Are Distinct

Because mAb 1223 inhibited calcium uptake into PMCs, we tested whether this could be attributed to inhibition of the association of calcium with the 1223 bearing pronase glycopeptide. We consistently found no inhibition of calcium binding to the 1223 glycopeptide or to the intact glycoprotein in the nitrocellulose $^{45}Ca^{+2}$ overlay assay (data not shown). Concentrations of mAb 1223 up to 20 μg/ml, which is well over the saturation level (5 μg/ml) found to decrease calcium uptake into cells, had no effect on calcium binding to the 1223 glycopeptide.

In vitro calcium binding overlays were also performed to test directly whether treatments that altered immunoreactivity of mAb 1223 with the 1223 glycopeptide also altered Ca^{+2} binding. As shown in Table III, neither mild acid hydrolysis nor treatment with neuraminidase destroyed Ca^{+2} binding to the 1223 glycopeptide. Likewise, mild base hydrolysis followed by neuraminidase digestion (see above) did not eliminate Ca^{+2} binding at pH 6.8. From these observations, we must conclude that removal of substituted sialic acid from the 1223 glycopeptide destroys immunoreactivity without destroying Ca^{+2} binding. This must mean that the 1223 epitope and the calcium binding site occupy distinct sites on the 3,200 M_r pronase glycopeptide.

The Calcium Binding Properties of the 1223 Glycopeptide Are pH Dependent

To estimate the pK_a of the residue(s) involved in Ca^{+2} binding to the 1223 glycopeptide, a series of experiments was performed in which $^{45}Ca^{+2}$ binding was measured using the overlay procedure at varying pH values. We found that lowering the pH of the binding solution to 5.0 reduced binding by ~50%. At pH 4.0, binding was barely detectable and at pH 3.0 or below, no binding was detectable (data not shown). Binding was stable between pH 6.8 and 8.0. These observations indicate that binding of Ca^{+2} is dependent on pH and suggest that the active group has a pK_a value between 4.0 and 5.0.

Discussion

Previous studies (Schneider et al., 1978; Mintz et al., 1981) have shown that gastrulation and spicule-formation by sea urchin embryos both are inhibited by the N-linked glycoprotein inhibitor tunicamycin. In addition, the incorporation of [^{3}H]glucosamine into glycoconjugates and of $^{45}Ca^{+2}$ into spicules are blocked in the presence of tunicamycin at concentrations that do not inhibit protein synthesis (Mintz et al., 1981). These earlier biochemical findings, coupled with morphological evidence obtained using Concanavalin A-gold (Decker et al., 1987), implicated a role for N-linked glycoproteins in skeleton formation by developing sea urchin embryos. These findings are consistent with those found during this study. In fact, our finding that mAb 1223, which inhibits calcium uptake and spicule formation in vitro, is directed against a carbohydrate epitope suggests that we have identified a key carbohydrate structure that might participate in calcium uptake and skeleton formation by spicule forming PMCs.

Primary mesenchyme cells have a unique task to accomplish during a very short interval of embryonic development:

assembly of a precisely organized skeletal structure within the confines of the embryonic blastocoel. The sea water surrounding the embryo is rich in calcium (~10 mM); however the calcium concentration in the blastocoel has not been well-defined. Almost certainly, however, it is lower than that in sea water. The strategy of the skeleton-forming embryo must be to import calcium from the sea water through the ectodermal cell layer and by some mechanism provide this calcium to the PMCs for uptake and conversion to $CaCO_3$. The molecular mechanisms involved in this complex process are completely unknown.

Several properties of the 1223 glycoprotein are of special interest. One is that this protein is extremely immunogenic when injected into mammalian cells. In fact, several laboratories, including our own, have obtained antibodies to this protein merely by injecting mixed populations of dissociated cells from gastrula-stage embryos (Carson et al., 1985; Anstrom et al., 1987) or intact spicule-forming cells (Shimizu et al., 1988). More interestingly, all of these antibodies have been shown to recognize carbohydrate epitopes (Anstrom et al., 1987; Shimizu et al., 1988; and this report). A second is that the expression of the carbohydrate structures recognized by these antibodies at the cell surface of the PMCs is coordinated with the differentiated functions of these cells; i.e., calcium uptake and skeleton formation. In particular, the 1223 epitope is developmentally regulated, appearing on the surface of the PMC at the time that uptake of $^{45}Ca^{+2}$ increases (Farach et al., 1987). A third point of interest is that the 1223 glycoprotein binds calcium. More specifically, the oligosaccharide bearing the 1223 epitope binds calcium in vitro under conditions that may resemble those in vivo. Whether this is related to its function remains a matter of speculation at present. Taken together, these findings strongly suggest that the 1223 glycoprotein plays a role in calcium uptake and skeleton formation by gastrula stage embryos of the sea urchin. We hypothesize that at least one function of the 1223 glycoprotein may be to facilitate the sequestration of calcium ions to the cell surface of the PMC such that they may be subsequently internalized for deposition into the growing embryonic skeleton.

