

# Inhibition of Glycoprotein Processing Blocks Assembly of Spicules during Development of the Sea Urchin Embryo

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**Abstract.** Previous studies have implicated an 130-kD glycoprotein containing complex, N-linked oligosaccharide chain(s) in the process of spicule formation in sea urchin embryos. To ascertain whether the processing of high mannose oligosaccharides to complex oligosaccharides is necessary for spiculogenesis, intact embryos and cultures of spicule-forming primary mesenchyme cells were treated with glycoprotein processing inhibitors. In both the embryonic and cell culture systems 1-deoxymannojirimycin (1-MMN) and, to a lesser extent, 1-deoxynojirimycin (1-DNJ) inhibited spicule formation. These inhibitors did not affect gastrulation in whole embryos or filopodial network formation in cell cultures. Swainsonine (SWSN) and castanospermine (CSTP) had no effect in either system. Further analysis revealed the following: (a) 1-MMN entered the embryos and blocked glycoprotein processing in the 24-h period before spicule formation as assessed by a twofold increase in endoglycosi-

dase H sensitivity among newly synthesized glycoproteins upon addition of 1-MMN; (b) 1-MMN did not affect general protein synthesis until after its effects on spicule formation were observed; (c) Immunoblot analysis with an antibody directed towards the polypeptide chain of the 130-kD protein (mAb A<sub>3</sub>) demonstrated that 1-MMN did not affect the level of the polypeptide that is known to be synthesized just before spicule formation; (d) 1-MMN and 1-DNJ almost completely abolished (>95%) the appearance of mAb 1223 reactive complex oligosaccharide moiety associated with the 130-kD glycoprotein; CSTP and SWSN had much less of an effect on expression of this epitope. These results indicate that the conversion of high mannose oligosaccharides to complex oligosaccharides is required for spiculogenesis in sea urchin embryos and they suggest that the 130-kD protein is one of these essential complex glycoproteins.

**E**ARLY studies on inhibition of glycoprotein synthesis and embryonic development of sea urchin revealed that tunicamycin blocked the process of gastrulation (Schneider et al., 1978; Heifetz and Lennarz, 1979). Subsequently, it was established that the sensitivity of the gastrulation process to tunicamycin could be related to a marked increase in the level of N-linked glycoprotein synthesis which occurs just before gastrulation (Lennarz, 1983). This requirement for glycoprotein synthesis was consistent with the finding that gastrulation could also be blocked by inhibiting synthesis of dolichyl phosphate (Carson and Lennarz, 1979, 1981), the lipid carrier required for oligosaccharide chain assembly in N-linked glycoprotein synthesis.

These early studies of the effect of tunicamycin on development also revealed that this drug blocked spiculogenesis when added at the late gastrula stage (Schneider et al., 1978). More recently this apparent requirement for N-linked glycoprotein synthesis during spicule formation has received greater attention. Carson et al. (1985) established that addi-

tion of a monoclonal antibody (mAb 1223) to a culture of primary mesenchyme cells caused a block in spiculogenesis. Immunofluorescence studies showed that the 1223 antigen was primary mesenchyme cell-specific. Immunoblot analysis revealed that one of the proteins containing the 1223 epitope was a 130-kD polypeptide whose level of expression correlated with the acquisition of the ability of primary mesenchyme cells to accumulate calcium during spicule formation. These observations, coupled with subsequent studies establishing that the 1223 antigen was a glycoprotein, and that the mAb 1223 was directed toward a carbohydrate chain on the glycoprotein (Farach et al., 1987; Farach-Carson et al., 1989), led us to study both the distribution of this epitope in the embryo and the nature of the oligosaccharide moiety in more detail. These studies have established that the epitope on the glycoprotein recognized by mAb 1223 is a complex, N-linked oligosaccharide chain (Farach-Carson et al., 1989) that is found in the cortical granules of the egg, disappears after fertilization and reappears in association with primary mesenchymal cells before spiculogenesis (Farach et al., 1987; Decker et al., 1988). Concurrently, Raff and co-workers (Angstrom et al., 1987) demonstrated that two other independently generated monoclonal antibod-

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ies, 1G8 (McClay et al., 1985) and B2C2 (Anstrom et al., 1987) also recognized an oligosaccharide group on the same 130-kD protein, suggesting that it is a highly immunogenic mesenchymal cell marker.

Given the complex nature of the carbohydrate chain of the 1223 antigen and its apparently essential role in a step in spiculogenesis, it seemed likely that inhibitors of the processing of oligosaccharide chains would block spicule formation. In this study, we report the results of biochemical and morphological studies with such inhibitors using both intact embryos and primary mesenchyme cells in culture. The results implicate complex oligosaccharide chains in general, and those associated with the 130-kD glycoprotein in particular, in the process of spiculogenesis in the developing sea urchin embryo.

## Materials and Methods

### Materials

*Strongylocentrotus purpuratus* were purchased from Pacific Bio Marine Supply (Venice, CA) or Marinus (Long Beach, CA). Artificial seawater (ASW),<sup>1</sup> (Instant Ocean) was obtained from Aquarium Systems (Mentor, OH). [2-<sup>3</sup>H]Mannose (30 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). L-[4,5-<sup>3</sup>H]-Leucine (156 Ci/mmol) and Na<sup>125</sup>I (carrier free) were from Amersham Corp. (Arlington Heights, IL). Goat anti-mouse IgG was obtained from Cappel Laboratories (Malvern, PA) and iodination was performed using the chloramine T method (Review 18, Amersham Corp.). Horse serum, penicillin-streptomycin, and gentamycin sulfate were purchased from Gibco Laboratories (Grand Island, NY). Endoglycosidase H (endo H), deoxymannojirimycin (1-MMN), deoxyojirimycin (1-DNJ), swainsonine (SWSN), and castanospermine (CSTP) were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals used were of reagent grade.

### Methods

**Processing Inhibitor Studies in Whole Embryos.** Eggs were collected and fertilized as described previously (Heifetz and Lennarz, 1979) and, after successive washings with ASW to remove sperm, the embryos were resuspended in ASW to a 1% suspension. 5-ml aliquots of this suspension were then cultured at 14°C in 9.5 cm<sup>2</sup>, 6-well Costar (Cambridge, MA) dishes. The indicated concentration of inhibitors were then added in a small volume of ASW. The addition was carried out within 1 h after fertilization, but before the first cleavage. At the stated times after fertilization, aliquots were removed for Western blot analysis as described below. Other aliquots were removed and the embryos fixed in 2% glutaraldehyde for subsequent photography using Nomarski optics and Kodak Panatomic X (ASA 32) film.

