Cell Biology International 1998, *Vol.* 22, *No.* 4, 321–326
Article No. cb980260, available online at http://www.idealibrary.com on IDEAL

THE OLIGOMERIC INTEGRITY OF TOPOSOME IS ESSENTIAL FOR ITS MORPHOGENETIC FUNCTION

GIOVANNI SCATURRO, FRANCESCA ZITO and VALERIA MATRANGA*

Istituto di Biologia dello Sviluppo, C.N.R., Via U. La Malfa 153, 90146 Palermo, Italy

Received 2 January 1998; accepted 28 April 1998

Sea urchin embryos are uniquely suitable for the study of morphogenetic cell interactions. Efforts to identify the molecules responsible for morphogenetic cell adhesion led to the isolation of a 22S glycoprotein complex from *Paracentrotus lividus* sea urchin embryo, that has been called toposome. The biological activity of toposome in mediating cellular adhesion has been fully documented. Its function in determining positional guidance during the development of the sea urchin embryo has been proposed. Here studies on the molecular structure of toposome are reported showing that, under non-reducing conditions, it is resolved in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a major band with an apparent molecular weight of 260 kDa, a doublet of 180–160 kDa and a lower band of 80 kDa. Digestion with EndoH endoglycosidase reduced the molecular sizes of the bands of 10%, 20% and 40%, respectively. In order to establish if the oligomeric integrity of toposome was essential for its function, the biological activity of each subunit on cells dissociated from sea urchin blastula embryos was tested. The resulting swimming embryoids were lacking skeleton, while reaggregating cells supplemented with native toposome developed into pluteus-like structures with skeletal elements.
© 1998 Academic Press

KEYWORDS: toposome adhesion molecule; glycoprotein complex; sea urchin embryo; cell reaggregation; skeletogenesis

INTRODUCTION

Cell–cell adhesion is a fundamental phenomenon that regulates all the stages of the developing embryo. In recent years, a large number of cell adhesion molecules (CAMs) have been identified (Damsky *et al.*, 1984), belonging to two general classes: those that require the presence of Ca^{2+} to mediate cell–cell adhesion, and those that do not (Takeichi, 1977). The presence of Ca^{2+} has been demonstrated to be of fundamental importance for cell adhesion by experiments where its withdrawal causes the dissociation of tissues and embryos. It has also been established that Ca^{2+} protects Ca^{2+}-dependent CAMs from enzymatic proteolysis (Takeichi, 1977). The possibility to easily obtain dissociated cells from sea urchin embryos, capable of reassociation into developing embryos (Giudice, 1962), offered a useful system to the study of cell-adhesion molecules and their role during morphogenesis. We have already described the characterization of a 22S glycoprotein complex from mesenchyme blastula embryos. The reducing SDS-PAGE pattern of the molecule showed the presence of six major bands ranging from 80 to 160 kDa. We have also demonstrated that the protein is involved in Ca^{2+}-dependent cell–cell adhesion. In fact, cells lacking the complex are not able to aggregate, even in the presence of Ca^{2+} and Mg^{2+} (Noll *et al.*, 1985). More recently the calcium-dependent homophilic interaction of the molecule has been demonstrated *in vitro* using sucrose isokinetic gradients (Cervello *et al.* 1992). The complex has been called toposome because of its putative role in specifying positional information as hypothesized from results with monoclonal

*To whom correspondence should be addressed: Dr Valeria Matranga, Istituto di Biologia dello Sviluppo, C.N.R., Via U. La Malfa 153, 90146 Palermo, Italy; E-mail: matranga@ibs.cnr.pa.it

1065–6995/98/040321+06 $30.00/0
© 1998 Academic Press

antibodies to the entire complex which stained, by indirect immunofluorescence, different territories in the embryo (Noll *et al.*, 1985).

In this report we further analysed the molecular structure of toposome and estimated the need of toposome integrity for its biological activity.

MATERIALS AND METHODS

Toposome purification

Large-scale preparations of toposome from *Paracentrotus lividus* embryos were performed according to Cervello and Matranga (1989).