The relationship of the 1223 epitope to calcium uptake is supported by other observations. In addition to the observation that mAb 1223 inhibits calcium uptake by PMCs (Carson et al., 1985), this monoclonal also alters the binding of Ca channel ligands to PMCs. Specifically, we found that in the presence of mAb 1223 (5 μg/ml), the binding of dihydropyridines to cultured PMCs was increased approximately twofold whereas the binding of verapamil was decreased to one-third of control levels (Farach-Carson, M. C., and W. J. Lennarz, unpublished observations). Unfortunately, attempts to study PMCs electrophysiologically using the patch-clamp technique proved unsuccessful because of their small size and elaborate extracellular matrix. Of many other tissues and cell lines that were surveyed, only the nonfusing embryonic mouse myoblast cell line designated BC3H1 expressed detectable amounts of the 1223 epitope (Caffrey and Farach, 1988). The relevance of these findings to the present study is that mAb 1223 also alters Ca^{+2} uptake into BC3H1 cells. More specifically, patch-clamp studies have shown that in BC3H1 cells, mAb 1223 interacts with the high threshold Ca channel protein. This evidence, coupled with the finding that mAb 1223 alters binding of Ca channel ligands to PMCs, indicates that the 1223 oligosaccharide epitope may be either part of or intimately associated with the voltage-dependent Ca channel in both spicule-forming cells and embryonic myoblasts.

In this study, we have partially characterized the epitope recognized by mAb 1223 on the 130-kD glycoprotein. The epitope appears to be part of an anionic N-linked oligosaccharide chain on the core protein. In light of its ability to bind Ca^{+2} ions, the finding that it bears a net negative charge is not surprising. On the other hand, several of the observations that we made regarding the nature of the sugar moieties were initially surprising. A previous report (Anstrom et al., 1987) found that the 1223 epitope was not expressed by sea urchin embryos grown in sulfate-depleted sea water, inferring that it might contain sulfated sugars. To test this more directly, we labeled cultures of isolated spicule-forming cells or gastrula-stage embryos with $^{35}SO_4$ and then isolated the 1223 pronase glycopeptide using a mAb 1223 immunoaffinity column. No mAb 1223-immunoreactive, sulfate-labeled material was detected in either gastrulae or PMCs, although dot blot analysis of the immunoaffinity purified material demonstrated that the 1223 epitope was present in both cases (data not shown). Thus we were unable to obtain direct evidence that the particular chain bearing the 1223 epitope is sulfated.

Selective chemical and enzymatic degradations before immunoblotting demonstrate that the 1223 epitope contains sialic acid residues that are likely to be *O*-acetylated. The resistance to periodate coupled with the susceptibility to neuraminidase only after treatment with mild base indicates the most likely point of substitution to be at the 9 position, although additional substitutions could occur at the 7 or 8 positions (see Cheresh et al., 1984; Varki and Diaz, 1984). The elution position of the 1223 glycopeptide from DE 52 is consisent with a proposed structure containing multiple negative charges. Comparison with standard anionic oligosaccharides with defined numbers of negative charges indicate that there must be either greater than three sialic acid residues or other anionic charge groups (Swiedler et al., 1983; Beeley, 1985). Based upon results showing a partial susceptibility to degradation by neuraminidases, including Endo N, we cannot eliminate the possibility that some polysialates may be present. Nonetheless, the complete susceptibility to neuraminidase after brief exposure to alkali provides strong evidence that the 1223 glycopeptide contains sialic acid. Unfortunately, the only way that the behavior of the 1223 glycopeptide could be monitored during anion exchange chromatography was with mAb 1223 binding. Since immunoreactivity was lost during enzyme digestion, we could not detect alterations in binding to DE 52 after neuraminidase digestion.

