

Localization and expression of msp130, a primary mesenchyme lineage-specific cell surface protein of the sea urchin embryo

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Summary

In this report, we use a monoclonal antibody (B2C2) and antibodies against a fusion protein (Leaf *et al.* 1987) to characterize msp130, a cell surface protein specific to the primary mesenchyme cells of the sea urchin embryo. This protein first appears on the surface of these cells upon ingress into the blastocoel. Immunoelectronmicroscopy shows that msp130 is present in the trans side of the Golgi apparatus and on the extracellular surface of primary mesenchyme cells. Four precursor proteins to msp130 are identified and we show that B2C2 recognizes only the mature form of msp130. We demonstrate that msp130 contains *N*-linked carbohydrate groups and that the B2C2 epitope is sensitive to endoglycosidase F digestion. Evidence that msp130 is apparently a sulphated glycoprotein is presented. The recognition of

the B2C2 epitope of msp130 is disrupted when embryos are cultured in sulphate-free sea water. In addition, two-dimensional immunoblots show that msp130 is an acidic protein that becomes substantially less acidic in the absence of sulphate. We also show that two other independently derived monoclonal antibodies, 1G8 (McClay *et al.* 1983; McClay, Matranga & Wessel, 1985) and 1223 (Carson *et al.* 1985), recognize msp130, and suggest this protein to be a major cell surface antigen of primary mesenchyme cells.

Key words: sea urchin embryos, primary mesenchyme cells, cell surface protein, msp130, sulphated glycoprotein, monoclonal antibody.

Introduction

The primary mesenchyme cells of sea urchin embryos are the progeny of the four micromeres formed at the fourth cleavage. Following hatching, the descendants of the micromeres, approximately 32 cells, ingress into the blastocoel and become primary mesenchyme cells. These cells begin to migrate by extending filopods which attach to the basal lamina and then contract, pulling the cell body toward the site of attachment (Gustafson & Wolpert, 1967). Following 6–8 h of apparently random migration, they arrange themselves into a ring of cells in the vegetal third of the blastocoel immediately subjacent to the ectoderm. During ring formation, primary mesenchyme cells extend filopods which fuse with one another to

form a syncytium. This syncytium will be the site of deposition of the calcium carbonate spicules that form the skeleton of the pluteus larva. These cellular events are determined as early as the 16-cell stage (Okazaki, 1975; Harkey & Whiteley, 1980).

The differentiation of primary mesenchyme cells is accompanied by the expression of specific proteins and cell surface antigens (Harkey & Whiteley, 1983; Carson *et al.* 1985; Benson, Benson & Wilt, 1986; McClay *et al.* 1983; Wessel & McClay, 1985). Little is known about the function of the majority of these proteins. Several primary mesenchyme-specific proteins have been identified as spiculins, the structural proteins of the organic matrix of the larval skeleton (Benson *et al.* 1986). The functions of the cell surface antigens may be related to cell motility, interactions

with the extracellular environment, or the uptake of calcium necessary for larval skeleton synthesis. Interestingly, this latter function has been inhibited by a primary mesenchyme-specific monoclonal antibody, 1223, that recognizes a $130 \times 10^3 M_r$ (130K) cell surface antigen (Carson *et al.* 1985).

To date, a limited number of primary mesenchyme-specific gene products have been analysed at the molecular level. These proteins include the spiculin protein SM50 for which cDNA clones have been isolated and characterized (Benson *et al.* 1987; Sucov *et al.* 1987) and msp130, a primary mesenchyme cell surface protein (Leaf *et al.* 1987). In addition, plus/minus screens have yielded other cDNA clones specific to or enriched in primary mesenchyme cells (Harkey, 1985; M. A. Harkey, unpublished data; D. A. Hursh & R. A. Raff, unpublished data).

Recently, we have used antibodies against a fusion protein derived from the cDNA clone 18C6 to demonstrate that this cDNA clone encodes a 130K cell surface protein, designated msp130, which is specific to primary mesenchyme cells (Leaf *et al.* 1987). In this report, we utilize two types of antibodies to characterize msp130. We employ a primary mesenchyme cell-specific monoclonal antibody B2C2, and the fusion protein antibodies to localize msp130 and to determine the nature of its post-translational modifications. In addition, we demonstrate that two independently derived primary mesenchyme cell-specific monoclonal antibodies, 1G8 (McClay *et al.* 1983) and 1223 (Carson *et al.* 1985), also recognize msp130.

Materials and methods

Sea urchins

Strongylocentrotus purpuratus were obtained from Pacific Biomarine, Venice, CA and were maintained in salt water aquaria at 13°C until used. *Lytechinus variegatus* were obtained from G. Noble, Panama City, FL and were maintained at 20°C. Eggs were collected by intracoelomic injection of 0.55 M-KCl. Embryos were cultured using artificial sea water (ASW; Cavanaugh, 1956).

Antibodies

The monoclonal antibody designated 1G8 was produced in the laboratory of Dr David McClay, Duke University (McClay *et al.* 1983; Wessel & McClay, 1985). The monoclonal antibody 1223 was prepared in the laboratory of Dr William Lennarz, M. D. Anderson Hospital, Houston, TX (Carson *et al.* 1985).

The monoclonal antibody designated B2C2 was produced by injecting into BALB/c mice the endoderm/mesoderm fraction of *S. purpuratus* gastrulae, produced according to Harkey & Whiteley (1980). Hybridomas were made according to Galfre *et al.* (1977), except that an alternative selection medium of azaserine and hypoxanthine was used (Foung, Sasaki, Grumet & Engleman,

1982), with the cell line P3X63-Ag8-653 as the fusion partner. Hybridomas were screened by staining sections of embryos with culture fluid.

The fusion protein antiserum is a rabbit polyclonal antiserum against an 88-amino-acid segment of msp130 expressed as a β -galactosidase fusion protein (Leaf *et al.* 1987). A monoclonal antibody (A3) against the fusion protein was selected and isolated as described above.