Electrophoretic analysis

SDS-PAGE was performed according to Laemmli (1970), under non-reducing and reducing conditions on 4–10% gradient gels. Protein standards (Pharmacia) were ferritin (220 kDa), albumin (67 kDa) and catalase (60 kDa). Gels were stained with Coomassie brilliant blue.

Enzymatic assay

Digestion of toposome with the endoglycosidases was performed as follow: 1 mg/ml of toposome in 0.1% SDS–0.05 M Tris–HCl pH 6 was incubated at 100°C for 1 min. A cocktail of protease inhibitors (1 μg/ml aprotinin, antipain, leupeptin, pepstatin A, 0.1 mM benzamidine and 0.1 mM PMSF) and 20 mU/ml of endoglycosidases were added and incubated at 37°C for 3 h. Samples were analysed by SDS-PAGE under non-reducing conditions.

Electroelution and SDS extraction

Electroelution of toposome bands 1, 2 and 3 was performed from gel slices in a BIO-RAD apparatus according to manufacturer instructions. Electroeluted bands were dialysed against 10 mM Tris, 0.1 mM PMSF and lyophilized by speed vacuum. The extraction of SDS bound to proteins was performed by the technique described by Konigsberg and Henderson (1983). Briefly, 1 ml of 85% acetone, 5% triethylamine, 5% acetic acid was added to each sample, which was incubated at 4°C for 5 min, and centrifuged at 14,000 rpm for 15 min. This procedure was repeated twice. Precipitated proteins were resuspended in sea water and used in the reaggregation assay.

Reaggregation assay

Dissociation of *Paracentrotus lividus* embryos into single cells was achieved by the technique fully documented by Matranga *et al.* (1986). Reaggregation of dissociated cells was scored as previously described in microtiter plates by using a cell concentration of 8×10^4 cells per well in a final volume of 100 μl.

RESULTS

Molecular structure of toposome

It has been demonstrated that toposome is a glycoprotein complex, with a sedimentation coefficient of 22S, consisting of six 160-kDa polypeptides. Upon embryonic development, each polypeptide is proteolytically cleaved into six major polypeptide chains which are held together within the complex by S–S bridges and non-covalent bonds. Cleaved polypeptides show different molecular weights in different sea urchin species (Noll *et al.*, 1985; Armant *et al.*, 1986; Lee *et al.*, 1989). In *Paracentrotus lividus* embryos polypeptides analysed under reducing conditions show a typical pattern of six major bands ranging in molecular mass from 80 to 160 kDa (Fig. 1A lane R). A major band at position 160 kDa is the original uncleaved polypeptide from which all the other bands originate (Cervello and Matranga, 1989). Following that, a sharp band and two doublets can be seen, which are the products of the 160-kDa specific cleavage. In some cases, when old preparations were run on SDS-PAGE under reducing conditions, smaller degradation products were visible (not shown).

To gain information on the molecular structure of toposome, we analysed the molecule by SDS-PAGE under non-reducing conditions. In this case associations between peptides due to S–S bridges would have been preserved. The pattern we observed showed a band corresponding to an apparent molecular weight of 260 kDa, a doublet at 180–160 kDa and a lower band of 80 kDa (Fig. 1A, NR). We named these bands 1, 2 and 3, respectively. To examine if the three bands were supramolecular aggregates, held together by weak hydrogen bonds, samples were denatured with increasing SDS concentrations, ranging from 0.5 to 4%, or increasing time at 100°C, ranging from 2 to 16 min. As shown in Figure 1B, in no case we observed a change in the pattern, that is band 1, 2 and 3 were at the same position in the gel and in the same relative amounts.

