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GP330 is specifically expressed in outer cells during epithelial differentiation in the preimplantation mouse embryo

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SUMMARY

During preimplantation development of the mouse embryo, a layer of outer cells differentiates into a perfect epithelium, the trophectoderm. The divergence between the trophectoderm and the inner cell mass takes place from the 8-cell stage to the 64-cell stage and precedes their commitment at the blastocyst stage. In this work, we have investigated the expression of gp330, a 330×10^3 M_r glycoprotein found in clathrin-coated areas of the plasma membrane of some epithelial cells characterized by a high level of endocytic activity. Our results show that gp330 is first synthesized in 16-cell stage embryos and that its appearance is restricted to outer cells until the blastocyst stage. Furthermore, its expression is repressed in inner cells at a post-transcriptional level, probably through the development of extensive cell-cell contacts.

Key words: preimplantation mouse embryos, compaction, gp330, epithelium, trophectoderm, cell polarity

INTRODUCTION

By the end of the preimplantation stage of mouse embryo development, a blastocyst has formed with two subpopulations of committed cells: an outer layer of trophectoderm cells surrounding the inner cell mass (ICM), located eccentrically within the blastocœlic cavity. The trophectoderm is morphologically and functionally a characteristic epithelium, with apical membranes facing outwards (for review see Fleming, 1992). It is responsible for blastocoel expansion at the 32-cell stage (Wiley, 1984) and is involved in the implantation of the blastocyst a few division stages later (for review see Strickland and Richards, 1992). After implantation, the trophectoderm will give rise to the major extraembryonic tissues of the conceptus and does not contribute to the formation of embryonic tissues (Rossant, 1986). These extraembryonic annexes provide physical protection for the embryo and regulate nutritional and hormonal exchanges between maternal and embryonic tissues.

Trophectoderm cells are derived exclusively from the outer cells of earlier stage embryos (Fleming, 1987). Segregation of inner and outer cells begins through asymmetrical cell divisions after compaction at the 8-cell stage (for reviews see Johnson and Maro, 1986; Gueth-Hallonet and Maro, 1992). Before compaction, blastomeres are non-polarized and equivalent. During compaction, they flatten against one another and become polarized: (i) a surface pole of microvilli forms (Reeve and Ziomek, 1981), (ii) cytoplasmic constituents, including cytoskeletal networks, endosomes and clathrin vesicles, reorganize and accumulate predominantly in the apical domain (Reeve, 1981; Johnson and Maro, 1984; Maro et al., 1985; Houliston et al., 1987) and (iii) focal tight-junctions form at the apicolateral cell border (Fleming et al., 1989). These polarization features are dependent largely on cell contacts mediated by the calcium-dependent cell adhesion molecule uvomorulin (Hyafil et al., 1980; Johnson et al., 1986; Vestweber et al., 1987). During the following cleavages, asymmetrical cell divisions give rise to inner and outer cells. Only blastomeres inheriting a sufficient part of the apical pole of microvilli display a polarized organization and remain in the outer position (Pickering et al., 1988). Outer cells continue their epithelial maturation, with restricted distribution of some membrane proteins, maturation of the endocytotic system at the 16-cell stage and desmosome formation at the 32-cell stage (for review see Fleming, 1992). Inner cells remain phenotypically apolar and undifferentiated although they do inherit some epithelial cell components, including the tight junction component ZO-1 (Fleming and Hay, 1991), and cytokeratins (Chisholm and Houliston, 1987) when they arise through differentiative divisions.

The divergence of the trophectoderm and ICM lineages is progressive (from the 8-cell stage to the 64-cell stage), slow (3 days in length) and precedes their commitment, since it is only in fully expanded blastocysts (after the 64-cell stage) that ICM and trophectoderm cells stop crossing lineages (Hillman et al., 1972; Ziomek et al., 1982). For example, experimentally moved 16- and 32-cell stage blastomeres convert phenotypically according to their new position and change their fate in consequence (Johnson, 1985). Early mouse embryo development thus provides a good system in which to study the spa-

Table 1. Summary of published work on immunodetection of epithelial markers in early mouse embryos

Markers	8-cell	Embryo stage: 16-cell Outer cells	16-cell Inner cells	32-cell Outer cells	32-cell Inner cells	64-cell Outer cells	64-cell Inner cells
Cytokeratins							
Endo A (1)	+	+	+/–	+	–*	+	–
Tight-junctions components							
ZO-1 (2)	+	+	+	+	–	+	–
Cingulin(3)‡	+	+	–	+	–	+	–
Desmosomal components							
dp3 (4)	+	+	+	+	+	+	–
dp1+2 (4)†	–	— + —		+	–	+	–
dg1, dg2+3 (4)†	–	— – —		+	–	+	–

+, presence; –, absence; +/–, presence in lower quantities in inner cells than in outer cells; –*, absence except in cells arising from an outer cell by differentiative division; †, determined only in whole embryos at the 16-cell stage, not in outer and inner cells separately;

‡Junctional and extra-junctional cingulin are considered as a whole.

(1) Chisholm and Houliston, 1987; (2) Fleming and Hay, 1991; (3) Fleming et al., 1993; (4) Fleming et al., 1991.

tiotemporal control of epithelial differentiation and its underlying cellular mechanisms. There is evidence for the expression of specific polypeptides in these two cell populations (Van Blerkom and Brockway, 1975; Van Berklom et al., 1976; Handyside and Johnson, 1978), although few molecular markers of the trophectoderm have been identified. Some cell markers seem to be down-regulated in the ICM lineage (Chisholm and Houliston, 1987; Fleming and Hay, 1991; Fleming et al., 1993), whereas others are expressed de novo in trophectoderm (Fleming et al., 1991) during epithelial differentiation (Table 1). To our knowledge, mechanisms controlling the expression of these markers have been investigated only in the case of ZO-1 (Fleming and Hay, 1991), where cellular interactions have been shown to play a major role.

In the present study, we examine the expression during early mouse development of a 330×10^3 M_r glycoprotein (gp330). gp330 is expressed in clathrin-coated areas of selected epithelia characterized by a high level of endocytic activity. It was initially identified in epithelial cells of the glomerus where it was shown to be the target of the antibodies responsible for Heymann's nephritis (Kerjaschki and Farquhar, 1982, 1983). Simultaneously, gp330 was detected in the brush border of epithelial cells of the proximal tubule where it is localized selectively on the luminal aspect of the intermicrovillar domain, which is in turn characterized by a cytoplasmic coat of clathrin (Kerjaschki et al., 1984). Subsequently, gp330 was detected with a similar subcellular localization in epithelial cells of the epididymis and visceral yolk sac and in trophectoderm cells (Chatelet et al., 1986; Buc-Caron et al., 1987). It has been found in all species studied, at least in proximal tubule cells. The exact function of gp330 is unknown, but its profile of expression and partial structural data which relate it to the α2-macroglobulin receptor (α2MR) and its binding properties suggest that it is involved in some aspect of endocytosis specific for the epithelial cells in which it is expressed (Christensen et al., 1992; Willnow et al., 1992).

Our results show that gp330 is first synthesized in 16-cell stage embryos and membrane-expressed in trophectoderm cells. Further investigations shows that its expression is repressed in cells of the inner cell mass at a post-transcriptional level, probably through the development of extensive cell-cell contacts.