**Processing Inhibitor Studies in Micromere-derived Primary Mesenchyme Cell Cultures.** Micromeres were isolated by the procedure of Okazaki (1975) as modified by F. Wilt (University of California at Berkeley; personal communication). Calcium-free (CFSW) and calcium-, magnesium-free sea water (CMFSW) were prepared as described by McClay (1986). Eggs were fertilized in the presence of 3 mM 3-amino-1,2,4-triazole (Sigma Chemical Co., St. Louis, MO) and passed through a 64- $\mu$ m Nitex filter to remove fertilization membranes. The embryos were then cultured in CFSW/ASW (2:1) until after the first cleavage. The embryos were allowed to settle at that point and were resuspended in CFSW until the 16-cell stage. The embryos were harvested and maintained at 4°C through the disaggregation step. After two washes with CMFSW, the embryos were incubated for 4 min in CMFSW with 1 mM EDTA. The embryos were pelleted by low speed centrifugation, resuspended in a small volume of CFSW and passed through a 10-ml serological pipette several times to disaggregate the embryos. The cells were diluted with CFSW to a 10% suspension and 10-ml aliquots were layered over 200-ml linear sucrose gradients ranging from 5% of 0.75 M sucrose in 95% CFSW to 25% 0.75 M sucrose in 75% CFSW with a 100-ml

1. **Abbreviations used in this paper:** ASW, artificial seawater; CFSW, calcium-free seawater; CMFSW, calcium- and magnesium-free seawater; CSTP, castanospermine; 1-DNJ, deoxyojirimycin; 1-MMN, deoxymannojirimycin; SWSN, swainsonine.

cushion of 25% sucrose. The cells were allowed to settle for 45–60 min and then the micromeres were harvested from just beneath the lysis layer at the top of the gradient. About 40 ml of micromere suspension were removed per gradient and 7-ml aliquots of the suspension plated per well of a 6-well dish. CaCl<sub>2</sub> was added from a 1-M solution to a final concentration of 10 mM, and the cells were allowed to attach for 1 h before removal of nonadherent cells. The adherent cells were washed once with ASW followed by addition of 5 ml of culture media consisting of 3% (vol/vol) horse serum, 1% (vol/vol) penicillin-streptomycin, 0.2 g/liter ticarcillin disodium (Sigma Chemical Co., St. Louis, MO), and 10 mg/liter erythromycin (Abbott Laboratories, Irving, TX) in ASW. The micromeres were allowed to attach firmly overnight and the following morning various concentrations of glycoprotein processing inhibitors were added in a small volume of ASW. Photographic records were made at various times using phase contrast and Kodak Panatomic X (ASA 32) film.

### Western Immunoblots

Aliquots of embryo cultures were removed at designated times and extracted overnight in Tris buffer containing 2% cholate and a mixture of protease inhibitors as described previously (Carson et al., 1985). Protein concentrations of the extracts were determined by the method of Lowry et al. (1951) using BSA as a standard. The extracts (200  $\mu$ g protein/lane) were then separated by SDS-PAGE (8.75% acrylamide). The transfer to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) and incubation with either mAb 1223, mAb A<sub>3</sub>, or <sup>125</sup>I-labeled goat anti-mouse IgG were as described by Farach et al. (1987) with the exception that the blocking buffer contained 3% nonfat dry milk (Carnation) instead of hemoglobin.

### Protein Synthesis

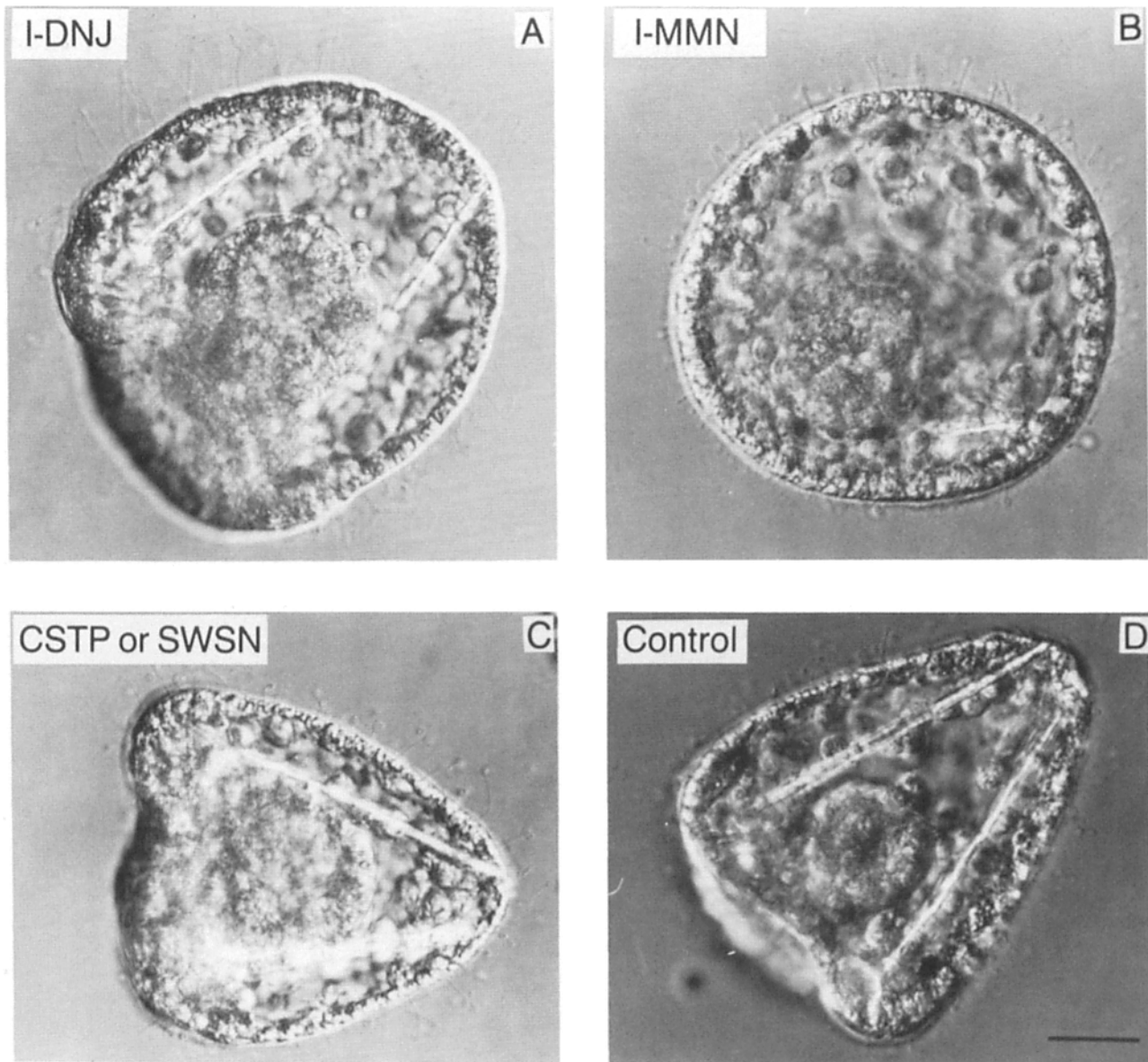
Rates of protein synthesis were measured at 24, 48, 72 h after fertilization. [<sup>3</sup>H]Leucine (10  $\mu$ Ci/ml of culture) was added to control and 1-MMN-treated embryos. At 5, 10, 15, and 20 min after addition of radiolabel, 1-ml aliquots were removed, placed on ice, and immediately precipitated with 10% TCA. After two washes with 10% TCA, the pellets were solubilized overnight in 0.1 N NaOH at 37°C. Subsequently, one aliquot was used for protein determination and another aliquot for measuring [<sup>3</sup>H]leucine incorporation. The rates of incorporation of label into protein were determined from the initial velocity of [<sup>3</sup>H]leucine incorporation into TCA precipitable material/mg protein plotted against the time of incubation.

