

The Establishment of Polarized Membrane Traffic in *Xenopus laevis* Embryos

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Abstract. Delineation of apical and basolateral membrane domains is a critical step in the epithelialization of the outer layer of cells in the embryo. We have examined the initiation of polarized membrane traffic in *Xenopus* and show that membrane traffic is not polarized in oocytes but polarized membrane domains appear at first cleavage. The following proteins encoded by injected RNA transcripts were used as markers to monitor membrane traffic: (a) VSV G, a transmembrane glycoprotein preferentially inserted into the basolateral surface of polarized epithelial cells; (b) GThy-1, a fusion protein of VSV G and Thy-1 that is localized to the apical domains of polarized epithelial cells; and (c) prolactin, a peptide hormone that is not polarly secreted. In immature oocytes, there is no polarity in the expression of VSV G or GThy-1, as shown by the constitutive expression of both proteins at the surface in the animal and vegetal hemispheres. At meiotic maturation, membrane traffic to the surface is blocked; the plasma membrane no longer accepts the vesicles synthesized by the oocyte (Leaf, D. L., S. J. Roberts, J. C. Gerhart, and H.-P. Moore. 1990. *Dev. Biol.* 141:1-12). When RNA transcripts are in-

jected after fertilization, VSV G is expressed only in the internal cleavage membranes (basolateral orientation) and is excluded from the outer surface (apical orientation, original oocyte membrane). In contrast, GThy-1 and prolactin, when expressed in embryos, are inserted or released at both the outer membrane derived from the oocyte and the inner cleavage membranes. Furthermore, not all of the cleavage membrane comes from an embryonic pool of vesicles—some of the cleavage membrane comes from vesicles synthesized during oogenesis. Using prolactin as a marker, we found that a subset of vesicles synthesized during oogenesis was only released after fertilization. However, while embryonic prolactin was secreted from both apical and basolateral surfaces, the secretion of oogenic prolactin was polarized. Oogenic prolactin was secreted only into the blastocoel (from the cleavage membrane), none could be detected in the external medium (from the original oocyte membrane). These results provide the first direct evidence that the oocyte synthesizes a cache of vesicles for specific recruitment to the embryonic cleavage membranes which are polarized beginning with the first cleavage division.

THE formation of the embryonic epithelium seals the interior fluid compartment of the embryo from the external environment and provides the earliest example of cellular specialization in *Xenopus* development. In the frog embryo, the outer layer of cells forms an epithelial sheet characterized by the formation of apical tight junctions and the polarized distribution of the NaK-ATPase on the interior membranes (Kalt, 1971; Sanders and Zalik, 1972; Slack and Warner, 1973; Morrill et al., 1975; deLaat et al., 1976; Peracchia, 1984). This embryonic epithelium vectorially pumps ions into the blastocoel which then inflates from the osmotic influx of water (Slack and Warner, 1973; Morrill et al., 1975). In addition, release of extracellular matrix proteins required for the migration of mesoderm during gastrulation is polarized; extracellular matrix is only found on the inner surfaces of the blastocoel (Keller and Winklbauer, 1990). Hence, the epithelialization of the outer layer of em-

bryonic cells is a critical step in the development of the embryo.

In cells of differentiated polarized epithelia, there are two membrane domains. One, the apical domain faces the lumen or external milieu, while the other, the basolateral domain faces the internal blood space. The apical and basolateral domains have distinct protein and lipid compositions that correlate with the different functions of these two surfaces. The apical surface prevents the influx of undesirable ions and toxins and regulates nutrient and water uptake while the basolateral surface is specialized for cell-cell adhesion and communication, signal transduction, and cell-substratum attachment. In some cell types, the polarized distribution of membrane proteins is set up by polarized membrane traffic from the *trans*-Golgi network and polarity is maintained by tight junctions which prevent the flow of protein and lipid between the two domains.

When are these epithelial characteristics first established during development? In the mouse, the first indication of the formation of the embryonic epithelium (which will become the trophoblast) is compaction. At compaction, the adhesion between blastomeres increases and the formerly round cells flatten against each other. This coincides with the formation of tight junctions, and the cell membranes become polarized into apical (external) and basolateral (internal) domains (reviewed by Fleming and Johnson, 1988 and Wiley et al., 1990). The localization of the E-cadherin (uvomorulin) to sites of cell-cell contact has been implicated in compaction (Johnson et al., 1986). When the activity of uvomorulin was impaired with an antibody, compaction was inhibited, no tight junctions formed and the polarized, basolateral insertion of Na⁺K⁺ ATPase was blocked (Fleming et al., 1989; Watson et al., 1990).

In *Xenopus*, another cadherin, U-cadherin, has recently been identified in early embryos that is restricted to the cleavage membranes as early as the first division. This cadherin is present in the egg but is not found at the cell surface until new membrane is inserted into the cleavage furrow (Choi et al., 1990; Ginsberg et al., 1991; Angres et al., 1991). Also, structures similar to epithelial tight junctions have been identified between blastomeres after the first cleavage (Bluemink, 1971; Sanders and Zalik, 1972). Consistent with these observations, measurements of ion currents in cleaving *Xenopus* eggs suggest that the cleavage furrow membranes have electrical properties and hence ion channels which are distinct from the old membrane (Kline et al., 1983). Therefore, by analogy to the development of polarity in the mouse embryo, cell polarity in *Xenopus* may be established as early as the first cell division.