MATERIALS AND METHODS

Recovery of oocytes and embryos

5- to 6-week-old Swiss female mice (Animalerie Spécialisée de Villejuif, Centre National de Recherche Scientifique, France) were superovulated by intraperitoneal injection of 6.5 IU of pregnant mare's serum gonadotrophin (PMSG, Intervet) and human chorionic gonadotrophin (hCG, Intervet) 48 hours apart. Unfertilized eggs were recovered at 12-14 hours post-hCG, by puncturing the ampullae of oviducts. Cumulus cells were dispersed by brief exposure to 0.1 M hyaluronidase (Sigma). In order to obtain embryos, the females were paired overnight with Swiss males and checked for vaginal plugs the next morning. In these conditions, fertilization is considered to occur 12 hours post-hCG. 2-cell stage embryos were recovered by flushing from the oviduct 48 hours post-hCG. Recovery of oocytes and embryos, and their manipulation were made in medium 2 containing 4 mg/ml bovine serum albumin (M2+BSA; Fulton and Whittingham, 1978). All buffers were at 37°C. Embryos were cultured in medium T6 containing BSA (T6+BSA; Howlett et al., 1987) under oil at 37°C with 5% CO_2 in air in Falcon plastic dishes. 8-cell stage embryos were recovered after about 12 hours in culture (60 hours post-hCG). They were examined hourly and considered as fully compacted when individual blastomeres were indistinguishable. 16-cell stage embryos were recovered after about 24 hours in culture (72 hours post-hCG), early blastocysts after about 36 hours (84 hours post-hCG) and fully expanded blastocysts after about 48 hours (96 hours post-hCG).

Preparation and handling of single cells

Zonae pellucidae were removed by brief exposure to acid Tyrode's solution (Nicolson et al., 1975). Decompaction of 8-cell stage embryos was achieved by 10-15 minute incubation in Ca^{2+}-free M2 medium containing 6 mg/ml BSA. For subsequent disagreggation, decompacted embryos were gently blown apart using flame-polished micropipettes, in the same Ca^{2+}-free medium. Single 1/8 blastomeres were cultured in isolated microdrops. To obtain natural 2/16 cell pairs, 1/8 cells were inspected every hour and newly formed pairs were collected. Some were fixed and stained during the first 2 hours, whereas others were cultured for a further 9 hours, either in T6+BSA, Ca^{2+}-free T6+BSA or T6+BSA containing ECCD-1 (diluted 1/20, gift from M. Takeichi, (Shirayoshi et al., 1983) before fixation and staining.

Preparation of inner cell masses by immunosurgery of early blastocysts

Inner cell masses (ICMs) were prepared according to Solter and Knowles (1975). Zona-free blastocysts at the 32-cell stage (corresponding to early cavitating embryos, see Gueth-Hallonet et al., 1993) were incubated for 30 minutes at 37°C in anti-gp330 antibodies (diluted 50 times in M2+PVP; Allegri et al., 1986; Sahali et al., 1993), rinsed 3 times in M2+PVP and finally incubated for 30 minutes at 37°C in the presence of guinea pig complement (Sigma, diluted 10 times in M2+PVP). After 3 washes in M2+PVP, they were further cultured in T6+BSA for 7 hours.

Cell fixation and immunocytological staining

Zona-free intact embryos, pairs of blastomeres or ICMs were placed in specially designed chambers as described by Maro et al. (1984). The chambers were coated previously with 0.1 mg/ml concanavalin A in phosphate-buffered saline (PBS) and rinsed with M2+PVP (containing α-amanitin when necessary). The chambers containing

samples were centrifuged at 450 ***g*** for 10 minutes at 37°C. When only anti-gp330 reactivity was assessed, cells were fixed with 3.7% formaldehyde (BDH) in PBS for 30 minutes at 37°C, neutralised with 50 mM NH_4Cl in PBS for 10 minutes and postpermeabilized with 0.25% Triton X-100 in PBS for 10 minutes. When cell surface polarity was also assessed, this protocol of fixation was preceded by a brief fixation of 1% paraformaldehyde in PBS for 2 minutes followed by a 2 minutes incubation in 700 μg/ml FITC-labeled Concanavalin A (ConA-FITC) at room temperature and three washes in M2+BSA. Immunological staining was performed using a polyclonal rabbit anti-gp330 serum (Allegri et al., 1986; Sahali et al., 1993), at a dilution of 1/500 followed by rhodamine-conjugated anti-rabbit antibodies (Cappel). To visualize chromatin, propidium iodide (5 mg/ml, Molecular Probes) was added to the second layer.

Photomicroscopy

Coverslips were removed from the chambers and mounted in Citifluor (City University, London). They were examined with a BioRad MRC 600 Confocal Laser Microscope, mounted on an Optiphot II Nikon microscope (60× objective Nikon plan apo; NA: 1.4). An argon ion laser adjusted to 488 nm wave length was used for fluorescein and an helium-neon ion laser adjusted to 543 nm for propidium iodide. The signal was averaged using a Kalman filter on 8 images. Micrographs were recorded using Kodak T-Max films on a VM 1710 Lucius & Baer black and white high resolution monitor.

Electron microscopy

The embryos, placed in chambers as for immunocytological staining, were fixed in periodate lysine phosphate fixative (containing 0.01 M $NaIO_4$, 0.75 M lysine-HCl, 0.0375 M $NaPO_4$, 2% paraformaldehyde, pH 6.2) for 3 to 5 hours at room temperature. They were permeabilized in 0.05% saponin in PBS with 0.3% BSA for 30 minutes. Immunoperoxidase staining was performed as described previously by Brown and Farquhar (1989) using the anti-gp330 serum (1/500) followed by peroxidase-conjugated Fab anti-rabbit antibody (1/50, Byosis). Samples were postfixed with 1% OsO_4 and 1.5% potassium ferrocyanate in 100 mM cacodylate buffer. After embedding on capsule beam, they were sectioned on a Reichert ultramicrotome. Sections were contrasted with uranyl acetate (0.1% in H_2O for 4 min) and lead citrate (0.1% in H_2O for 1.5 minutes) before observation under a Philips EM410 at 80 kV.

Immunoblotting

Eggs and embryos were washed three times in a small drop of M2 with 4 mg/ml polyvinyl-pyrrolidone, mixed with the same volume of double-strength SDS sample buffer and boiled for 2 minutes (Laemmli, 1970). Proteins were separated using a 4% polyacrylamide gel of 0.75 mm on a microgel apparatus (Biorad). Proteins were transferred electrophoretically (Biorad) onto nitrocellulose. Gp330 was detected after incubation with anti-gp330 (dilution 1/100 for oocytes and embryos and 1/5000 for lysed vitellin carcinoma cells of Brown Norway rats) followed by alkaline phosphatase-labeled anti-rabbit antibodies (dilution 1/7500, Promega). Incubation and washing buffers contained 3% milk powder and 0.1% Tween-20.