### Glycopeptide Analysis

Embryo cultures (2% suspension) of 5 ml in 6-well dishes were prepared as described above. [2-<sup>3</sup>H]Mannose (20  $\mu$ Ci/ml of culture) was added to 2–4 wells of 1-MMN-treated and control embryos at either 24 or 48 h after fertilization. After incubation for 24 h with radiolabel, cultures of like treatment were pooled and concentrated by gentle centrifugation. The pellets were washed three times with CHCl<sub>3</sub>/MeOH (2:1) and three times with CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O (10:10:3). The protein pellet was resuspended in 1 vol of 0.1 M Tris, pH 8.0, by sonication. Pronase (Calbiochem-Behring Corp., San Diego, CA) was dissolved in the Tris buffer (10 mg/ml) and incubated at 50°C for 30 min. An aliquot of the Pronase mixture (0.1 vol) was added to the resuspended sea urchin protein pellet along with a drop of toluene and the mixture was incubated for 72 h at 37°C, with fresh additions of Pronase at 24 and 48 h. After 72 h, the mixture was boiled for 20 min and clarified by centrifugation. The supernatant was chromatographed on Biogel P-4 column (1  $\times$  40 cm) equilibrated with 0.1 M ammonium bicarbonate. Aliquots of each fraction were assayed for [<sup>3</sup>H]mannose and the glycopeptide containing fractions (*V*<sub>E</sub>, 11–25 ml) were pooled, lyophilized, and resuspended in 50 mM Na citrate, pH 5.5, with a drop of toluene. Two equal portions, with and without endo H (10 mU), were incubated at 37°C for 24 h with a subsequent addition of fresh endo H at 12 h. After the incubation, the mixtures were boiled for 10 min and clarified by centrifugation. The aliquots were subsequently rechromatographed on the Biogel P-4 column and the fractions were assayed for [<sup>3</sup>H]mannose to determine sensitivity of the glycopeptides to endo H.

## Results

Inhibitors of both the initial removal of glucose residues and the subsequent trimming of mannose residues during oligosaccharide chain processing (Elbein, 1987) were tested for



**Figure 1.** Effect of processing inhibitors on embryogenesis. Eggs were fertilized and embryos cultured as described in Materials and Methods. At 72 h postfertilization, embryos treated with 4 mM 1-DNJ (A), 2 mM 1-MMN (B), 2 mM CSTP or 80  $\mu$ M SWSN (C), and untreated controls (D) were fixed in 2% glutaraldehyde, and photographed using Nomarski optics. Bar, 5  $\mu$ m.

their effect on spicule formation. The inhibitors used were 1-DNJ and CSTP which block both glucosidase I and II; 1-MMN which blocks mannosidase I; and SWSN which inhibits mannosidase II. The site of action of these inhibitors is shown in scheme I. The inhibitors were added to embryo cultures in concentrations previously shown to be effective in mammalian systems (Elbein, 1987). The results shown in Fig. 1 clearly establish that two of the inhibitors, 1-MMN and 1-DNJ, affected normal embryonic development between gastrula and prism stage by impairing or completely blocking spicule formation. In contrast, embryos treated with CSTP or SWSN were indistinguishable from untreated control embryos. The inhibitory effect was most dramatic in 1-MMN-treated cultures, where the embryos maintained a rounded appearance more associated with gastrula stage embryos rather than the triangular shape associated with prism

stage. Spicule formation in 1-MMN-treated embryos was markedly impaired; in many embryos no spicules could be detected whereas in others only partial spicules were evident. The spicules in 1-DNJ treated embryos were generally longer, but still incomplete. In neither group were embryos observed with full-length spicules meeting at the posterior of the embryo. The effect on spicule formation was detectable as early as 48 h after fertilization; i.e., at the mid to late gastrula stage when nascent spicules are normally first detectable in the embryos.

To insure that the observed effect was due to inhibition of processing of the oligosaccharide chains on glycoproteins and not to a general toxic effect of the inhibitors, the rate of protein synthesis was determined in control and 1-MMN-treated embryos. From the results shown in Table I, it is clear that there was no significant difference between the two

**Table I. Effect of 1-MMN on the Rate of Protein Synthesis**

Drug treatment	Rate of incorporation per time (h) of development		
	24 h	48 h	72 h
None	3.6	5.8	6.0
1-MMN (2 mM)	3.6	5.4	4.2

Rate of incorporation into protein was measured in embryo cultures as described in Materials and Methods and is expressed as cpm of [<sup>3</sup>H]leucine incorporated/mg protein/min.

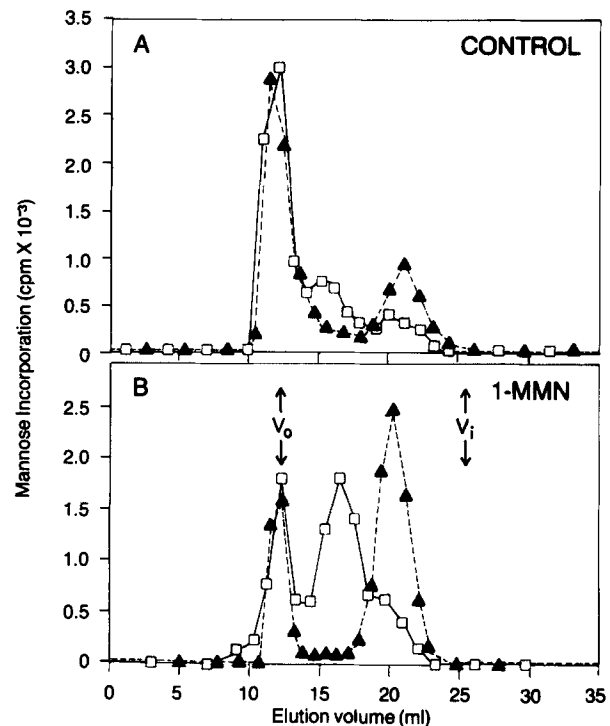
groups of embryos until after 72 h. Thus, at a stage when an impairment of spiculogenesis had already occurred (48 h), there was no measurable effect on protein synthesis. These findings suggest that the primary effect of 1-MMN is not general toxicity. At 72 h, which corresponds to early pluteus stage in control embryos, the rate of protein synthesis in the 1-MMN-treated embryos declined by ~30% relative to the untreated embryos. Presumably, the decline in rate at this late stage is a secondary consequence of impaired development caused by inhibition of glycoprotein processing.