The residue(s) involved in calcium binding to the 1223 glycopeptide have an apparent pK_a value of 4.0–5.0. Negatively charged oligosaccharide structures that might promote Ca^{+2} binding with this pK_a include phosphate or carboxyl-containing structures. Depending upon the properties of the microenvironment including neighboring groups, any of a number of other charged moieties could function with this pK_a. Sialic acid residues have been demonstrated to function as calcium binding sites in other systems (Jaques et al., 1977; Kouvonen and Grasbeck, 1984). Physicochemical measurements based upon chemical shifts in ^{13}C and ^{1}H nuclear magnetic resonance spectra indicate that Ca^{+2} binds to sialic acid in free solution as a 1:1 complex with an associa-

tion constant of 121 ± 5 M^{-1} (Jaques et al., 1977). The sulfate groups in heparin have also been shown to function as Ca^{+2} binding sites, as have other sulfated glycosaminoglycans (Ayotte and Perlin, 1986; Vannucchi et al., 1978). Based upon earlier findings coupled with the results presented here, we propose that at least one site of calcium binding on the 130-kD glycoprotein resides on the 3,200-M_r oligosaccharide chain recognized by mAb 1223. However, because mAb 1223 does not inhibit calcium binding to the 1223 pronase glycopeptide and because treatments that destroy immunoreactivity do not destroy Ca^{+2} binding activity, it is unlikely that the sites of antibody binding and calcium binding are identical.

Our observation that mAb 1223 binds with greater efficiency to the aggregated form of the 1223 glycopeptide (Fig. 3) may indicate that mAb 1223 binds better to the calcium salt as compared to the free acid form of the epitope. The tendency of both the isolated 1223 glycopeptide (Fig. 3) and the intact glycoprotein (Fig. 6) to aggregate in solution is probably because of the formation of protein–calcium complexes that form stable aggregates. This type of behavior has been previously observed for other membrane proteins such as the asialoprotein receptor (for review, see Ashwell and Harford, 1982).

Understanding the structure of the 1223 oligosaccharide is complicated by our results following fucosidase treatment, where we found that immunoreactivity increased linearly with the time of digestion. This implies that removal of fucose residues from the 1223 oligosaccharide either increases binding affinity or exposes cryptic binding sites. The specificity of this enzyme indicates that these residues would occupy terminal positions (Carlsen and Pierce, 1972). In this case, fucosylation of the oligosaccharide chain would lead to masking of the epitope recognized by mAb 1223. This type of "epitope masking" by fucose residues is well precedented in living cells. The blood group antigens, for example, were found to be frequently interconverted by the addition or removal of fucose residues (Feizi and Childs, 1985). It is even possible that the fucose residues occupy terminal positions as capping sugars after internal sialic acid moieties. This type of structure was reported to occur in the sea cucumber, in which fucopyranosyl-(1,4)-*N*-glycolylneuraminic acid was identified (Van der Meer et al., 1983). The functional significance of the fucosylation of the 1223 glycoprotein is not clear at present.

In spite of the growing body of information that is available concerning the variety of carbohydrate structures assembled by living cells, very little is known about the functions of the oligosaccharides themselves. With rare exception, such as the mannose-6-phosphate receptor recognition system for uptake and targeting of lysosomal proteins (Kaplan et al., 1977; Ullrich et al., 1978; Fischer et al., 1980) or the asialoglycoprotein receptor mechanism for removal of galactose-terminating glycoproteins from the circulation (see Ashwell and Harford, 1982), the functions of most oligosaccharide structures remain unknown. Monoclonal antibodies directed against discrete carbohydrate epitopes on cell surface glycoproteins have expanded our knowledge about the role of carbohydrates in processes such as fertilization, intercellular adhesion, tumorigenesis, and host–parasite interaction (for review, see Ivatt, 1984; Sharon, 1984; Feizi, 1985). It is also becoming apparent that negatively charged oligosaccharide structures might play a role in binding of divalent cations such as calcium to the cell surface (Long and Mouat, 1971; Kouvonen and Grasbeck, 1984). Such a function might provide a new direction for the exploration of structure–function relationships for carbohydrates. The results of this study support the notion that certain anionic oligosaccharides are involved in calcium sequestration to the cell surface of spicule-forming cells. Experiments are underway to further test this hypothesis and to elucidate the exact structure of the 1223 oligosaccharide.

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