RESULTS

Characterization of the anti-gp330 serum

When 400 blastocysts were probed by immunoblotting with the anti-gp330 serum, a single polypeptide was observed (Fig. 1, lane 3). A protein of the same molecular mass was also detected in rat vitellin carcinoma cells, known to contain large quantities of gp330 (Fig. 1, lane 1). The use of molecular mass markers allowed us to evaluate the molecular mass at 360 kDa.

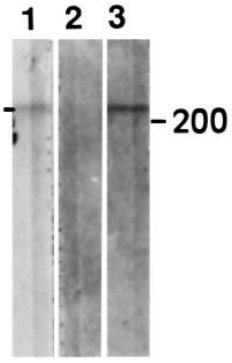

Fig. 1. Immunoblot with the anti-gp330 antiserum on rat vitelline carcinoma cells (lane 1), mouse oocytes (lane 2) and mouse fully expanded blastocysts (lane 3). 200, molecular mass marker.

No signal was observed when the same number of oocytes were probed with the anti-gp330 serum (Fig. 1, lane 2).

gp330 reactivity appears de novo at the 16-cell stage in outer cells

No fluorescent staining was detected with the anti-gp330 serum in oocytes or embryos prior to the 16-cell stage. Positive staining was first detected after about 77 hours post-HCG, in 16-cell embryos, when outer cells surrounding inner apolar cells are present. At this stage, epifluorescence microscopy showed an intense diffuse fluorescence in the cells, although some blastomeres did not seem to be stained (data not shown). Using confocal microscopy, it was possible to remove background staining due to the large size of the embryo (80 μm in diameter) so as to analyze anti-gp330 reactivity of the individual blastomeres in relation to their position within the embryo. At the morula stage, internal cells were never stained with the anti-gp330, whereas outer cells were often positive (Fig. 2A,B). The staining was distributed in aggregates found throughout the cytoplasm and which were more abundant and larger in the apical domain of the cells (Fig. 2A,B). The number of negative outer cells decreased rapidly with time and, at the end of the 16-cell stage, almost all outer cells were stained (Fig. 2C).

At the blastocyst stage, only trophectoderm cells were fluorescently stained (Fig. 3A,B). The staining pattern after permeabilization was restricted to the apical domain and apical cell surface of the cells. At high magnification, a tangential view of the apical cell surface allowed us to detect positive dots that were sometimes clustered into larger aggregates (Fig. 3C). When the embryos were not permeabilized before immunolabeling, the cytoplasmic staining was largely reduced, whereas the apical membrane staining persisted (Fig. 3D), indicating that gp330 is a component of the apical membrane of trophectoderm cells.

Immunoperoxidase electron microscopy was also performed on 16-cell stage embryos and fully expanded blastocysts (Fig. 4A-E). At the 16-cell stage, gp330 was detected only in outer cells and never in inner cells, thus confirming our immunofluorescence results. The peroxidase reaction product was detected within the cytoplasm in vesicles of different sizes similar to endocytic vesicles, and within organelles resembling Golgi saccules and endoplasmic reticulum (Fig. 4A,B). Membrane staining was not observed, not even in apical membrane invaginations representing potential coated pits. In fully expanded blastocysts, staining for gp330 was restricted strictly to the trophectoderm (Fig. 4C). The distribution of the

gp330 antigen was different from that observed in 16-cell stage embryos, since the staining was found both on apical membranes and in large apical cytoplasmic vacuoles and the Golgi saccules and reticulum were less densely stained. On the apical membrane, the staining was discontinuous (Fig. 4D), due to staining of clumps of intermicrovillous membrane but not the microvilli which were negative, or only very slightly labeled. The dense staining observed at the cell surface was often underlined by a fine lattice-like structure, likely corresponding to the clathrin coat. This reactive cell surface was undergoing frequent invaginations (Fig. 4E).

Following our observation that the gp330 protein was found first at the 16-cell stage, we attempted to determine whether the mRNA coding for gp330 was expressed in the embryo prior to the 16-cell stage. Compacted 8-cell embryos were cultured to the late 16-cell stage (13 hours later) and 11 μg/ml α-amanitin (Levey and Brinster, 1978), an inhibitor of RNA synthesis, was added at different times (0, 3, 6 and 9 hours) after the beginning of culture (Table 2). In these conditions, the transition between the 8- and 16-cell stages took place after 4-6 hours of culture. gp330 expression, assessed by anti-gp330 reactivity, was inhibited only when the drug was added during the first 6 hours of culture (compare lane 2 and 3 of Table 2), showing that the synthesis of the mRNA coding for gp330 took place at the early 16-cell stage.

Table 2. Percentage of 16-cell stage embryos expressing gp330 in outer cells when cultured either in normal medium (T6) or in medium containing α-amanitin for various periods of time

Culture conditions (hours)*		Number of embryos	Number of gp330 positive embryos† (%)
T6	T6 + α-amanitin		
0-13		29	22 (76)[1]
0-9	9-13	27	17 (63)[1]
0-6	6-13	28	3 (11)[2]
0-3	3-13	28	3 (11)[2]
	0-13	20	1 (5)[2]

*8-cell embryos which had undergone compaction within one hour of each other were cultured for a further 13 hours at which point they had reached the late 16-cell stage (in these experiments, the transition between the 8 and 16-cell stages took place after about 4-6 hours of culture). α-amanitin (11 μg/ml) was added at various time after the beginning of culture (0, 3, 6 and 9 hours).

†Embryos with more than one gp330-positive cell.

[1]The difference observed between the two first lines is not significant (P=0.3863 using Fisher's exact test).

[2]The differences observed between the last three lines is not significant (P≥0.6309).

The difference observed between groups 1 and 2 is statistically significant (P<0.0001).

Relation between distribution of gp330 and extent of cellular contacts in 16-cell stage blastomeres: evidence for down-regulation in inner cells

In order to analyze the importance of intercellular contacts in controlling the expression of gp330, we inhibited intercellular adhesion. Inhibition of adhesion could not be induced with certainty in whole 16-cell stage embryos, since newly formed tight junctions between outer cells would maintain inner cells in an inner position and thus a different environment than the outer cells. We chose to study the expression of gp330 in pairs of cells obtained after division in culture of isolated 8-cell blastomeres (2/16 pairs) in which the phenotype can be determined by staining with rhodamine-labeled concanavalin A (Con A; Handyside, 1980): a stained surface pole demonstrates that the blastomere is polar (P) and would have been in an outside position in the whole embryo whereas an homogeneous surface staining indicates that the blastomere is apolar (A) and would have been an inner cell of the embryo. The fact that gp330 appeared in isolated pairs of blastomeres during prolonged culture and did not preexist is essential when testing for the modulation of gp330 expression due to the