To determine whether these processing inhibitors affected glycoprotein processing *in vivo*, we examined the endo H sensitivity of [2-<sup>3</sup>H]mannose-labeled glycopeptides prepared from 1-MMN or control embryo cultures by Pronase digestion. If 1-MMN blocked processing to complex oligosaccharide chains, one would expect a greater proportion of the glycopeptide (which elutes near the  $V_0$  on a Biogel P-4 column) would be sensitive to treatment with endo H, a glycosidase with specificity towards high mannose glycopeptides. Such sensitivity would result in the release of smaller molecular weight oligosaccharides eluting nearer the  $V_i$  of the column. Cultures were incubated with [2-<sup>3</sup>H]mannose for a 24-h period starting at either 24- or 48-h postfertilization. As shown in Fig. 2, in the 24- to 48-h labeling interval a much larger fraction of the glycopeptides recovered from the 1-MMN-treated cultures was sensitive to endo H; 72% of the glycopeptides from the drug-treated culture were endo H sensitive, whereas only 30% were sensitive in the control culture. In the 48- to 72-h labeling experiment, the results were quite similar, with 64% of the labeled glycopeptides being endo H sensitive for treated embryos and only 28% for control embryos. Thus, it is clear that relatively early in development 1-MMN entered the embryos because it affected glycoprotein processing by the early gastrula stage.

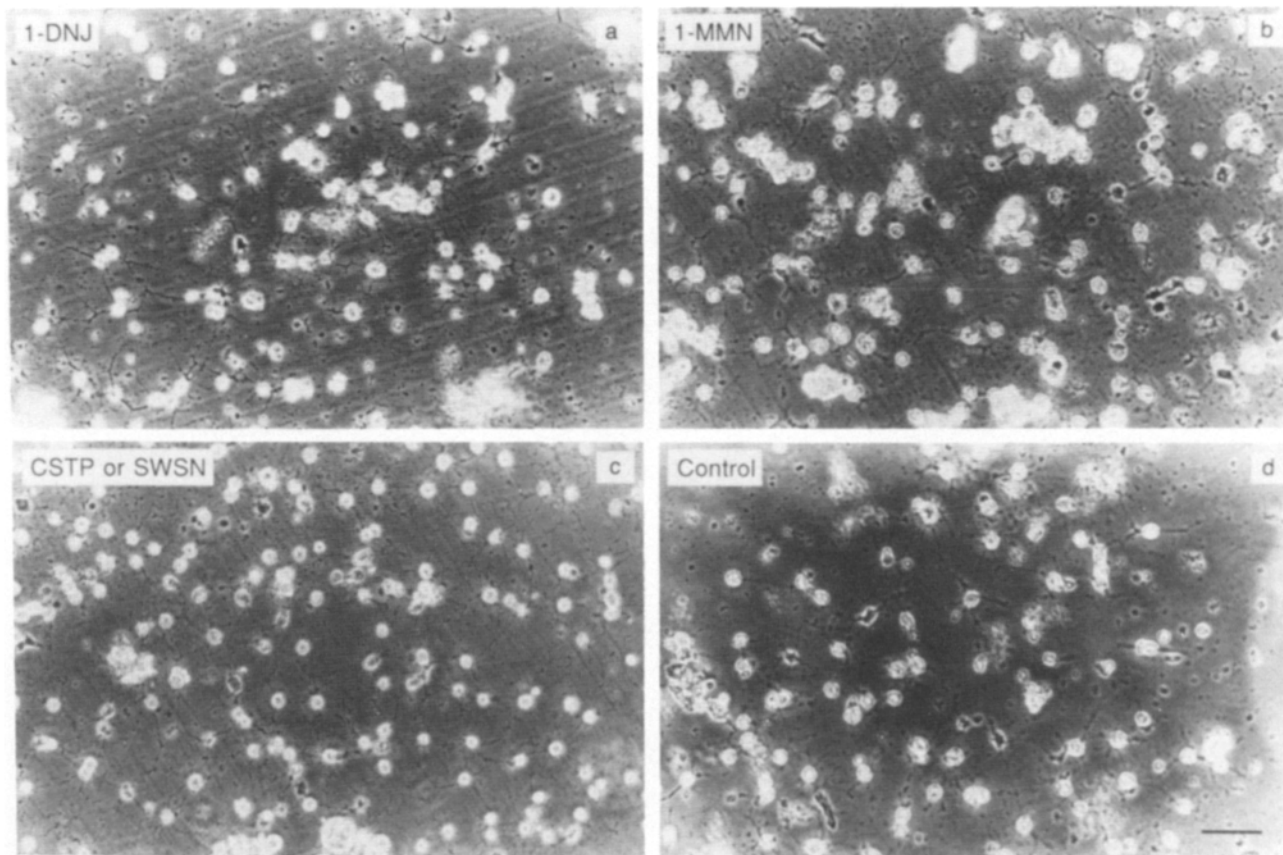
Because the major morphological effect resulting from 1-MMN and 1-DNJ treatment appeared to be related to spiculogenesis, the effect of these drugs was further examined in a cell culture system in which isolated micromeres differentiate into spicule-forming primary mesenchyme cells. After the isolated micromeres became firmly attached to the culture dishes, concentrations of processing inhibitors identical to those used in the whole embryo studies were added to the culture media. As shown in Fig. 3, no differences in appearance of the cultured cells were detectable for the first 48 h of culture. However, the results shown in Fig. 4 indicated that by 96 h of culture when spicules normally become fully elongated, there were striking effects that exactly paralleled those observed in whole embryos (Fig. 1). Thus, addition of SWSN or CSTP had little effect on spiculogenesis,

whereas 1-DNJ and 1-MMN inhibited this process, with 1-MMN being the most effective. This impairment of spicule formation was noticeable as early as 72 h after culture, when nascent spicules first appear (data not shown). It should be noted that despite the presence of 1-MMN or 1-DNJ, the cells still produced extensive networks of filopodia (Fig. 5). This observation indicates that neither 1-MMN nor 1-DNJ were toxic in these cultures, and that extensive processing of glycoproteins is not necessary for production of the filopodial networks that form before generation of syncytia. Because of the small amounts of material in these primary mesenchyme cell cultures, we did not attempt parallel labeling studies with respect to [<sup>3</sup>H]leucine incorporation or endo H sensitivity of glycopeptides. However, given the striking similarity of the results with those observed in whole embryos and the lack of effect of the drugs on either cell attachment or filopodial network formation, it is reasonable to assume that the inhibitors directly affected glycoprotein processing and did not exert some general toxic effect in these cell cultures.

Previous work by Farach et al. (1987) demonstrated that a glycoprotein containing an N-linked complex carbohydrate epitope recognized by mAb 1223 plays an essential role in spiculogenesis. Consequently, it was of interest to determine if the inhibitory effect of the processing inhibitor on spiculo-



**Figure 2.** Effect of 1-MMN treatment on endo H sensitivity of mannose-labeled N-linked oligosaccharide chains. Eggs were fertilized and embryos cultured as described in Materials and Methods. [2-<sup>3</sup>H]mannose was added (20  $\mu$ Ci/ml culture) to untreated control cultures (A) and cultures treated with 2 mM 1-MMN (B). After incubation for 24 h, the proteins were extracted, washed, pronase digested, and glycopeptides isolated as described in Materials and Methods. One portion of the glycopeptide preparation remained untreated ( $\square$ --- $\square$ ) while another portion was treated with endo H ( $\blacktriangle$ --- $\blacktriangle$ ). The two preparations were subsequently chromatographed on Biogel P-4 (1  $\times$  40 cm) to assess the effect of endo H treatment.