Immunofluorescence microscopy

Embryos were fixed for 30 min in ASW containing 2.0% formalin, washed in ASW, dehydrated in an ethanol series and embedded in Paraplast. Prior to incubation with primary antibody, sections were incubated for 20 min in 10% normal goat serum (NGS), 1.5% ovalbumin and 1.5% bovine serum albumin in phosphate-buffered saline (PBS: 20 mM- Na_2HPO_4 , 140 mM- NaCl , pH 7.6). Undiluted culture fluid was used as the primary antibody and incubation was carried out for 1 h at room temperature in a humid chamber. Slides were washed in PBS and goat anti-mouse IgG-conjugated fluorescein isothiocyanate (GAM-FITC) diluted 1:500 in PBS was added for 1 h followed by washing. Nuclei of some embryos were then stained with 0.3 mg ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI) in PBS for 10 min and destained 10 min in PBS. Slides were mounted with Gelutol and viewed with epifluorescence optics. Photographs were taken with Kodak Tri-X Pan film using a setting of ASA 1600.

Immunostaining of live cells

For staining live primary mesenchyme cells, gastrulae were disaggregated by first washing in calcium-free ASW (CFSW; Cavanaugh, 1956) and then incubating for approximately 1 min in 1.0 M-glycine, 2.0 mM-EDTA. The disaggregated embryos were pelleted and washed twice with ASW. The disaggregated cells were then incubated for 20 min on ice in either culture fluid containing B2C2 diluted 1:25 in ASW, in rabbit anti-yolk antiserum (Shyu, Raff & Blumenthal, 1986) diluted 1:100 in ASW or in an anti-tubulin monoclonal antibody (Amersham). The cells were washed and then incubated on ice for 20 min in the appropriate secondary antibody diluted 1:50 in ASW. Following two washes in ASW, the cells were fixed in 3.0% formaldehyde in ASW for 20 min, washed, mounted and photographed.

Immunoelectron microscopy

Embryos were fixed for 10 min in ASW containing 0.5% glutaraldehyde, followed by washing in ASW. The embryos were then dehydrated in dimethylformamide, infiltrated with Lowicryl K4M (Polysciences, Inc., Fort Washington, PA), and polymerized, all according to Altman, Schneider & Papermaster (1983). Sections were cut with a diamond knife and placed on 100-mesh, formvar-coated, nickel grids. Grids were incubated three times, 10 min each in 0.5 mg ml⁻¹ sodium borohydride followed by washing in Tris/saline (20 mM-Tris [hydroxymethyl] aminomethane, 140 mM- NaCl , pH 8.2) before incubation for 30 min in 10% NGS in Tris/saline. Tissue culture fluid containing either B2C2 or 1G8 was used undiluted as the primary antibody. Grids were incubated for 1 h and then washed with Tris/saline. Again, grids were incubated for 30 min in 10% NGS

in Tris/saline followed by goat anti-mouse IgG conjugated to 10 nm colloidal gold particles (Janssen Pharmaceutica, Inc., Piscataway, NJ) diluted 1:10 in Tris/saline. Following a wash in Tris/saline, grids were incubated in 1.0% glutaraldehyde followed by staining in 0.5% aqueous uranyl acetate and 0.2% lead citrate. Specimens were examined in a Philips 300 electron microscope and photographed on Kodak SO.163 film.

Immunoblotting

To prepare protein samples, embryos were pelleted and immediately resuspended in 10 vol. of Laemmli sample buffer (10% glycerol, 5% β -mercaptoethanol, 2.3% sodium dodecyl sulphate, 62.5 mM-Tris-HCl, pH 6.8). Following vigorous mixing, the samples were boiled 4 min, clarified by brief centrifugation and stored at -80°C . Proteins were separated on SDS-polyacrylamide gels according to Laemmli (1970) and electrophoretically transferred to nitrocellulose according to Towbin, Staehelin & Gordon (1979). The nitrocellulose blots were incubated with PBS containing 0.05% Tween-20 and 5.0% nonfat dry milk for 1 h. This was followed by incubation for 1 h to overnight with primary antibodies. The blots were stained using the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) method of Hsu, Raine & Fanger (1981), with 4-chloro-1-naphthol used as the chromagen. Undiluted culture fluid was used when blots were stained with the monoclonal antibodies. The fusion protein antibody was used at a dilution of 1:100 in PBS. Molecular weights were determined using BRL (Gaithersburg, MD) protein molecular weight standards.

Embryo labelling

For labelling embryos to the steady state, mesenchyme blastula stage embryos were dissociated with 1.0 M-glycine. The cells were resuspended at a 10% concentration in CFSW containing [^{35}S]methionine at $100\ \mu\text{Ci ml}^{-1}$ for 4 h. The cells were homogenized in 10 vol. of TENT buffer (0.01 M-Tris, pH 7.4, 0.05 M-NaCl, 3.0 mM-EGTA, 0.5% triton X-100, 0.1 mM-phenylmethylsulphonyl fluoride, $10\ \mu\text{g ml}^{-1}$ pepstatin A, $5\ \mu\text{g ml}^{-1}$ aprotinin, $10\ \mu\text{g ml}^{-1}$ leupeptin [Sigma Chemical Co., St Louis, MO]) and clarified by a 19000g centrifugation for 10 min. $100\ \mu\text{l}$ of the clarified supernatant was added to $900\ \mu\text{l}$ of TENT buffer and immunoprecipitated with B2C2 or A3.

For the pulse/chase labelling, mesenchyme blastula embryos were dissociated, resuspended at a 20% concentration in CFSW, and labelled for 30 min with [^{35}S]methionine ($200\ \mu\text{Ci ml}^{-1}$). After labelling, the cells were washed once in CFSW and chased in CFSW containing 5 mM-methionine for 1 h or 2.5 h. The cells were homogenized in 5 vol. of TENT buffer and clarified by a 19000g centrifugation for 10 min. 1.0 ml of the clarified supernatant was immunoprecipitated with B2C2 or A3.