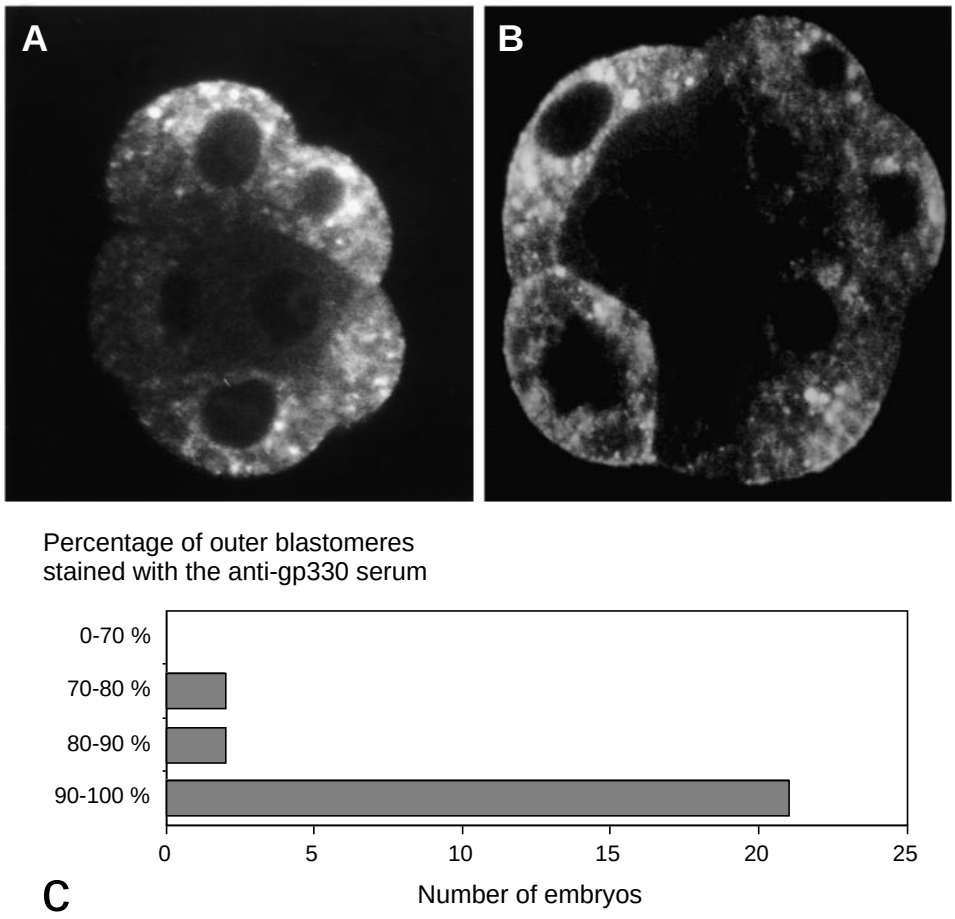

Fig. 2. Immunofluorescence staining with the anti-gp330 antiserum at the 16-cell stage (A) and at the 32-cell stage (B) observed under a confocal microscope. Magnification is ×650 in A and ×800 in B. (C) Percentage of outer cells stained by the anti-gp330 antibody in late 16-cell stage embryos (81 hours post-hCG).

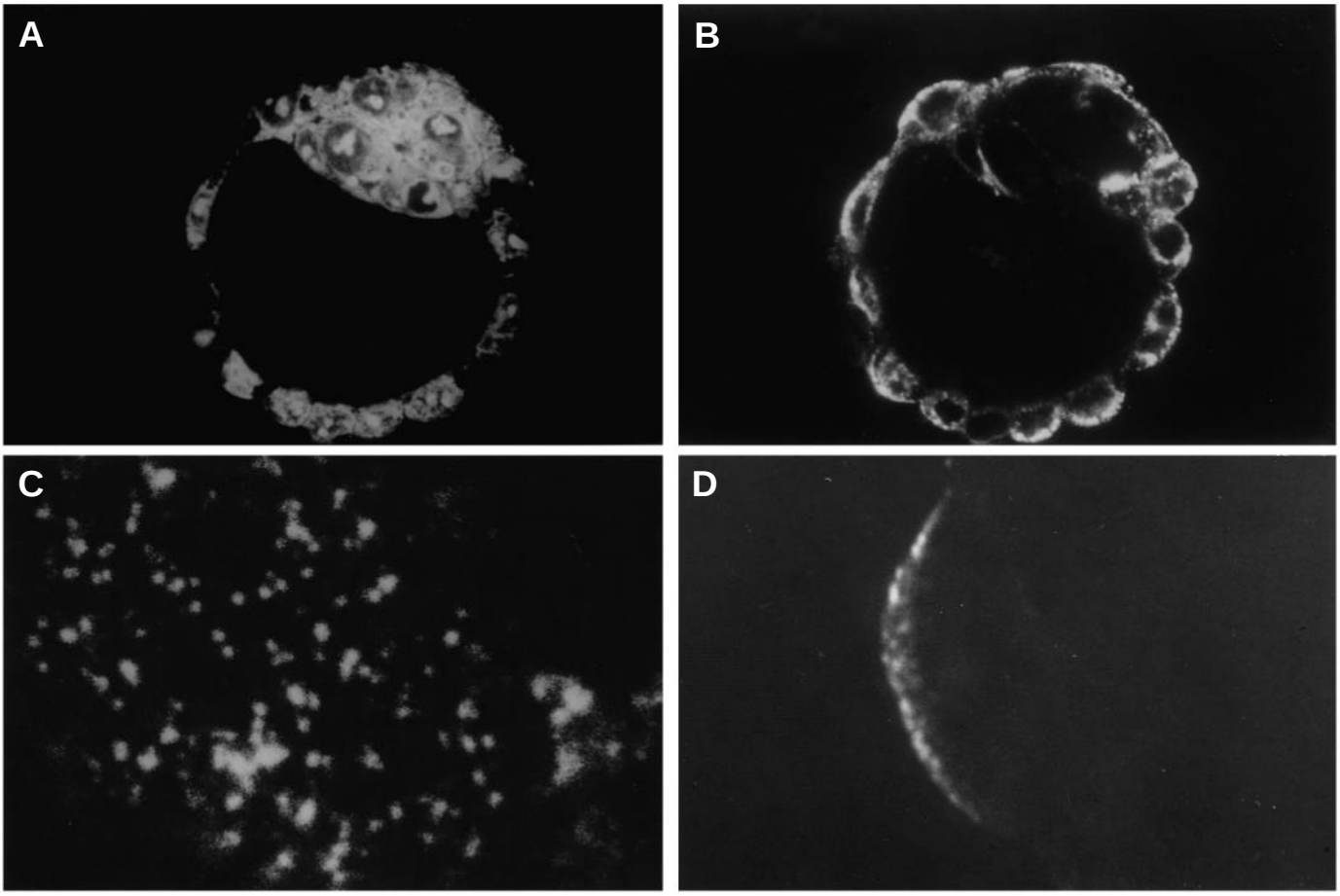

Fig. 3. Immunofluorescence staining with the anti-gp330 antiserum at the fully expanded blastocyst stage observed under a confocal microscope. (A,B) Blastocyst stained with propidium iodide (A) and corresponding image stained with anti-gp330 antiserum (B) after permeabilization (Note the absence of staining in the inner cell mass). (C) Optical section tangential to the surface (note the punctate staining at the cell surface). (D) Detail of one blastomere in a blastocyst not permeabilized prior to staining (note the absence of cytoplasmic staining). Magnification is ×200 in A,B, ×2000 in C and ×1000 in D.

extent of intercellular adhesion. Therefore we checked that no gp330 staining was observed in 2/16 pairs cultured 1 to 3 hours postdivision in control medium (T6+BSA), whatever the phenotype. This result also confirmed the time course of gp330 expression observed in whole embryos.