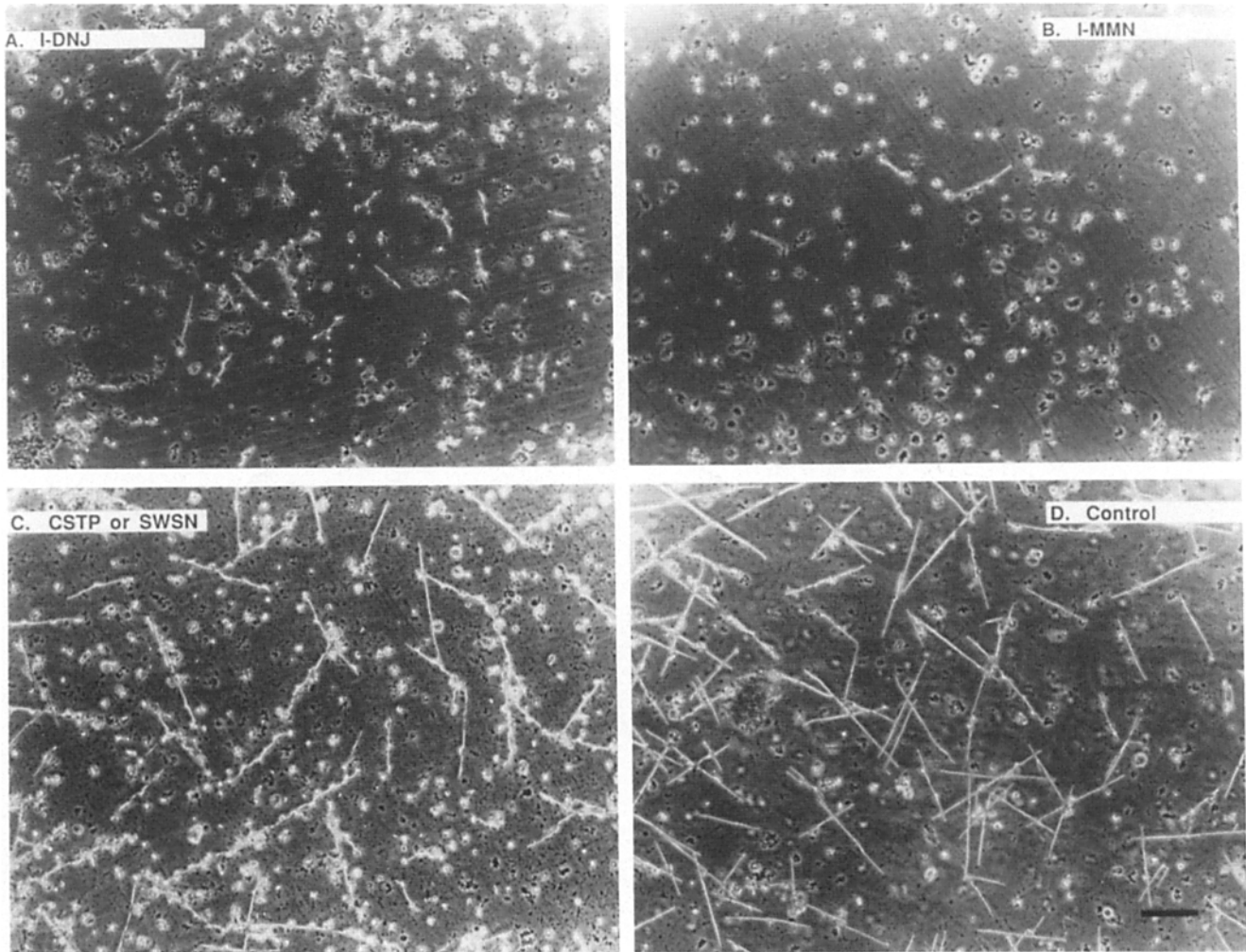
**Figure 3.** Effect of processing inhibitors on primary mesenchyme cell cultures at 48 h. Micromere-derived mesenchyme cell cultures were prepared and inhibitors added as described in Materials and Methods. Photographic records were made at 48 h of cultures treated with 4 mM 1-DNJ (a), 2 mM 1-MMN (b), 2 mM CSTP or 80  $\mu$ M SWSN (c), and untreated control cultures (d). Bar, 10  $\mu$ m.

genesis correlated with a block in expression of the 1223 epitope. To do this, Western blot analysis with mAb 1223 was performed on protein extracts from the variously treated embryo cultures. As shown in Fig. 6, two major protein bands (130- and 205-kD) bearing the 1223 epitope consistently appeared in control cultures; the presence of 1-DNJ and 1-MMN greatly diminished (>95%) the amount of this epitope in both protein species. In contrast, SWSN and CSTP, two processing inhibitors that had virtually no effect on spiculogenesis, exhibited much less of an effect on expression of this epitope. The availability of an antibody (mAb A<sub>3</sub>) to the protein portion of the 130-kD species (Anstrom et al., 1987), kindly provided by R. Raff, Indiana University, made it possible to determine if the processing inhibitors had an effect on the level of expression of that polypeptide chain associated with the 1223 antigen. The results of the Western blot analysis shown in Fig. 7 revealed that there was no decrease in the intensity of the band upon 1-MMN treatment. Thus, inhibition of processing had no effect on the synthesis of the polypeptide chain of the 130-kD glycoprotein.

From the results shown in Fig. 6 it is evident that besides a diminishment of the intensity of the immunoreactive bands, an apparent decrease in molecular weight; i.e., a higher mobility was observed for the 1223 antigen upon 1-MMN, 1-DNJ, or SWSN treatment. This shift is readily apparent for the 205-kD protein upon treatment with the aforementioned inhibitors and for the 130-kD protein upon SWSN treatment.

Overexposure of the autoradiogram in Fig. 6 a shows a similar shift in the 130-kD protein upon 1-MMN and 1-DNJ treatment (data not shown). The availability of mAb A<sub>3</sub> also enabled us to establish whether or not the band of higher mobility in the vicinity of the 130-kD protein represented the same polypeptide moiety. The results in Fig. 7 show that mAb A<sub>3</sub> recognized only the 130-kD protein from control embryos and a species of slightly lower molecular weight from the 1-MMN-treated embryos. Having confirmed that the two bands of different mobility represent the same polypeptide species in the 130-kD protein, the autoradiograms in Fig. 6 were densitometrically scanned to quantitate the relative amounts of the immunoreactive carbohydrate epitope expressed on the glycoprotein with different inhibitor treatments. The results, presented in histogram form in Fig. 8, are consistent with the observed effects of the processing inhibitors on spiculogenesis in whole embryo and primary mesenchyme cell cultures and clearly support the conclusion that a block in processing of the oligosaccharide chain of the 1223 antigen blocks spiculogenesis.

In contrast, very little is known about the 205-kD protein. The work of Leaf et al. (1987) suggests that it shares a common posttranslational modification (i.e., the same N-linked oligosaccharide), but that the polypeptide chain is different from that of the 130-kD protein. No antibodies are available to the polypeptide portion of the 205-kD protein, and, therefore, we cannot conclude with certainty that the lower mo-



**Figure 4.** Effect of processing inhibitors on spicule formation. Micromere-derived primary mesenchyme cell cultures were prepared as described in Materials and Methods. Photographic records were made at 96 h of cells treated with 4 mM 1-DNJ (A), 2 mM 1-MMN (B), 2 mM CSTP or 80  $\mu$ M SWSN (C), and untreated control cultures (D). Bar, 20  $\mu$ m.