Immunoprecipitations

For immunoprecipitations using B2C2, Sepharose CL-4B beads conjugated with protein A (Pharmacia, Inc., Piscataway, NJ) were incubated for 1 h with rabbit anti-mouse kappa light chain and then for 3 h with culture fluid

containing B2C2. When 1223 or the fusion protein monoclonal antibody (A3) were used in immunoprecipitation experiments they were added directly to the protein A-conjugated Sepharose beads. Beads were then added to the clarified embryo supernatant and incubated overnight at 4°C . Following washing in TENT buffer the beads were suspended in Laemmli sample buffer and boiled for 2 min. The beads were then pelleted by centrifugation and the clarified supernatant was collected and proteins separated on SDS-polyacrylamide gels (Laemmli, 1970).

Endoglycosidase F digestion

Protein A-conjugated Sepharose beads (17.0 mg) were incubated with the fusion protein monoclonal antibody (A3). The conjugated beads were incubated for 8–12 h with gastrula proteins homogenized in TENT buffer. The beads were then resuspended in $200\ \mu\text{l}$ of an endo F reaction mixture (100 mM-sodium phosphate, pH 6.1; 50 mM-EDTA; 1.0% Nonidet P-40; 0.1% SDS; 1.0% β -mercaptoethanol) with or without 1.0 unit of endoglycosidase F per $100\ \mu\text{l}$ of reaction mixture and incubated at 37°C for 12–18 h according to Elder & Alexander (1982). Following digestion, $200\ \mu\text{l}$ of $2\times$ Laemmli sample buffer was added and the sample was boiled for 2 min. The solubilized proteins were precipitated with acetone and redissolved in $20\ \mu\text{l}$ of $1\times$ Laemmli sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. The nitrocellulose sheets were stained with either B2C2 or fusion protein antiserum.

Culture in sulphate-free sea water

Strongylocentrotus purpuratus eggs were shed, fertilized and cultured in sulphate-free sea water (in g l^{-1} : NaCl, 27.72; KCl, 0.67; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.36; MgCl_2 , 9.32; NaHCO_3 , 0.18; pH 8.2).

Isoelectric focusing gels

Two-dimensional isoelectric focusing gels were run according to O'Farrell (1975). Embryos were lysed in Laemmli sample buffer. Proteins were precipitated by adding 3 vol. of -20°C acetone. The dried pellet was dissolved in lysis buffer (9.5 M-urea, 2.0% Nonidet P-40, 2.5% dithiothreitol, 2.0% ampholines consisting of a mixture of four parts pH 4–6 and one part pH 3.5–10 [LKB Produkter AB, Bromma, Sweden]) and loaded onto a 3.0 mm diameter gel consisting of 5.0% acrylamide, 0.27% bis-acrylamide, 13.9 M-urea, 19% ampholine mix (four parts pH 4–6 and one part pH 3.5–10) and run for a total of 9300 volt hours. Before running the second dimension the gels were equilibrated in a solution of 5.0% β -mercaptoethanol, 2.3% sodium dodecyl sulphate and 62.5% Tris, pH 6.8. A 7.5% SDS-polyacrylamide gel was used in the second dimension.

Results

The monoclonal antibody, B2C2, recognizes a primary mesenchyme-specific surface antigen

We have conducted a screen for primary mesenchyme cell-specific monoclonal antibodies and have isolated

one such monoclonal antibody, termed B2C2. We have demonstrated recently that B2C2 recognizes msp130, a cell surface protein encoded by the cDNA clone 18C6 (Leaf *et al.* 1987). B2C2 stains the primary mesenchyme cells from the time they ingress into the blastocoel until after they have produced the skeletal spicules. At no period of embryonic development are any cells other than primary mesenchyme cells stained by B2C2.

The indirect immunofluorescence staining with B2C2 of fixed primary mesenchyme cells indicates that msp130 is present on their surface. To determine if the epitope of msp130 recognized by B2C2 is on the extracellular surface of living primary mesenchyme cells, gastrulae were collected and incubated in calcium-free ASW followed by washing in 1.0 M-glycine. This treatment disaggregates the ectoderm leaving the primary mesenchyme cells trapped within the basal lamina. The primary mesenchyme cells were incubated in ASW containing B2C2, washed and incubated in ASW containing GAM-FITC. The cells were then fixed in 3.0% formaldehyde in ASW and examined. Fig. 1 demonstrates that under these conditions primary mesenchyme cells are still recognized by B2C2. To determine if the disaggregation process

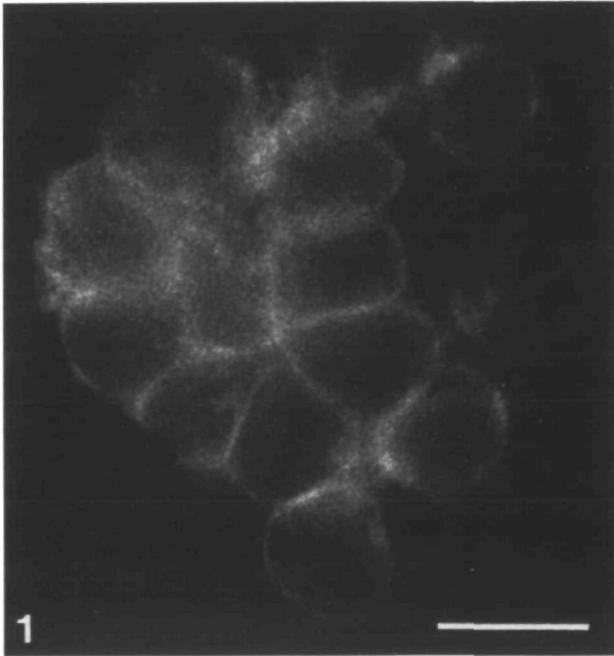


Fig. 1. Staining of the extracellular surface of live primary mesenchyme cells. Living primary mesenchyme cells from a dissociated *S. purpuratus* gastrula were incubated with B2C2. At the gastrula stage, primary mesenchyme cells are arranged in a ring which contains two distinct clusters of cells. Shown in this figure are the primary mesenchyme cells in one of these clusters. Bar, 10 μm .

disrupted cell membranes thereby enabling antibodies to enter the cytoplasm, identically treated embryos were also incubated with either anti-yolk or anti-tubulin antibodies. Neither of these antibodies stained the cells (data not shown). Therefore, the epitope of msp130 recognized by B2C2 is present on the extracellular surface of the cells.