After 9 hours postdivision in control medium, 56% of the pairs were identified as polar:polar pairs (P:P) and 44% as apolar:polar pairs (A:P). This ratio is consistent with the scoring of 2/16 pairs surface phenotype performed by scanning electron microscopy (Pickering et al., 1988). P:P pairs were flattened on their basal surface, whereas in A:P pairs the polar blastomere enveloped partially (55%) or completely (45%) the apolar cell. In 83% of the P:P pairs, the anti-gp330 serum stained both blastomeres (Table 3; Fig. 5E,F). The staining pattern was similar to that described for whole 16-cell stage embryos. In 17% of the P:P pairs, neither blastomeres was stained. This result is consistent with a progressive increase in the number of cells positive for gp330 observed in whole embryos during the 16-cell stage. We did not observe P:P pairs in which only one of the blastomeres was positive. In contrast, in all but one of the A:P pairs

Table 3. Percentage of 2/16 pairs expressing gp330 when cultured either in normal medium (T6) or in a medium in which flattening is inhibited (T6+ECCD-1)

Pair phenotype	T6	T6+ECCD-1
*P/P pairs**	*56%*	*55%*
P(+)/P(+)	83% (*n*=20)	92% (*n*=24)
P(–)/P(–)	17% (*n*=4)	8% (*n*=2)
P/A pairs†	*44%*	*45%*
P(+)/A(+)	0% (*n*=0)	90% (*n*=19)
P(+)/A(–)	95% (*n*=18)	10% (*n*=2)
P(–)/A(–)	5% (*n*=1)	0% (*n*=0)

The cell polarity of each blastomere is scored using concanavalin A reactivity on pairs double-stained with anti-gp330 antiserum. Cumulated results from 2 experiments.

P, polar phenotype; A, apolar phenotype

(+), gp330 immunoreactivity; (–), no gp330 immunoreactivity

*The difference observed between the two columns is not significant (P=0.4092 using Fisher's exact test).

†The differences observed for apolar cells between the two columns is statistically significant (P<0.0001).

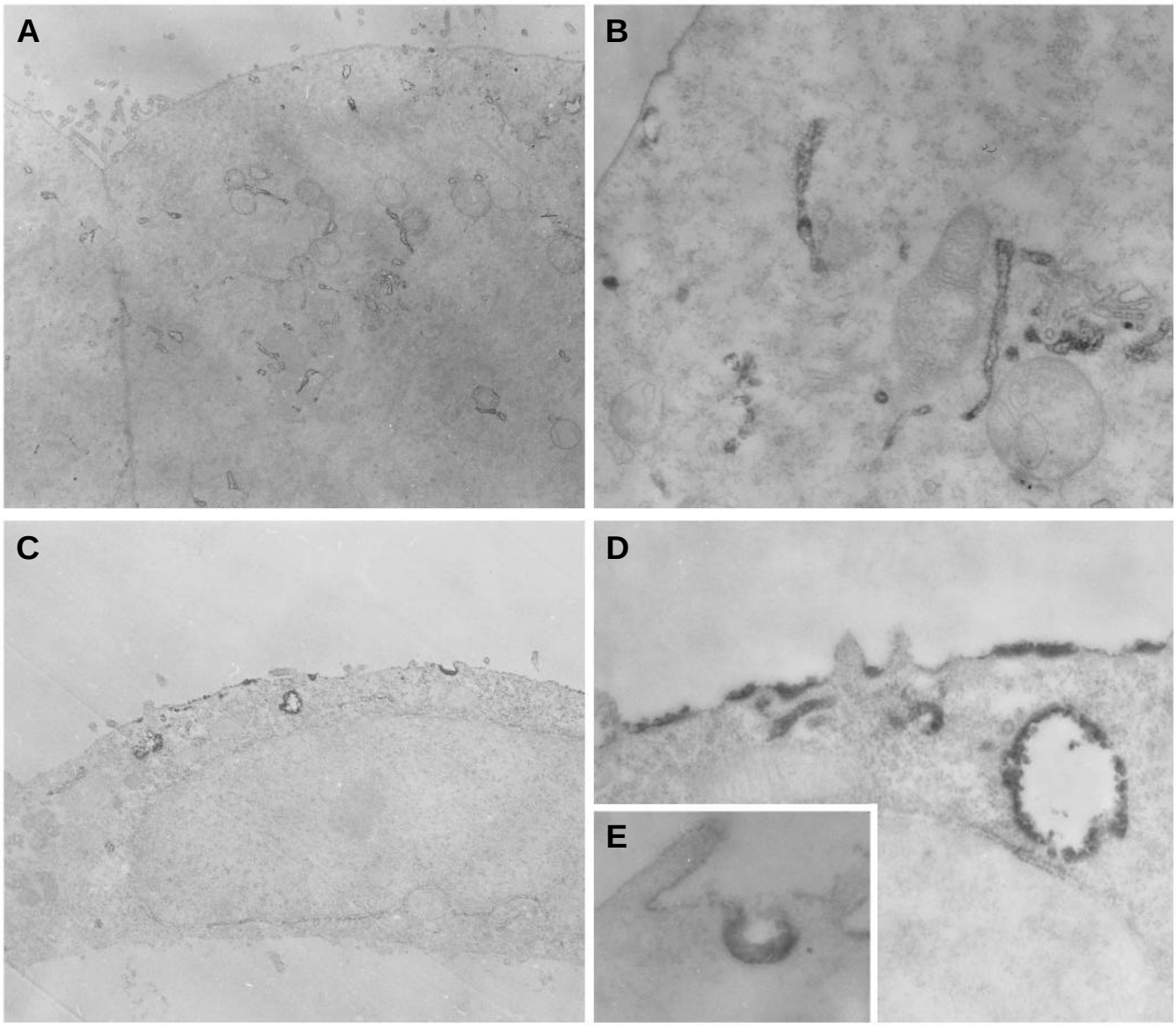

Fig. 4. Immunoperoxidase staining with the anti-gp330 antiserum at the 16-cell stage (A,B) and blastocyst stage (C-E) observed by electron microscopy. Magnification is ×9100 in A, ×14500 in B, ×3600 in C and 1×4500x in D and ×24000 in E.

only the outside cell (surrounding the inner cell) was positive (Table 3; Fig. 5G,H). This absence of gp330 staining in the apolar cell is consistent with the absence of staining observed in the inner cells of whole embryos at the 16-cell stage.