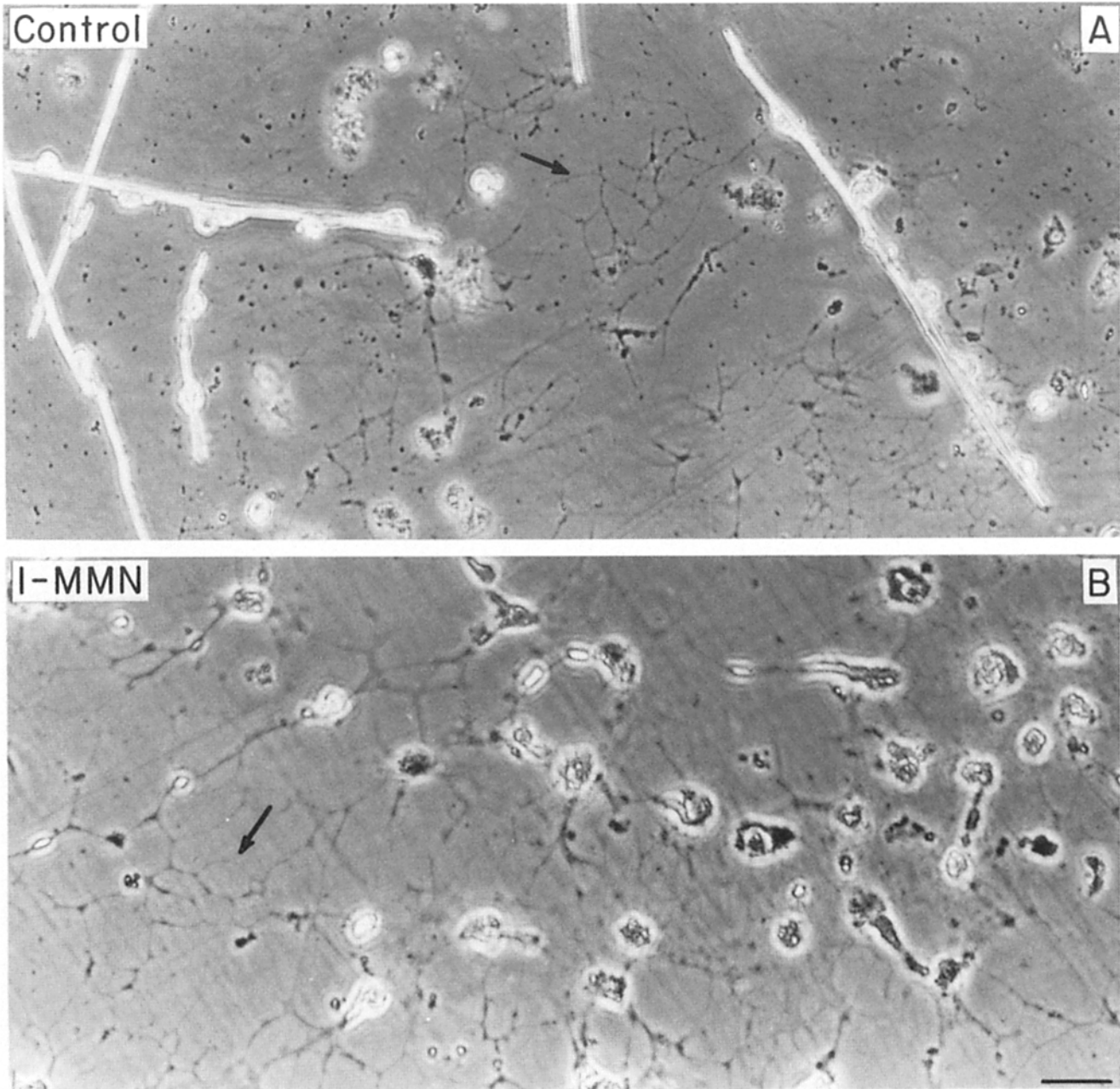
lecular mass species in the inhibitor-treated sample is the same protein. Because of this uncertainty no further analysis was done of the 1223 epitope on the 205-kD glycoprotein.

### Discussion

Earlier studies from this laboratory established that N-linked glycoprotein synthesis was required for two major morphogenetic events in the development of the sea urchin embryo, gastrulation and subsequent skeleton formation (Schneider et al., 1978). This relationship between glycoproteins and formation of the skeleton was later confirmed and extended using an *in vitro* cell culture system containing the spicule, forming primary mesenchyme cells (Mintz et al., 1981; Mintz and Lennarz, 1982). The serendipitous isolation of a mAb 1223, enabled us to further explore this relationship. It was found that the mAb 1223 bound to the surface of primary mesenchyme cells, blocked  $Ca^{2+}$  deposition and caused a subsequent arrest of spiculogenesis in cell culture (Carson et al., 1985). On the basis of these observations, and the finding that an 130-kD protein bearing the 1223 antigen began to appear during the mesenchyme blastula stage and

peaked during late gastrula stage (Farach et al., 1987), we proposed that this glycoprotein was involved in spiculogenesis.

As mentioned in the introduction, two additional primary mesenchyme cell-specific monoclonal antibodies, designated IG8 (McClay et al., 1985) and B2C2 (Anstrom et al., 1987) exhibit the same cell type specificity and developmental expression observed with the 1223 antigen. Anstrom et al. (1987) established that all three monoclonal antibodies recognized the same protein and Leaf et al. (1987) cloned a cDNA encoding for msp130 and reported a partial sequence. Very recently the complete deduced sequence and the existence of a phospholipid anchor on msp130 have been reported (Parr et al., 1990). By use of various glycosidase treatments, it has been shown that the epitope recognized by B2C2 (Anstrom et al., 1987) and 1223 (Farach et al., 1987) is an N-linked oligosaccharide chain (Farach-Carson, 1989). Additionally, Farach-Carson et al. (1989) have demonstrated that the 1223 epitope is an acidic, complex oligosaccharide chain capable of binding calcium. Indirect evidence also suggested that the oligosaccharide epitope contains O-acetylated sialic acid residues.

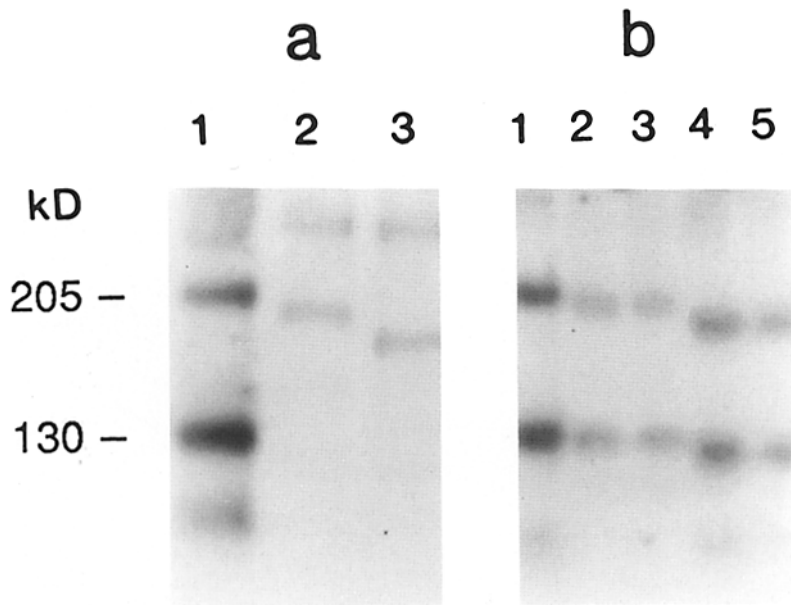


**Figure 5.** Effect of processing inhibitors on filapodial network formation. The untreated cell cultures (A) and 1-MMN-treated cell cultures (B) at 96 h are depicted at higher magnification for observation of filapodial networks. The arrows point to examples of such networks. Bar, 5  $\mu$ m.