Immunoblot analysis using B2C2

A 130K protein (msp130) is the major antigen recognized by B2C2 (Leaf *et al.* 1987). This molecule is not detected prior to the ingress of the primary mesenchyme cells into the blastocoel, but appears at the mesenchyme blastula stage and persists throughout early development.

We have examined whether two other independently derived monoclonal antibodies, 1G8 (McClay *et al.* 1983) and 1223 (Carson *et al.* 1985), which also specifically stain the surface of primary mesenchyme cells, recognize the same protein as B2C2. As shown in Fig. 2, the 130K protein immunoprecipitated by 1223 is recognized by B2C2, and the 130K antigen immunoprecipitated by B2C2 is recognized by 1G8. In addition, a minor $205 \times 10^3 M_r$ band, immunoprecipitated by B2C2, is also recognized by 1G8. Leaf *et al.* (1987) have used similar experiments to show that B2C2 and antibodies against a fusion protein derived from the primary mesenchyme cell-specific cDNA clone, 18C6, recognize an identical 130K protein, msp130. Taken together, these experiments establish that B2C2, 1G8 and 1223 recognize msp130.

Electron microscopic immunolocalization of msp130

To determine more precisely the cellular localization of msp130 in the primary mesenchyme cells, we have carried out electron microscope immunolocalization studies with B2C2 and 1G8 (Fig. 3). Both antibodies stained primary mesenchyme cells specifically and both showed identical subcellular staining patterns, although the density of the colloidal gold particles when B2C2 was used as the primary antibody was less than when 1G8 was used. Only background levels of colloidal gold staining were observed over ectoderm cells or when the primary antibody was omitted (data not shown).

Consistent with the immunofluorescence studies, colloidal gold is concentrated uniformly at the surface of primary mesenchyme cells stained with B2C2 (Fig. 3A). This observation confirms the extracellular location of msp130 and its distribution over the entire extent of the plasma membrane. Colloidal gold is also found concentrated in the Golgi apparatus. The staining of the Golgi apparatus is more clearly shown in Fig. 3B. This panel shows a *Lytechinus variegatus* primary mesenchyme cell stained with 1G8. Based on the orientation of the Golgi apparatus relative to the

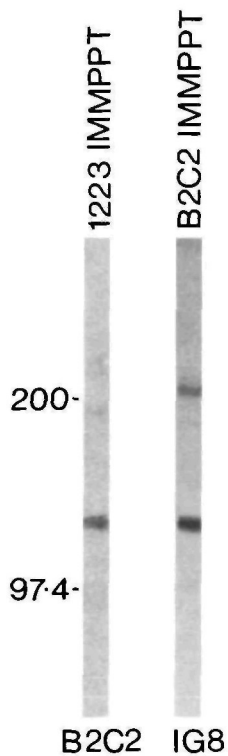


Fig. 2. Two independently derived monoclonal antibodies, 1223 and 1G8, recognize the identical 130K protein as B2C2. (A) Immunoprecipitation was done using the monoclonal antibody, 1223. The immunoprecipitated proteins from a homogenate of *S. purpuratus* gastrulae were separated on a polyacrylamide gel and electroblotted to nitrocellulose. The nitrocellulose was then incubated with B2C2. The 130K band immunoprecipitated by 1223 is also recognized by B2C2. (B) Immunoprecipitation was done using B2C2. The immunoprecipitated proteins from a homogenate of gastrula-stage embryos were separated on a polyacrylamide gel and electroblotted to nitrocellulose. The nitrocellulose was then incubated with the monoclonal antibody, 1G8. The 130K band immunoprecipitated by B2C2 is also recognized by 1G8. In addition, the 205 K that is recognized by B2C2 also stains with 1G8.

nucleus, the colloidal gold appears to be concentrated in the trans-Golgi compartment. Specific reactions known to occur in the trans-Golgi compartment include addition of sialic acid, sulphate and *N*-acetyl glucosamine to carbohydrate chains (reviewed in Farquhar, 1985). We suggest that the epitope(s) recognized by B2C2 and 1G8 is a post-translational modification that is added to a carbohydrate moiety of msp130 as it traverses the trans-Golgi compartment.

B2C2 recognizes only the mature-sized msp130

Immunoprecipitations of labelled proteins strongly suggest that B2C2 recognizes a post-translational

modification of msp130. In Fig. 4, mesenchyme blastula embryos labelled to the steady state with [³⁵S]methionine have been immunoprecipitated with B2C2 or a monoclonal antibody, A3 [generated against the fusion protein derived from the cDNA clone 18C6 as described in Leaf *et al.* (1987)]. Msp130 is immunoprecipitated by A3 and B2C2. A3 also immunoprecipitates four smaller proteins ranging from 110 to 120 × 10³ M_r.