Detection of oligosaccharides in the toposome subunits

It has been known that toposome is a glycoprotein; however, the exact nature of oligosaccharide chains

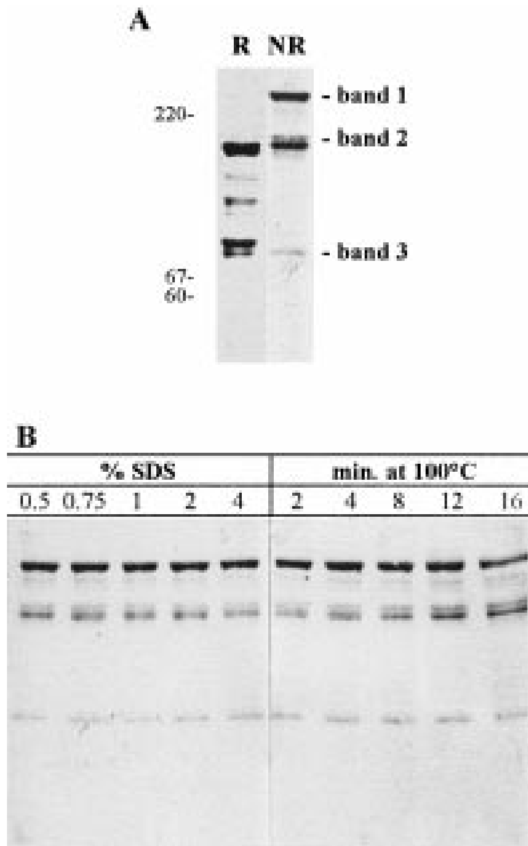

Fig. 1. Electrophoretical analysis of toposome structure. (A) SDS-PAGE of toposome under reducing (R) and non-reducing (NR) conditions. (B) SDS-PAGE under non-reducing conditions of toposome with increasing concentrations of SDS (left panel) and increasing time at 100°C (right panel).

has never been estimated as well as the percentage of toposome molecular mass due to carbohydrate moieties. To analyse the presence of some specific oligosaccharides in the molecule, experiments of digestion with endoglycosidases were carried out. The enzymes used were Endo-*N*-acetylglucosaminidase D (Endo D) and Endo-*N*-acetylglucosaminidase H (Endo H), each hydrolysing mannose-containing *N*-linked oligosaccharides possessing one or at least three mannose residues respectively (Maley *et al.*, 1989). Digestion with Endo H gave rise to a reduction of molecular mass of the 3 major bands, measured to be about 10%, 20% and 40% for band 1, 2 and 3, respectively (Fig. 2). On the contrary, digestion with Endo D did not cause any visible variation of the molecular mass of the 3 major bands (Fig. 2).

Biochemical analysis of toposome subunits

To investigate on the molecular composition of toposome subunits, the three bands were electro-

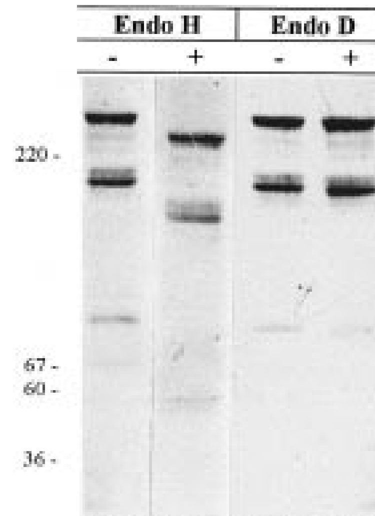

Fig. 2. Detection of oligosaccharides in toposome subunits. SDS-PAGE under non-reducing conditions of toposome after incubation in the presence (+) or absence (−) of endoglycosidases Endo H and Endo D.