Inhibition of intercellular contacts was performed using either T6+BSA medium completely depleted in Ca^{2+} (T6-Ca^{2+}) or T6+BSA containing ECCD-1 (T6+ECCD-1), a monoclonal antibody directed against uvomorulin (Shirayoshi et al., 1983). The polarity is less evident when the pairs are cultured 9 hours postdivision in T6+ECCD-1 medium, but the ratio of P:P (55%) and A:P (45%) pairs observed after Con A-staining in these conditions was consistent with the controls. Intercellular adhesion was inhibited in all pairs independently of their surface polarity, as shown by the minimal surface of contact established between the two blastomeres. 100% of the blastomeres in the P:P pairs and 93% in the A:P pairs expressed gp330 in these conditions (Table 3; Fig. 5I-L). This result showed that inhibition of cell flattening had a drastic effect on the expression of gp330 in apolar cells in vitro. Inhibition of adhesion also perturbed the intracellular localization of gp330, since the staining was observed throughout the cytoplasm with no, or reduced, apical polarization. In pairs cultured 9 hours postdivision in T6 lacking Ca^{2+}, no gp330 staining was observed. This inhibition of expression of gp330 was also observed in whole embryos cultured in the absence of Ca^{2+} (data not shown).

gp330 is synthesized in exposed inner cell mass cells in the absence of new mRNA synthesis

To determine whether mRNA synthesis is required for gp330 appearance in inner cells when exposed at the periphery of the embryo, we prepared inner cell masses (ICMs) by immunosurgery from 32-cell stage embryos. After immunosurgery, most ICMs were devoid of gp330 reactivity (Fig. 6A; 7/10=70%). The

residual reactivity is likely to be due to some trophectoderm blastomeres resistant to the immunosurgery. After 7 hours of culture in T6+BSA, gp330 was detected at the periphery of most ICMs (Fig. 6B; 11/13 = 85% significantly different from the control group P=0.0131 using Fisher's exact test). This staining was not modified when ICMs were cultured in the presence of 11 μg/ml α-amanitin, the same percentage of gp330-positive ICM being observed (Fig. 6C; 11/13 = 85%). This result showed that RNA synthesis is not required for gp330 appearance in inner cells in which cell contacts have been reduced.

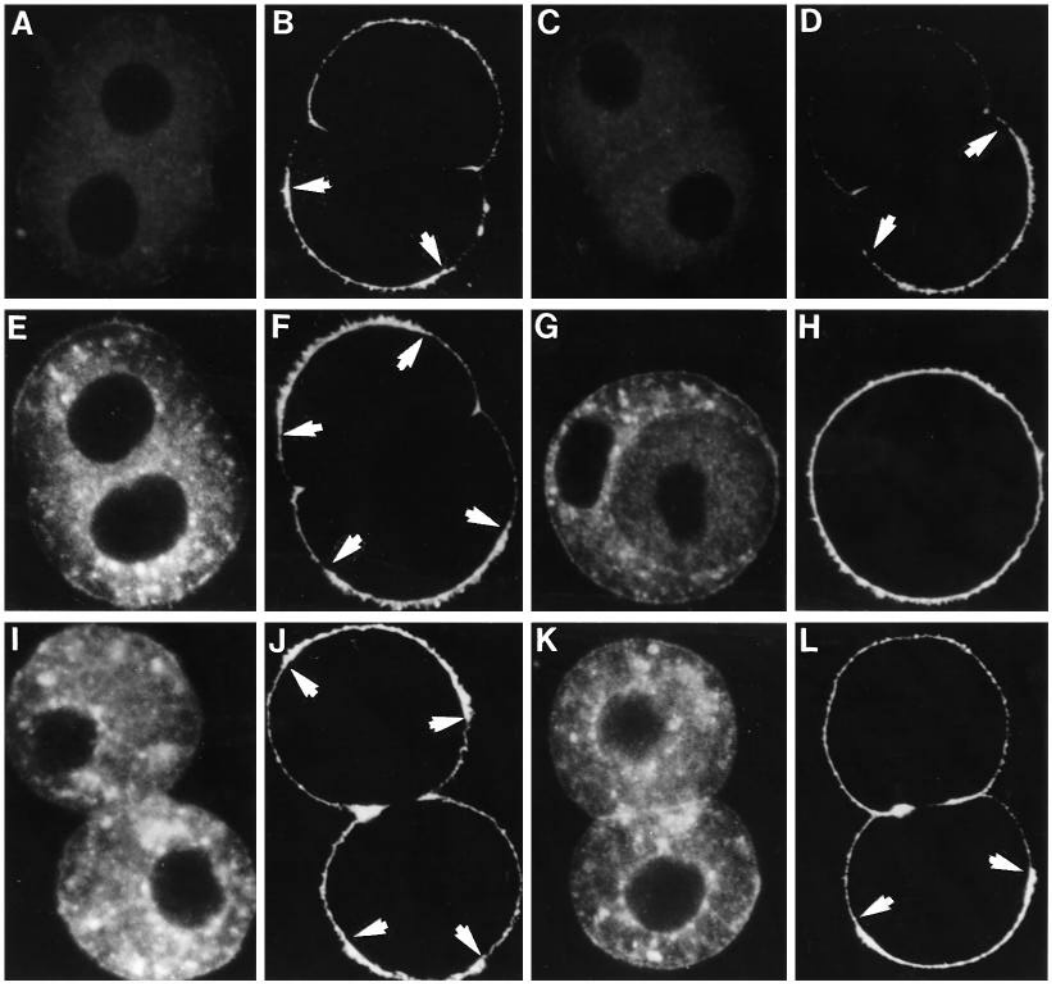

Fig. 5. Cell polarity and immunofluorescence staining with anti-gp330 antiserum in 2/16 pairs obtained after 3 hours (A-D) or 9 hours (E-L) culture of isolated 8-cell stage blastomeres. (A-H) Pairs cultured in control medium; (I-L) pairs cultured in medium containing the monoclonal anti-uvomorulin antibody ECCD-1. Immunofluorescence staining with anti-gp330 antiserum (A,C,E,G,I,K), corresponding to fluorescent concanavalin A staining in (B,D,F,H,J,L) observed by confocal microscopy. Fluorescent concanavalin A staining (surface pole delimited by arrows) allows the identification of the pairs on the left as P/P pairs and of the pairs on the right as A/P pairs.

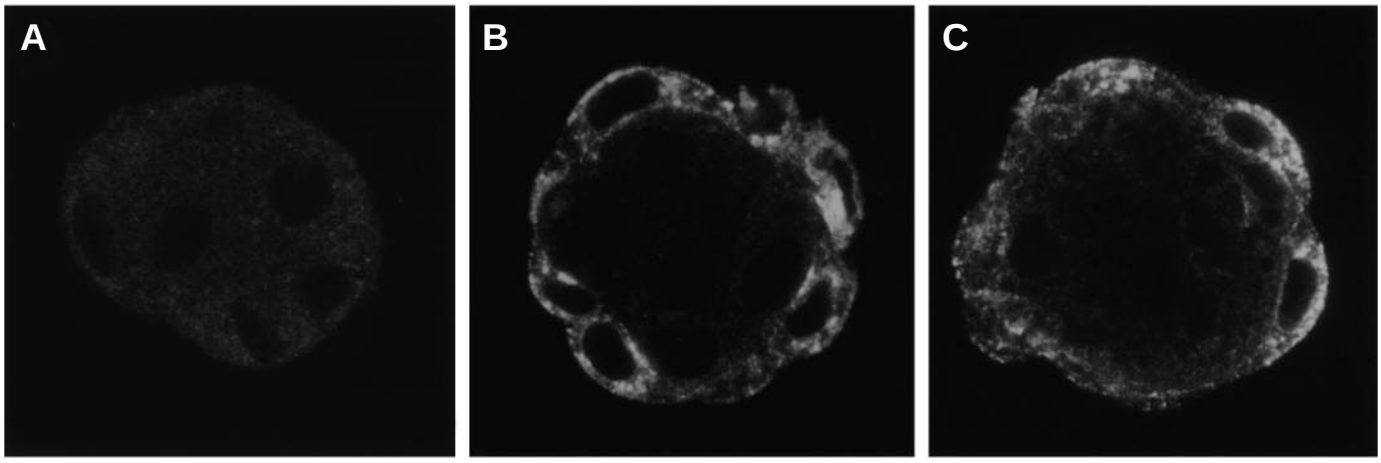

Fig. 6. Immunofluorescence staining with anti-gp330 antiserum of a control inner cell mass fixed immediately after immunosurgery (A) or inner cell masses cultured for 8 hours in the absence (B) or presence (C) of 11 μg/ml α-amanitin. Confocal optical sections of the embryos stained with the anti-gp330 antiserum. Magnification, ×700.