We have performed a pulse/chase experiment with [³⁵S]methionine on mesenchyme blastula embryos to demonstrate that these smaller proteins are precursors to msp130 (Fig. 4). After a 1 h chase, the only labelled proteins immunoprecipitated by the monoclonal antibody A3 are the four presumptive precursor proteins. By 2.5 h of the chase, the label has disappeared from the smaller proteins and the 130K protein is labelled. It is evident that B2C2 also fails to immunoprecipitate a significant amount of the 130K protein until 2.5 h of the chase. To verify that B2C2 fails to recognize the precursor proteins, we show an overexposure of the B2C2 immunoprecipitated samples in lanes 7 and 8. These data demonstrate that B2C2 only recognizes a highly matured form of msp130 and thus suggest that B2C2 recognizes a post-translational modification of msp130.

Endoglycosidase F digestion of msp130

The above data suggest that msp130 may be a glycoprotein. To test for the presence of *N*-linked carbohydrate chains on msp130, we digested immunoprecipitated msp130 with endoglycosidase F and stained immunoblots of the digestion products with the polyclonal antibodies to the fusion protein and with B2C2 (Fig. 5). Digestion of msp130 with endoglycosidase F has two effects. First, it yields a single product detectable by the fusion protein antiserum. This product is of lowered molecular weight and is heterogeneous in size (lane C). This heterogeneity may reflect incomplete removal of the *N*-linked carbohydrate chains from msp130. The second result of endoglycosidase digestion of msp130 is the pronounced reduction in the staining of the protein by B2C2 (lane F). Treatment of msp130 under identical conditions without endoglycosidase F shows no effect on the molecule (Fig. 5, lanes B & E). These data suggest that the epitope recognized by B2C2 is part of an *N*-linked carbohydrate chain attached to msp130.

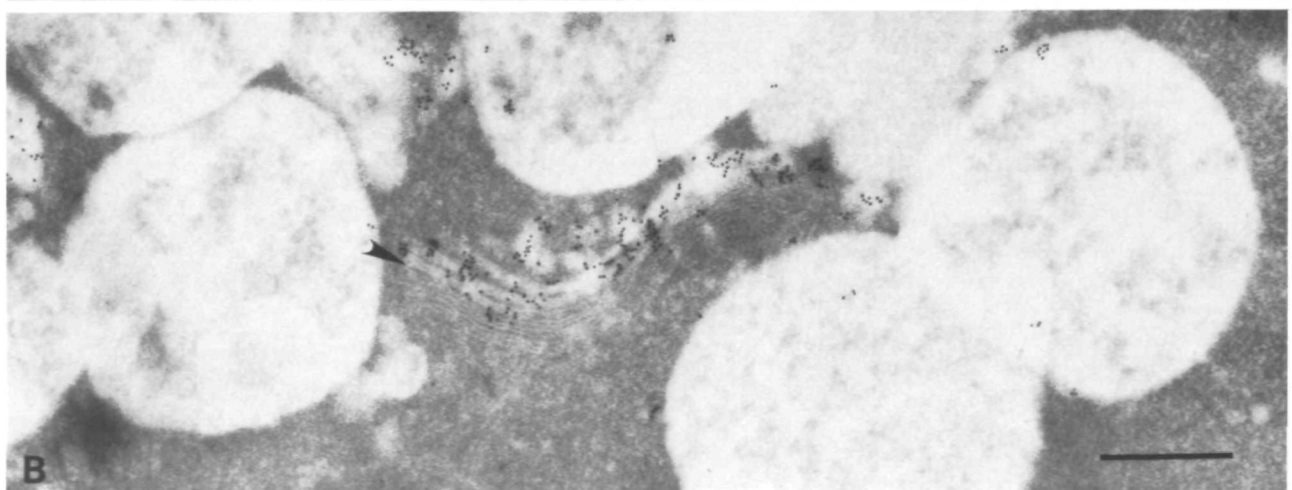
Expression of msp 130 in embryos cultured in sulphate-free sea water

It has been demonstrated that during ingress primary mesenchyme cells express sulphated molecules which are important for the migratory behaviour of these cells. Embryos cultured in sulphate-free sea water show abnormal primary mesenchyme cell

migration (Karp & Solursh, 1974). We have examined the effect of sulphate-free sea water on the expression of *msp130*.

In sulphate-free sea water (Fig. 6), primary mesenchyme cells ingress into the blastocoel but are not able to migrate and therefore do not form the typical

primary mesenchyme cell ring. They do make spicules, though these are not located properly in the blastocoel. These spicules have an abnormal morphology and there are often more than two present per embryo. Fig. 6C shows that primary mesenchyme cells in embryos cultured in sulphate-free sea water



do not express the epitope of msp130 recognized by B2C2. However, immunofluorescence analysis using the fusion protein antiserum shows that the protein moiety of msp130 is present (Fig. 6D). Furthermore, despite the absence of the epitope recognized by B2C2, msp130 appears to be properly located at the primary mesenchyme cell surface.

If msp130 is a sulphated glycoprotein, the failure to add sulphate ions to the molecule when embryos are cultured in sulphate-free sea water should be reflected in an increased isoelectric point of msp130 synthesized under these experimental conditions. Fig. 7 shows immunoblots of proteins isolated from control and sulphate-free-treated embryos and separated by two-dimensional isoelectric focusing gels. The results demonstrate that msp130 synthesized by the control cultures has a significantly lower (i.e. more acidic) isoelectric point than msp130 synthesized by embryos cultured in the absence of sulphate. This shift in isoelectric point is consistent with the addition of sulphate groups to the msp130 synthesized in control embryos.