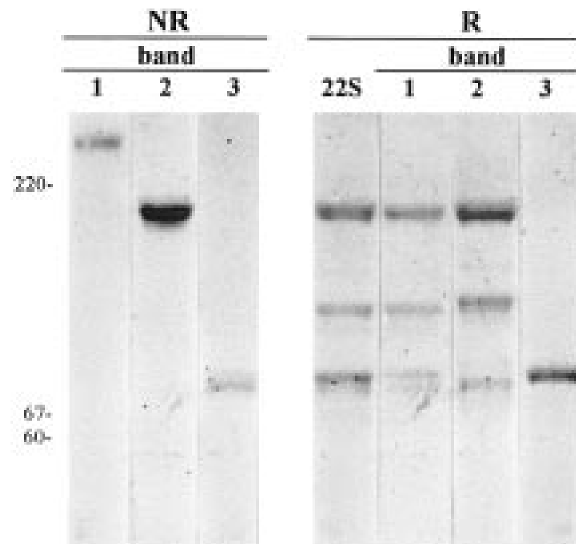

Fig. 3. Biochemical analysis of toposome subunits. Native toposome (22S) and electroeluted bands 1, 2 and 3 of toposome analysed by SDS-PAGE under non-reducing (NR) and reducing (R) conditions.

eluted from the gel, precipitated and analysed by SDS-PAGE under reducing conditions. As a control we run bands 1, 2 and 3 on SDS-PAGE under non-reducing conditions (Fig. 3, NR), to verify that the three bands were not damaged. The right panel of Figure 3 (R) shows that both bands 1 and 2, when under reducing conditions, were resolved into bands having the typical pattern of toposome analysed under the same conditions, that is six major bands corresponding to molecules ranging in molecular mass from 80 to 160 kDa. On the

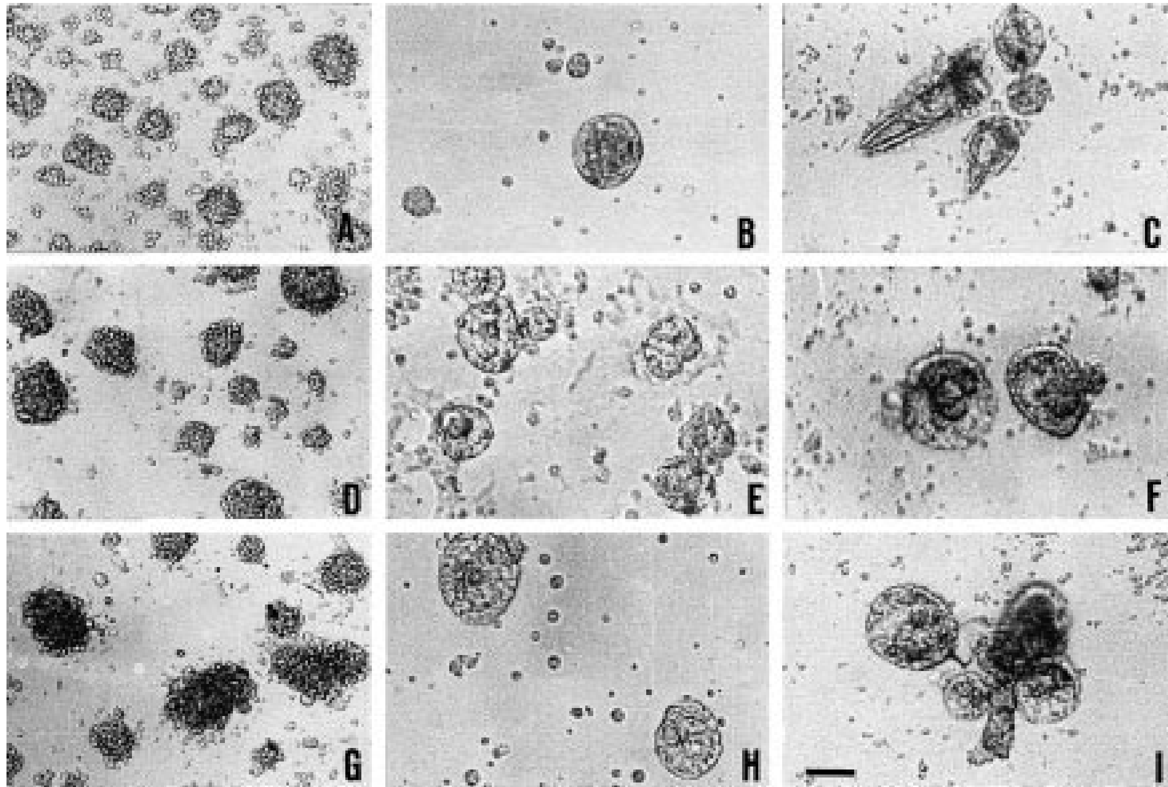

Fig. 4. Functional characterization of toposome subunits. Dissociated blastula cells cultured in the presence of 22S (A, B, C), band 2 (D, E, F) and band 3 (G, H, I), observed after 3 h (A, D, G), 24 h (B, E, H) and 6 days (C, F, I). Bar=100 μm.

contrary, band 3 gave rise only to one single band, corresponding to an approximate molecular mass of 85 kDa.