The cytoplasmic distribution of gp330 in blastocysts is dependent upon the microtubule network

A redistribution of the gp330 antigen in epithelial kidney cells after injection of colchicine in vivo has been reported (Gutmann et al., 1989). In order to study directly the involvement of microtubules in the apical localization of gp330, expanded blastocysts were treated 3 hours with 10 μM nocodazole. The treatment resulted in a significant dispersion of the gp330-staining throughout the cytoplasm of trophectoderm cells (Fig. 7). However, the apical polarization was not lost completely.

DISCUSSION

Expression of gp330 during mouse preimplantation development

Gp330 expression begins at the 16-cell stage and is restricted to the outer blastomeres, undergoing epithelial differentiation. At this stage, it is present in cytoplasmic vesicles likely corresponding to endoplasmic reticulum and Golgi. At the blastocyst stage, gp330 is observed in coated pits at the plasma membrane as has been reported for glomerular and proximal tubule epithelial cells (Kerjaschki and Farquhar, 1983). Therefore, we assume that the anti-gp330 serum that we used recognized in early mouse embryos the same antigens as those characterized in renal epithelial cells. The slightly higher molecular mass we estimated (360 kDa versus 330 kDa) is not significant, since discrepancy in the evaluation is more likely in such large proteins, as observed by other authors studying gp330 (Orlando et al., 1992).

It is likely that the cytoplasmic localization of gp330 observed at the 16-cell stage corresponds to its synthetic pathway, before becoming addressed to the plasma membrane, rather than to an alternative localization and function. Secondary lysosomes form and distribute in the basal domain during the 16-cell stage (Fleming and Pickering, 1985; Maro et al., 1985). Thus, gp330 appears during the final phase of maturation of the endocytic system, which is essential for the polarized activities that characterize the epithelial trophectoderm (Fleming, 1986). Dispersion of gp330 in trophectoderm cells after nocodazole treatment is consistent with the colchicine-induced redistribution of gp330 observed in renal proximal tubules 6 hours after injection in vivo (Gutmann et al., 1989). These data support the conclusion that microtubules are involved in the accumulation of gp330-containing vesicles in the apical domain and, indirectly, in the targeting of gp330 to the apical membrane. In addition, depolymerization of micro-

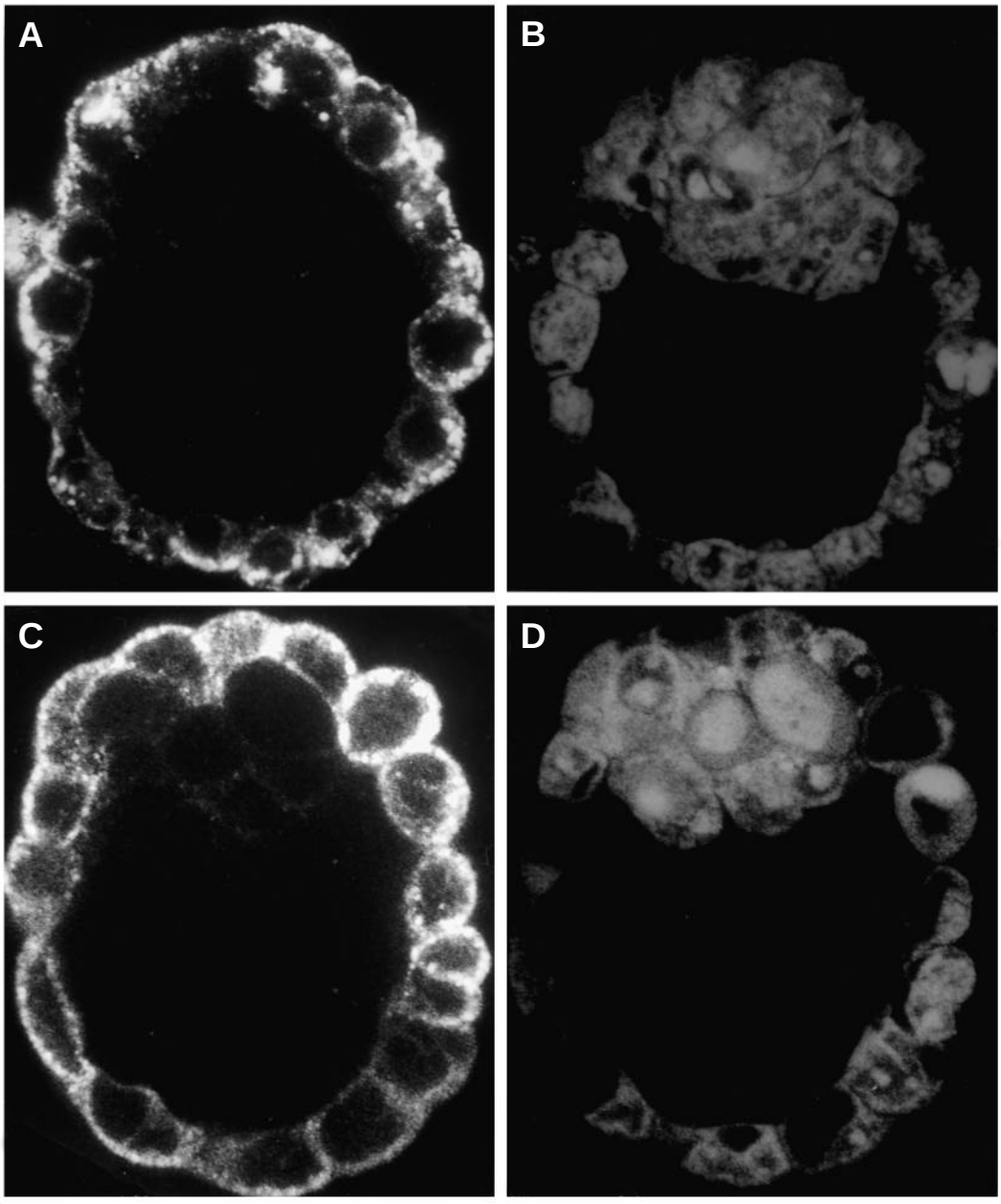

Fig. 7. Immunofluorescence staining with anti-gp330 antiserum of control (A,B) or nocodazole-treated (10 mM for 3 hours) blastocysts. Confocal optical sections of the embryos stained with the anti-gp330 antiserum (A,C) and corresponding staining with propidium iodide to visualize all the cells (B,D). Magnification, ×700.

tubules has been reported previously to disturb the apicobasal polarity of the endocytic route in the mouse preimplantation embryo (Fleming and Pickering, 1985; Fleming et al., 1986).