Given (a) the complex nature of the oligosaccharide chain of the 130-kD glycoprotein and (b) the evidence that this 130-kD glycoprotein is involved in the process of spiculogenesis, it became of interest to study the developmental effects of drugs that inhibit processing and thereby prevent complex oligosaccharide chain formation. The studies were carried out using two inhibitors of the initial glucosidase activities, 1-DNJ and CSTP, and two inhibitors of the subsequent mannosidase activities, 1-MMN and SWSN. None of these drugs had any inhibitory effect on morphogenesis during the first 48 h in culture; blastulation, primary mesenchyme cell ingress and gastrulation proceeded normally. However, skeleton formation at the late gastrula-prism stage

(48 to 72 h) was markedly impaired by 1-MMN and 1-DNJ, with 1-MMN being most effective. The other two drugs had no effect on the spicule formation process. In contrast, treatment with tunicamycin, which inhibits all N-linked glycoprotein synthesis, blocks both gastrulation and skeletogenesis. This would suggest that high mannose but not complex type N-linked oligosaccharides are required for gastrulation, whereas certain complex type oligosaccharide chains are required for spiculogenesis.

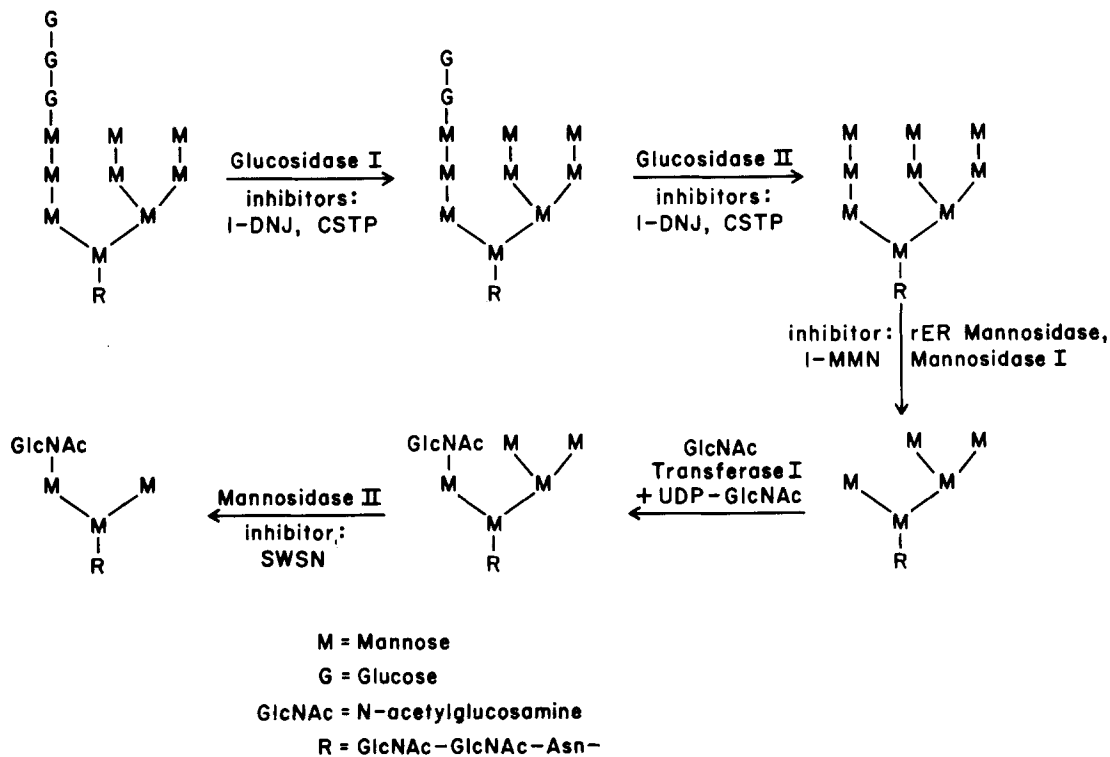
There are many possible explanations for the observation that some of the processing inhibitors are more effective than others in blocking spiculogenesis. If complex oligosaccharide formation is crucial for spiculogenesis, then it is not sur-



**Figure 6.** Effect of processing inhibitors on expression of the 1223 epitope in gastrula stage embryos. Cultures were prepared, inhibitors added, proteins extracted at 54 h, and immunoblots performed with mAb 1223 as described in Materials and Methods. In experiments 1 (a), embryos were untreated (lane 1) or treated with either 4 mM 1-DNJ (lane 2) or 2 mM 1-MMN (lane 3). In experiment 2 (b), embryos were untreated (lane 1), or treated with 1 mM CSTP (lane 2), 2 mM CSTP (lane 3), 40 μM SWSN (lane 4), or 80 μM SWSN (lane 5). All lanes contained 200 μg of protein.

prising that 1-MMN is the most effective inhibitor. By blocking mannosidase I this drug inhibits all possible processing of either branch of the oligosaccharide chain (Fuhrmann et al., 1985). In contrast, use of the other inhibitors is usually accompanied by "leakage" (see Scheme I), resulting in some level of complex oligosaccharide chain formation (Fuhrman et al., 1985). This is especially true of SWSN, which has been clearly established to induce formation of hybrid oligosaccharides with one high mannose branch and one processed

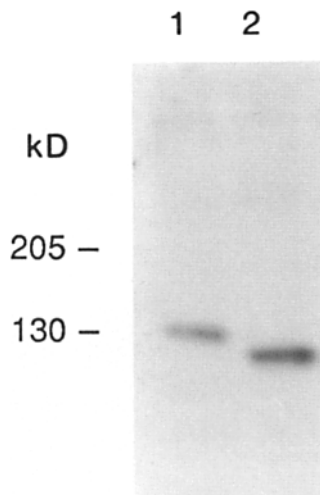
complex branch (Tulsiani and Touster, 1983; Elbein, 1987). In a large number of studies, SWSN had no effect on glycoprotein function, and it has been suggested that partial complex chain formation may be sufficient for the activity of such glycoproteins (Elbein, 1987). The results obtained in this study in which SWSN treatment clearly causes a distinct shift in mobility of the 1223 epitope and yet only decreases the intensity of the immunoreactive band by ~50% strongly supports this possibility. The ineffectiveness of CSTP and



**Scheme I**

*Scheme I.*



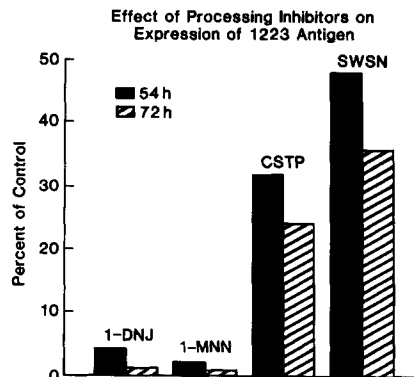


**Figure 7.** Effect of 1-MMN on expression of the polypeptide chain of the 130-kD protein in gastrula stage embryos. Embryos were cultured, 1-MMN added, proteins extracted at 52 h, and immunoblots with mAb A<sub>3</sub> were performed as described in Materials and Methods. Embryos were either untreated (lane 1) or treated with 2 mM 1-MMN (lane 2). Each lane contained 200 μg of protein.