Functional characterization of toposome subunits

In order to establish if the oligomeric integrity of toposome was essential for its function, we tested the biological activity of each subunit on cells dissociated from sea urchin blastula embryos. Bands 2 and 3 were separated on SDS-PAGE under non-reducing conditions, electroeluted from the gel and used for the bioassay after detergent removal. We were not able to use band 1 preparations in reaggregation experiments, due to the SDS contamination, which caused cell lysis. As shown in Figure 4, an enhancement of cellular reaggregation was observed at 3–5 hours after addition of either band 2 or 3 (Fig. 4D,G) as compared to control where intact toposome was used to stimulate reaggregation (Fig. 4A). The presence of a smooth epithelium on swimming ciliated reaggregates was recorded at 24 h after addition of either band 2 or 3 (Fig. 4E,H), similar to what observed in controls supplemented with native toposome (Fig. 4B). After a longer observation period, 6 days, while controls reaggregates developed into swimming pluteus-like structures, with the presence of skeletal elements (Fig. 4C), reaggregates supplemented with either of the bands did not develop further and did not show any skeletal elements (Fig. 4F,I).

DISCUSSION

Toposome is a glycoprotein complex having a sedimentation coefficient of 22S, which owes its name to its putative role in specifying positional information (Noll *et al.*, 1985). It has been shown that 22S complex purified from *Paracentrotus lividus* eggs is constituted of six polypeptides, each polypeptide having a molecular mass of 160 kDa, that undergo limited proteolysis throughout development (Noll *et al.*, 1985; Cervello and Matranga, 1989). At the blastula stage the processing is complete, but the molecule does not change its sedimentation value, because all the polypeptides generated by the proteolysis remain assembled in the complex due to intercatenary or intracatenary covalent S–S bridges and non-covalent bonds. In this paper we have further

analysed the molecular structure of toposome by SDS-PAGE, showing that it is composed of three major components: a major band with an apparent molecular weight of 260 kDa, a doublet of 180–160 kDa and a lower band of 80 kDa, which are held together by non-covalent bonds. The analysis, by SDS-PAGE under reducing conditions, of each of the components has shown that the 260 kDa band and the 180–160 kDa doublet are resolved into six polypeptides, ranging from 80 to 160 kDa, typical of reduced native toposome. The 80-kDa band, when analysed under reducing conditions, gives rise to a polypeptide having a molecular mass of about 85 kDa. This apparent paradox can be explained by the fact that the polypeptide is rich in cysteine groups. Under non-reducing conditions the polypeptide has a more compact shape and migrates faster in the gel, whereas under reducing conditions the molecule is more expanded and migrates slower. This suggests extensive intrachain disulfide bonding as reported for integrins beta subunits (Hynes, 1987).

The presence of some specific oligosaccharides in the toposome was analysed by experiments of digestion with the Endo D and Endo H endoglycosidases. The results obtained suggest the presence of mannose-containing *N*-linked oligosaccharides possessing at least three mannose residues in the toposome. No removal of carbohydrates is obtained when using Endo D, possibly because of the presence of sialyl-galactosyl-*N*-acetylglucosaminyl chains, which are known to hinder the action of the enzyme (Koide and Muramatsu, 1974).