Discussion

msp130 is recognized by three independently derived monoclonal antibodies

In this report, we characterize msp130 using the primary mesenchyme cell-specific monoclonal antibody, B2C2. We show that the 130K protein recognized by B2C2 is also recognized by two independently derived monoclonal antibodies, 1223 (Carson *et al.* 1985) and 1G8 (McClay *et al.* 1983). Leaf *et al.* (1987) have shown that both B2C2 and antibodies generated against a fusion protein derived from the primary mesenchyme-specific cDNA clone, 18C6, recognize the identical 130K protein which has been designated msp130. We conclude that each of the primary mesenchyme-specific monoclonal antibodies

Fig. 3. Electron microscope immunolocalization of msp130. Embryos embedded in Lowicryl K4M were stained with monoclonal antibodies B2C2 or 1G8. The secondary antibody was conjugated to 10 nm colloidal gold spheres. (A) A primary mesenchyme cell in an *S. purpuratus* gastrula-stage embryo stained with B2C2. Gold spheres are present in two regions; the cell periphery and a perinuclear region containing a Golgi complex. (B) Golgi region of a primary mesenchyme cell in an *L. variegatus* gastrula stained with 1G8. The midpoint of the membrane stacks of the complex are indicated by the arrow. Since osmium cannot be used in immunostained sections, the membranes appear as white tracks. With respect to the nucleus of this cell, the trans-Golgi is above the arrow and the cis-Golgi below. Thus, the heavy concentration of gold spheres marks the trans side of the Golgi. Bars, 0.5 μ m.

1223, 1G8 and B2C2 recognize msp130. Therefore, msp130 is a prominent cell surface antigen for primary mesenchyme cells. Although each of these monoclonal antibodies may recognize a different

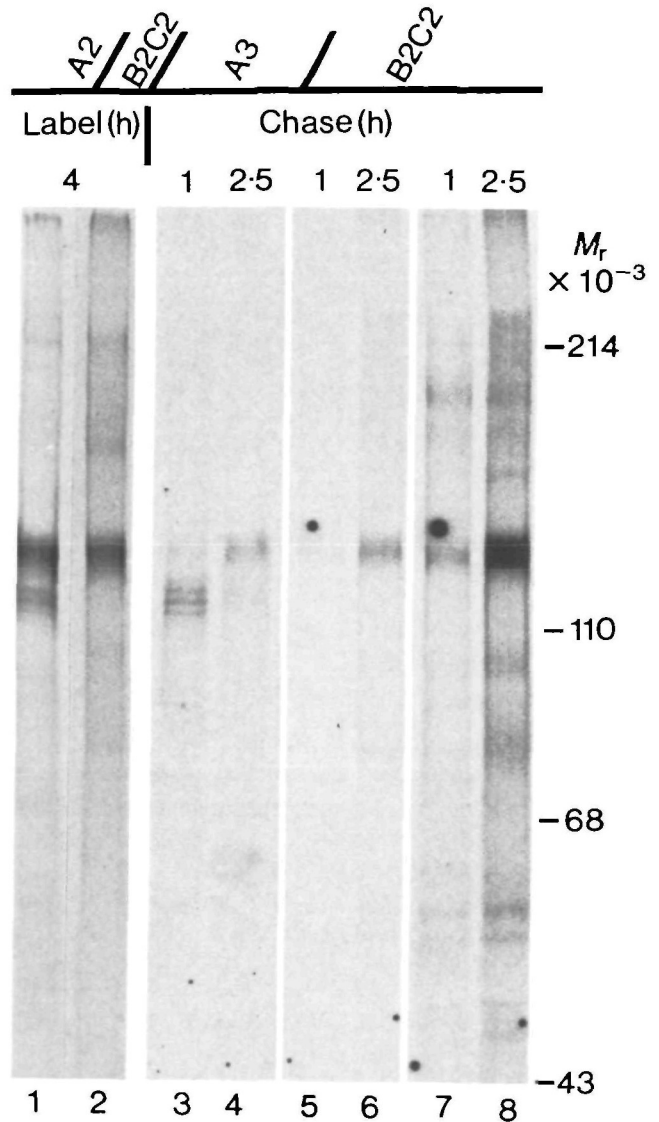


Fig. 4. Steady-state and pulse/chase labelling of msp130. Immunoprecipitations of embryos were labelled to the steady state or pulse/chased with [35 S]methionine. Lanes 1 and 2 are immunoprecipitations of mesenchyme blastula stage embryos labelled for 4 h in CFSW with 100 μ Ci ml $^{-1}$ [35 S]methionine and immunoprecipitated with B2C2 or A3, a monoclonal antibody against a fusion protein. Lanes 3–8 show a pulse/chase experiment. Mesenchyme blastula embryos were labelled for 30 min in CFSW with 200 μ Ci ml $^{-1}$ [35 S]methionine, resuspended in CFSW with unlabelled methionine, and immunoprecipitated at either 1 h (lanes 3, 5 and 7) or 2.5 h (lanes 4, 6 and 8) after the midpoint of the labelling. The immunoprecipitates were subjected to electrophoresis on 7.5% SDS-polyacrylamide gels and autoradiographed. Lanes 1, 3, 4, 7 and 8 were exposed 18 days. Lanes 2, 5 and 6 were exposed 2.5 days.

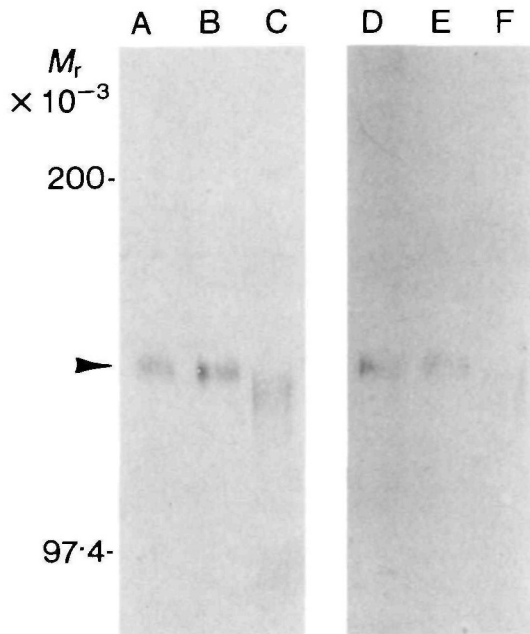


Fig. 5. Endoglycosidase F digestion of msp130. Msp130 was immunoprecipitated from *S. purpuratus* gastrulae using the fusion protein monoclonal antibody (A3) as described in Materials and methods, digested with endoglycosidase F and stained by antibodies on an immunoblot. Lanes A and D, untreated gastrula protein. Lanes B and E, immunoprecipitated msp130 incubated in the reaction mixture without endoglycosidase F. Lanes C and F, immunoprecipitated msp130 incubated with endoglycosidase F (equal samples from the same reaction). Lanes A, B, and C are stained with the fusion protein antiserum, Lanes D, E, and F are stained with monoclonal antibody B2C2.

epitope on msp130, it is noteworthy that they all fail to stain primary mesenchyme cells in embryos grown in sulphate-free sea water (unpublished observations).