SWSN may also simply reflect the poorer inhibitory properties of these compounds relative to 1-DNJ and 1-MMN, respectively, thus allowing enough complex carbohydrate formation to allow spicule formation to occur. In fact the potency of each of these inhibitors has been shown to vary widely depending on the particular biological system (Fuhrman et al., 1985), although in general the concentration of 1-DNJ required for 50% inhibition of glucosidase activity is much lower than that for CSTP. It is possible that, by further raising the concentrations of SWSN and CSTP, a similar effect on spiculogenesis would be observed, but the concentrations used for these drugs were already quite high and further elevation would probably introduce general toxicity effects.

Because it was possible that the inhibitory effects of 1-MMN and 1-DNJ on spicule formation might be unrelated to an impairment in complex oligosaccharide chain synthesis, in separate experiments we carried out metabolic labeling of the polypeptides with [<sup>3</sup>H]leucine and the oligosaccharide chains with [<sup>3</sup>H]mannose. The polypeptide labeling experiments revealed that there was little effect of the processing inhibitors on protein synthesis until after the onset of inhibition of spiculogenesis. In contrast, when the glycopeptides generated from [<sup>3</sup>H]mannose-labeled glycoproteins were digested with endo H and then analyzed by gel filtration, it was found that in the presence of the processing inhibitors the proportion of polymannose chains was markedly increased relative to that of complex chains. Thus, the drugs do impair processing of the oligosaccharide chains of glycoproteins in the developing embryo and this inhibition occurs before spiculogenesis.

The observations discussed above with respect to spicule formation in the intact embryos were confirmed by similar studies in primary mesenchyme cells in culture. None of the drugs had any effect on the formation of filopodial networks that occurs before assembly of the syncytia in which the spicule is formed. However, as was observed in the intact embryos, both 1-MMN and 1-DNJ blocked spiculogenesis in the cultured cells with 1-MMN being most effective. Immunoblot analysis of protein extracts from intact embryos with mAb 1223 revealed that the level of the immunoreactive 1223 carbohydrate epitope of the 130- and 205-kD proteins markedly decreased in the presence of the processing inhibitors. The level of the decrease in the 130-kD protein was



**Figure 8.** Quantitation of the effect of processing inhibitors on the level of 1223 epitope expression. The autoradiograms (54 h) presented in Fig. 6 (solid bars), as well as those obtained from extracts at 72 h (hatched bars) from the same cultures (not shown), were densitometrically scanned. The levels of the 1223 epitope associated with the 130-kD protein were expressed as a percentage relative to control levels for the corresponding time point. The measurements for CSTP and SWSN correspond in Fig. 6 b to lane 3 (2 mM) and lane 5 (80 μM).

found to be consistent with the observed effects of the inhibitors on spiculogenesis; i.e., the decrease was much more marked with 1-MMN (>98%) and 1-DNJ (96%), as compared to CSTP (68%) and SWSN (50%). In contrast, the immunoblot analysis using mAb A<sub>3</sub>, which is directed toward the polypeptide chain of the 130-kD protein demonstrated that the level of the protein backbone was unaffected by drug treatment. This result, when combined with the fact that synthesis of this protein begins roughly 24 h subsequent to the addition of inhibitors (Farach et al., 1987; Leaf et al., 1987; McClay et al., 1985) further corroborates the findings of the labeling experiments in intact embryos that demonstrated the specificity of the effect of these drugs on glycoprotein processing. Additionally, the analysis with mAb A<sub>3</sub> revealed that the apparent molecular mass of the 130-kD protein was reduced upon inhibitor treatment, as would be expected if the addition of *N*-acetylglucosamine, galactose, sialic acid, and/or other sugar residues did not occur because of a block in glycosidase-mediated processing of the newly formed glycosylated, high-mannose oligosaccharide chains. The fact that the mAb 1223 probe for the complex oligosaccharide chain (Farach-Carson, 1989) recognizes a fainter band of lower molecular mass reveals further information about the glycoprotein. If there is only one glycosylation site, the inhibition of processing of that oligosaccharide should cause complete disappearance of the band when mAb 1223 is used as a probe. Sequencing of the cloned msp130 gene has revealed six consensus sequences for possible *N*-linked glycosylation (Parr, et al., 1990). Our results suggest the possibility that more than one of these sites is actually glycosylated and that most, but not all of the chains on the glycoprotein are blocked from being processed by 1-MMN and 1-DNJ.

Our conclusion from these studies with oligosaccharide processing inhibitors is that processing of certain oligosaccharide chains from the high-mannose to the complex type is essential for spiculogenesis in sea urchin embryos. Although complex oligosaccharide chains have been shown to

be required in human B lymphocyte differentiation (Tulp et al., 1986), to the authors' knowledge, this is the first report of the necessity of complex oligosaccharide chain formation for proper embryonic development. This is in contrast to the majority of the other reports in which the role of oligosaccharide chain processing in normal cell differentiation has been examined. Studies with differentiating myoblasts (Holland and Herscovics, 1986; Spearman et al., 1987; Simard and Connolly, 1987; Trudel et al., 1988), embryonic brain cells (Bhat, 1988), and embryonic corneal cells (Overton, 1988) have all led to the conclusion that glucosidase inhibitors blocked differentiation, whereas mannosidase inhibitors did not. From this it has been inferred that the removal of the terminal glucose residues to form high-mannose oligosaccharides, but not the further processing to form complex oligosaccharides, is necessary for normal differentiation in these systems. Interestingly, in the study of abnormal cells undergoing differentiation, it has been reported that complex oligosaccharide chain formation is necessary for both murine sarcoma L-1 cells to become metastatic (Pulverer et al., 1988) and for murine P815 tumors to be recognized and lysed by activated mouse macrophages (Mercurio, 1986).

Overall, the results of these studies on the effect of 1-MMN and 1-DNJ in intact embryos and isolated primary mesenchyme cells have established a correlation between a block in processing of the oligosaccharide chain of glycoproteins and impairment of spicule formation. Although these results do not exclude the possibility that a number of different glycoproteins are involved in spiculogenesis, they do provide strong additional support for the earlier proposal that the 130-kD glycoprotein in particular plays a role in the  $Ca^{2+}$  deposition process (Carson et al., 1985). Current studies on structural characterization should enable us to gain insight into how the oligosaccharide chain of this glycoprotein might participate in this important process.

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