The biological activity of each single subunit of toposome was tested using a morphogenetic cell aggregation assay on dissociated blastula cells. The main criterion to verify the cell-adhesion activity of the tested molecules is to look for the formation of a smooth epithelial sheet and of a rudimentary gut in reaggregates, after 7–24 h of culture (Matranga *et al.*, 1986). In addition, for longer periods the presence of skeletal rods is a sign of resumption of morphogenesis. Since we were not able to remove traces of SDS from preparations of band 1 to be tested on cells, at the moment we cannot exclude the possibility that band 1 alone is sufficient to promote skeleton formation and elongation. Results obtained testing bands 2 and 3 separately show that, although both of them enhanced reaggregation of cells in a short-term period, the development of the newly formed embryoids was highly inhibited if the oligomeric integrity of toposome is not preserved. This has been known for long time for integrins, which bind to their counterpart extracellular matrix (ECM) molecules only when the heterodimers are formed (Buck *et al.*, 1986). The abnormal pattern of the gastrula-like embryoids and the total absence of spicules suggest that the oligomeric integrity of toposome is needed for its function during morphogenesis of the sea urchin embryo.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Cervello for helpful comments and suggestions on experimental procedures. This work was partially supported by a CNR Research and Training Program for Third Mediterranean Countries to VM titled: ‘Monitoring of sea coasts pollution by biological indicators and biomolecular probes’.

REFERENCES

Armant DR, Carson DC, Decker GL, Weply JK, Lennarz WJ, 1986. Characterization of yolk platelets isolated from developing embryos of *Arbacia punctulata*. *Dev Biol* **113:** 342–355.

Buck CA, Shea E, Duggan K, Horwitz AF, 1986. Integrin (The CSAT antigen): functionality requires oligomeric integrity. *J Cell Biol* **103:** 2421–2428.

Cervello M, Matranga V, 1989. Evidence of a precursor-product relationship between vitellogenin and toposome, a glycoprotein complex mediating cell adhesion. *Cell Differ* **26:** 67–76.

Cervello M, Di Ferro D, D’Amelio L, Zito F, Matranga V, 1992. Calcium-dependent self-aggregation of toposome, a sea urchin embryo cell adhesion molecule. *Biol Cell* **74:** 231–234.

Damsky CH, Knudsen KA, Buck CA, 1984. Integral membrane glycoprotein in cell–cell and cell-substratum adhesion. In: Ivatt RJ, ed. *The Biology of Glycoprotein* Plenum Publishing Corporation, New York, 1–64.

Giudice G, 1962. Reconstitution of whole larvae from disaggregated cells of sea urchin embryos. *Dev Biol* **5:** 402–404.

Hynes RO, 1987. Integrins: a family of cell surface receptors. *Cell* **48:** 549–554.

Koide N, Muramatsu T, 1974. Endo-beta-N-acetylglucosaminidase acting on carbohydrate moieties of glycoproteins. Purification and properties of the enzyme from Diplococcus pneumoniae. *J Biol Chem* **249:** 4897–4901.

Konigsberg WH, Henderson L, 1983. Removal of sodium dodecyl sulfate from proteins by ion-pair extraction. *Methods Enzymol* **91:** 254–259.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lee GF, Fanning EW, Small MP, Hille MB, 1989. Developmentally regulated proteolytic processing of a yolk glycoprotein complex in embryos of the sea urchin, *Strongylocentrotus purpuratus*. *Cell Differ Dev* **26:** 5–17.

Maley F, Trimble RB, Tarentino AL, Plummer TH, 1989. Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal Biochem* **180:** 195–204.

Matranga V, Kuwasaki B, Noll H, 1986. Functional characterization of toposomes from sea urchin blastula embryos by a morphogenetic cell aggregation assay. *EMBO J* **5:** 3125–3132.

Noll H, Matranga V, Cervello M, Humphreys M, Kuwasaki B, Adelson D, 1985. Characterization of toposomes from sea urchin blastula cells: a cell organelle mediating cell adhesion and expressing positional information. *Proc Natl Acad Sci USA* **82:** 8062–8066.

Takeichi M, 1977. Functional correlation between cell adhesive properties and some cell surface proteins. *J Cell Biol* **75:** 464–474.