To our knowledge, the only primary mesenchyme cell-specific monoclonal antibody that apparently does not recognize msp130 is MESO-1. Wessel & McClay (1985) report that the antigen recognized by MESO-1 is a $380 \times 10^3 M_r$ cell surface protein. This protein shows the same pattern of localization as msp130 (Wessel & McClay, 1985).

Relationship of the $205 \times 10^3 M_r$ (205K) protein to msp130

Antibody staining of embryonic proteins on immunoblots shows that B2C2 recognizes a major 130K protein (msp130) and a minor 205K protein (Fig. 2). The fusion protein antibodies which are generated against an 88-amino-acid region of msp130 fail to stain the 205K protein (Leaf *et al.* 1987). This suggests that the 205K protein lacks this 88-amino-acid region and thus is not a precursor to msp130. Since

B2C2 recognizes a post-translational modification of msp130 (Figs 4, 5), the 205K protein may have no relationship to msp130 other than a similar post-translational modification. The 205K protein is apparently specific to primary mesenchyme cells.

Expression of msp130 during embryogenesis

Several experiments suggest that the expression of msp130 on the surface of primary mesenchyme cells corresponds with ingress into the blastocoel. The monoclonal antibodies B2C2, 1G8 and 1223 (this report; Carson *et al.* 1985; McClay *et al.* 1983; McClay *et al.* 1985) and the fusion protein antibodies (Leaf *et al.* 1987) stain primary mesenchyme cells soon after they have ingressed. The low prevalence of the msp130 transcript before ingress (Harkey, 1985; Leaf *et al.* 1987) may limit the abundance of msp130 at early periods of development.

Although B2C2 fails to stain embryos prior to the ingress of primary mesenchyme cells, it has been observed that B2C2 stains the cortical granules of unfertilized eggs. Antibody staining with the fusion protein polyclonal and monoclonal antibodies suggest that the cortical granule antigen protein is apparently unrelated to msp130 (Anstrom, Chin, Parks & Raff, 1987).

Localization of msp130

The monoclonal antibodies B2C2 and 1G8 unequivocally demonstrate the cell surface localization of msp130. Immunoelectron microscopy shows that the epitope(s) of msp130 recognized by B2C2 is present on the extracellular surface of the plasma membrane (Fig. 3). This orientation has been confirmed by binding B2C2 to live primary mesenchyme cells (Fig. 1). Since no patching is observed when B2C2 is applied to living cells, we suggest that msp130 is not a freely diffusible membrane protein. The extracellular orientation of both msp130 and the 205K protein have been confirmed also by cell surface iodination experiments (unpublished observations).

Post-translational modifications of msp130

We have performed several experiments to examine the post-translational modifications of msp130. Four precursor forms to msp130 have been discovered. B2C2 recognizes only the mature form of msp130. The structure of the precursors is presently unknown.

Immunoelectron microscopy using B2C2 and 1G8 suggested their epitope(s) are added to msp130 at the trans-Golgi compartment (Fig. 3). This observation implies that msp130 may be a glycoprotein, since the trans-Golgi is a site of carbohydrate modification. Endoglycosidase F digestion of msp130 confirms that it is glycosylated.

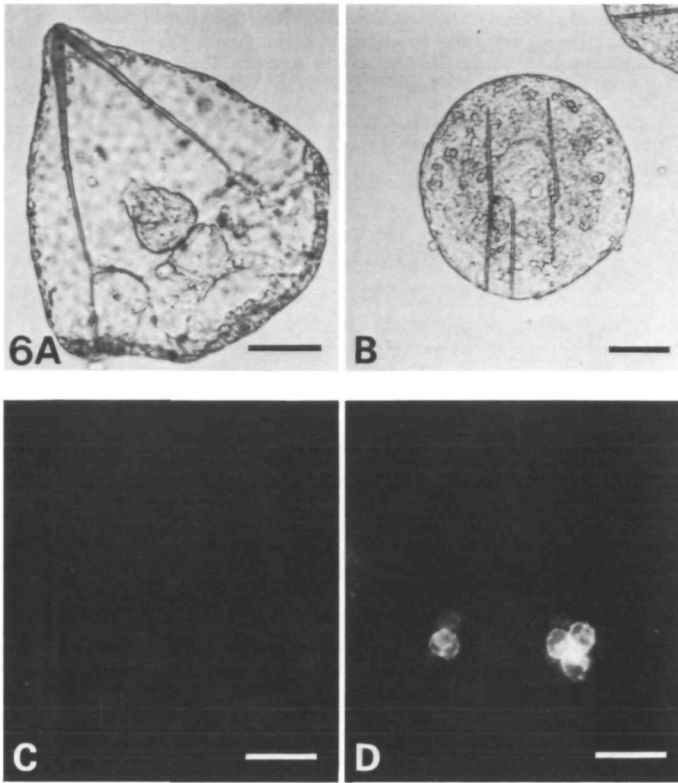


Fig. 6. Embryos cultured in sulphate-free sea water. (A) A control *S. purpuratus* embryo after 72 h of development. (B) A 72 h *S. purpuratus* embryo grown in sulphate-free sea water. (C,D) Indirect immunofluorescent staining of mesenchyme blastulae grown in sulphate-free sea water. (C) Embryos were stained with B2C2. The mesenchyme cells do not react with this antibody. (D) Embryos stained with the fusion protein antiserum. Bars, (A) 40 μm ; (B-D) 20 μm .