The pattern of gp330 appearance during mouse preimplantation development differs from that observed for cytokeratins (Chisholm and Houliston, 1987), tight-junction components (Fleming and Hay, 1991; Fleming et al., 1993), or the desmosomal component dp3 in association with desmosomes (Fleming et al., 1991). gp330 appears de novo in outer embryonic cells only, whereas these other epithelial markers become restricted progressively to outer cells after expression initially in both inner and outer cells (Table 1). The possibility of missing dispersed anti-gp330 reactivity in inner cells is unlikely since this antigen was already detected during the biosynthesis process in outer cells (likely due to the high sensitivity of our antibodies and to the fact that gp330 remains concentrated in the membranes of organelles before becoming addressed to the plasma membrane). The pattern of gp330 expression is more reminiscent of that observed for some other desmosomal components (Knudsen and Wheelock, 1992; Table 1). Appearance of gp330 starts at the same stage as that of dp1+2 (desmoplakins) and one stage earlier than dg1 (desmoglein) and dg2+3 (desmocollins). However, the difference between outer and inner cells in the rate of biosynthesis of these components has not be analyzed.

There are thus two possible ways for a molecule to segregate into one particular lineage: (1) appearance only in outer cells undergoing epithelial differentiation, or (2) progressive restriction to outer cells following appearance into both outer and inner cells lineages. The behaviour of gp330 in the first category confirms that the segregation of a cellular marker into one of the two lineages of the preimplantation mouse embryo is not always the consequence of a differential inheritance and that the expression of a protein can be modulated without prior cytoplasmic segregation (Ziomek and Johnson, 1982; Fleming, 1987).

Inhibition of gp330 appearance in inner cells is mediated by intercellular contacts

gp330 is expressed in cells that have already begun epithelial differentiation, suggesting that a positive control could be involved in the triggering of its expression. We have observed that gp330 expression shows plasticity and can be modulated by changing the environment of the blastomeres. An inner position, where the blastomere is surrounded by other cells, leads to a drastic down-regulation of the gp330 expression. This inhibition takes place at the post-transcriptional level as shown by the absence of any effect by α-amanitin on gp330 appearance at the periphery of ICMs after immunosurgery and further culture. This down-regulation, controlled by cell interactions, can account perfectly for the restricted expression of gp330 observed in vivo. Thus, the control of gp330 expression is similar to that observed in the case of ZO-1 (Fleming and Hay, 1991), even if their mode of appearance is different.

Such a mechanism of down-regulation is likely to be important for the maintenance of the lineages and to allow flexibility in case of disturbances (for example epithelialization of inner cells when outer cells have been injured). Trophectodermal processes have been shown to provide a cellular cover to the ICM cells on the blastocœlic surface (Fleming et al., 1984) before this layer of ICM cells undergoes an epithelial differentiation to give rise to the primitive endoderm at 4-5 days of pregnancy (Gardner, 1985). Expression of gp330 has been reported in rat primitive endoderm cells (Sahali et al., 1993). Our results are thus in complete accordance with the hypothesis that the trophectoderm processes are involved in the suppression of ICM differentiation in situ.

It is likely that the decisive stimulus allowing the expression of these epithelial markers is not the extent of intercellular contacts established by the cell, but rather the absence of contacts on part or all of its surface. Indeed, the extent of cellular contacts established by polar cells in apolar :polar 2/16 pairs is significant (complete envelopment of the inner cell), but is not sufficient to inhibit gp330 expression.

The apical localization of gp330 reactive cytoplasmic structures at the end of the 16-cell stage may ensure that gp330 is not inherited by the inner cells derived from outer cells by differentiative divisions. This phenomenon may thus reinforce the inhibition of gp330 expression mediated by intercellular contacts.

Regulation of gp330 expression by Ca^{2+}

gp330, as revealed by our anti-gp330 serum, is not expressed in the absence of Ca^{2+} in the medium. gp330, like other members of the low density lipoprotein receptor-related protein (LRP) family, is a Ca^{2+}-binding protein (Christensen et al., 1992). Ca^{2+} is a cytoplasmic second messenger produced by activation of the phosphoinositide pathway after external stimulation (Berridge and Irvine, 1989) and plays important roles in a variety of cellular processes (Murray and Weber, 1974; Schulman, 1993). Intracellular Ca^{2+} has also been reported to regulate the gadd153 gene (a CCAAT /enhancer-binding protein related gene) expression through transcriptional control (Bartlett et al., 1992). Thus, the observed effect of removing Ca^{2+} from the extracellular medium could be mediated through very different mechanisms and this has to be investigated further.

Possible functions of gp330

In contrast to the other epithelial components analysed during trophectoderm differentiation (cytokeratins, tight-junctions and desmosomal components), gp330 is present in a restricted number of epithelia, which share an intense endocytotic activity associated with the degradation of the internalized proteins (Chatelet et al., 1986). At the subcellular level, gp330 localization is restricted to the clathrin-coated areas that give rise to the endocytic vesicles (Pastan and Willingham, 1983). This cellular- and subcellular-restricted distribution suggests that gp330 is involved in specific receptor-mediated endocytosis. Partial sequencing of a gp330 encoding cDNA indicates that it is highly homologous to the LRP/α2-macroglobulin receptor (α2MR; Raychowdhury et al., 1989; Pietromonaco et al., 1990). They also bind to several common ligands including plasminogen activator (PA)/inhibitor complexes (Christensen et al., 1992; Kounnas et al., 1992; Willnow et al., 1992; Bu et al., 1992; Moestrup et al., 1993). It is however important to note that LRP/α2MR has a larger tissue distribution compared to the epithelia-restricted expression of gp330 (Moestrup et al., 1992).

gp330 might be involved in implantation as suggested by its restriction to the clathrin-coated intermicrovillar areas first detected at the blastocyst stage and because plasminogen activator activity has been demonstrated in cultured mouse

blastocysts at the time of embryo implantation in vivo (Strickland et al., 1976; Sappino et al., 1989). Maintenance of such enzymatic activity requires efficient elimination of the complexes formed by active enzymes and their inhibitors. This function is carried out very efficiently in the liver by LRP/α2MR through internalization of circulating PA/PAI1 complexes and can be performed by gp330 in tubular cells (Bu et al., 1993; Moestrup et al., 1993). A recent study based on disruption of the *LRP/α2MR* gene suggested that this receptor has a similar scavenger role in trophectoderm cells, since implantation and mouse embryonic development before 13.5 days postcoïtum were partly disturbed (Herz et al., 1992, 1993). The incomplete effect of this disruption might be explained by a partial rescue by gp330, which can mediate the internalization of the same complexes. The role of gp330 may be even more complex, since it is also able to bind plasminogen (Kanalas and Makker, 1991) and components of the extracellular matrix in vitro (Mendrick et al., 1990). Our observations show that the mouse preimplantation embryo, resilient to in vitro manipulations, represents another convenient system for further analysis of gp330 function. Furthermore, this model allows the study of the onset of gp330 expression during epithelial differentiation.

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