The epitope of msp130 recognized by B2C2 is not detectable in embryos cultured in sulphate-free sea water (Fig. 6). Thus, msp130 is not completely processed when embryos lack an exogenous source of sulphate. It is likely that msp130 in embryos cultured in sulphate-free sea water follows the normal course of synthesis and glycosylation except for the final addition of sulphate groups. Proteins isolated from embryos cultured in sulphate-free sea water and stained with the fusion protein antibodies on one-dimensional immunoblots (unpublished observations) and two-dimensional immunoblots (Fig. 7) reveal that msp130 remains a 130K protein in the absence of sulphate.

Our data suggest that msp130 is sulphated. Two-dimensional gels show that msp130 isolated from embryos cultured in sulphate-free water is less acidic than the normal msp130 (Fig. 7). However, this experiment does not prove the epitope itself is sulphated. We do not know the potential sulphation site(s) of msp130, but it is likely that the sulphate moieties are added to *N*-linked carbohydrate chains. Other glycoproteins containing sulphated *N*-linked carbohydrate chains have been characterized (Green, Morishima, Boime & Baenziger, 1985) including the adhesion molecule, N-CAM, which incorporates sulphate that can be released by digestion with endoglycosidase F (Sorkin, Hoffman, Edelman & Cunningham, 1984).

Importance of sulphated molecules to primary mesenchyme cells

Primary mesenchyme cells express sulphated molecules on their surface during ingress into the blastocoel (Motomura, 1960; Sugiyama, 1972; Karp & Solursh, 1974). Such sulphated molecules include sulphated proteoglycans (Karp & Solursh, 1974; Solursh & Katow, 1982; Solursh, Mitchell & Katow, 1986) and sulphated glycoproteins (Heifetz & Lennarz, 1979). The importance of the presence of sulphate to primary mesenchyme cells has been demonstrated by culturing embryos in sulphate-free sea water. Under these conditions, primary mesenchyme cells are able to ingress into the blastocoel but fail to migrate (Karp & Solursh, 1974; this report). Heifetz & Lennarz (1979) have suggested that the expression of sulphated glycoproteins in particular is necessary for proper primary mesenchyme cell differentiation. One striking effect of the inhibition of glycosylation of *N*-linked glycoproteins (including sulphated glycoproteins) with tunicamycin is that primary mesenchyme cells are still able to ingress into the blastocoel, but fail to migrate and elongate spicules (Schneider, Nguyen & Lennarz, 1978; Heifetz & Lennarz, 1979). Carson *et al.* (1985) have demonstrated that the monoclonal antibody 1223, which we demonstrate recognizes msp130, inhibits

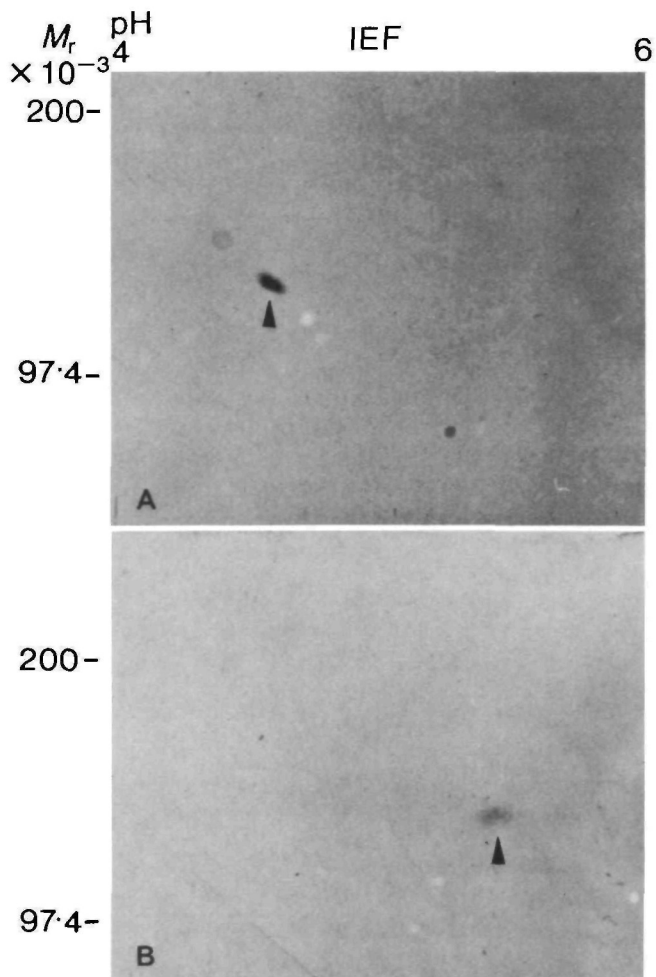


Fig. 7. Isoelectric focusing of msp130 from control embryos and from embryos cultured in sulphate-free sea water. Proteins from *S. purpuratus* gastrula-stage embryos were separated in the first dimension on an isoelectric focusing gel and in the second dimension on an SDS-polyacrylamide gel. The proteins were electroblotted to nitrocellulose which was then incubated with the fusion protein antiserum. (A) Immunoblot of proteins from embryos cultured in normal sea water. (B) Immunoblot of proteins from embryos cultured in sulphate-free sea water. In both samples msp130 migrates as a 130K protein.

calcium uptake and spicule synthesis by cultured primary mesenchyme cells. In this report, we show msp130 is an acidic glycoprotein and that an epitope of msp130 is sensitive to sulphate-deprivation.

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