One issue that has not been directly addressed by previous studies is whether the cleavage membrane is synthesized during oogenesis or after fertilization or a combination of both. In *Xenopus* embryos, cleavage occurs every 30 min (at 20°C) and each radial division requires the addition of new membrane that is equivalent in area to ~25% of the membrane of the unfertilized egg. Since the oocyte stores many of the other components required for these rapid cell divisions (Gilbert, 1988), it has been hypothesized that the oocyte also synthesizes stores of membrane vesicles for insertion into the cleavage furrow (Singal and Sanders, 1974). The observation that the total pool of glycoproteins does not change in either quantity or composition from fertilization to gastrulation provides further evidence for a maternal pool of membrane proteins in the egg (Servetnick et al., 1990).

Another issue is how the apical-basal polarity of embryonic membranes is established. One possibility is that polarity is generated by the production of apically and basolaterally targeted vesicles from the *trans*-Golgi as has been proposed for epithelial cells (Kelly, 1985; Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990). Alternatively, polarity could be generated if the original oocyte plasma membrane is inert after fertilization (no vesicle fusion) and new membrane inserts exclusively at the cleavage furrows. The apical surface would then be defined by the membrane of the egg and the basolateral surface would consist of the new membrane inserted during cleavage. A third possibility is that the embryo may employ a combination of both mechanisms.

To approach these questions we have used proteins produced from injected RNA as markers for following mem-

brane traffic in embryos. Using this technique we have obtained evidence that oocytes synthesize secretory proteins that are stored in vesicles and targeted specifically for the internal, basolateral surfaces of the embryo. Also, we find that membrane traffic after fertilization is polarized as indicated by the localization of VSV G, a membrane protein targeted to the basolateral domain of epithelial cells, exclusively to the internal cleavage membranes of the embryo. Another membrane marker, GThy-1, which is apically directed in epithelial cells, is found in both the internal and external membranes, possibly a consequence of the demand for new membrane during cleavage. Hence, the targeting of secretory and membrane proteins to the basolateral domain is established before the first cleavage division.

Materials and Methods

Supplies

Restriction endonucleases, SP6 polymerase, 7-methyl GpppG cap analog, RNase-free DNase, were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). T4 DNA ligase was purchased from New England Biolabs (Beverly, MA). Rabbit antiserum proactin antiserum was obtained from U.S. Biochemicals (Cleveland, OH). Affinity-purified FITC Goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR). *Staphylococcus aureus* cells were purchased from Calbiochem-Behring Corp. (La Jolla, CA) and Bethesda Research Labs (Bethesda, MD). Ascites fluid of a mouse mAb against VSV G was a gift of Dr. Leo LeFrancis, Upjohn Co. (Kalamazoo, MI). [³⁵S]methionine was obtained from Amersham Inc. (Arlington Heights, IL). Diethylpyrocarbonate, progesterone, and pregnant mare serum gonadotropin were purchased from Sigma Chemical Co. (St. Louis, MO).

Handling of Oocytes and Eggs

Xenopus laevis females were injected with pregnant mare serum gonadotropin 1-2 d before removal of ovaries. Ovary tissue was surgically removed from females anaesthetized with 0.12% benzocaine. Full grown stage 6 oocytes were manually dissected from the ovary. Unless otherwise indicated oocytes were maintained in MR (MR refers to modified Ringer's, full strength MR contains: 100 mM NaCl, 1.5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, buffered with 2.5 mM Hepes, pH 7.5). To induce meiotic maturation, immature stage 6 oocytes were incubated in 1 μg/ml progesterone in MR for 1-2 h.

The procedure for obtaining eggs from *Xenopus* females is essentially as described in Vincent et al. (1986). Females were induced to ovulate by injection of human chorionic gonadotropin (600 U) 12 h before ovulation. After stripping, the eggs were fertilized with a suspension of macerated testis in 33% MR. After fertilization, the eggs were dejellied with 2% cysteine, pH 8.0.

Plasmids

A bovine prolactin cDNA clone in pSP64T was a gift from P. Walter (Dept. of Biochemistry and Biophysics, University of California at San Francisco). pSP64T is described in Krieg and Melton (1984).

VSV G was subcloned from pSV2 G (Rose and Bergmann, 1982) into pSP64 (Promega Biotec, Madison, WI) as previously described in Leaf et al. (1989). A HindIII-BglII fragment containing VSV G from pSV2 G (Rose and Bergmann, 1982) was initially cloned into the HindIII and BamHI sites of Bluescript. Since only SP6 transcripts were efficiently translated in oocytes we subcloned a HindIII-XbaI fragment of VSV G from the Bluescript construct into pSP64.

The fusion construct of VSV G and Thy-1 was obtained from J. K. Rose (Yale University School of Medicine, New Haven, CT) and subcloned into pSP65 (Promega Biotec) as follows: an XbaI-XhoI restriction fragment from pVS GThy-1 (Crise et al., 1989) was filled in with Klenow and blunt end ligated into the SmaI site of pSP65 to create pSP65 GThy-1.

In Vitro Transcriptions

SP6 transcripts capped with 7-methyl GpppG were generated according to

Krieg and Melton (1986). Plasmid DNA was prepared from transformed JM103 cells by alkaline lysis and purified by cesium chloride banding according to Maniatis et al. (1982). pSP64T-prolactin DNA was linearized by EcoRI digestion, pSP64-VSV G DNA was linearized by BamHI digestion, and pSP65-GThy-1 DNA was linearized with HindIII. 5 mg of template DNA were used per transcription reaction which generates approximately the same mass of SP6 transcripts. After the transcription reactions, the template DNA was digested with RNase-free DNase, and extracted three to five times with equal volumes of phenol/chloroform, one time with an equal volume of chloroform, and precipitated with 1/2 vol of 7 M ammonium acetate, and 2 vol of ethanol. After pelleting and drying, the SP6 transcripts were reprecipitated with ethanol. The pellet was resuspended in a final volume of 10 μ l diethylpyrocarbonate-treated water. 40–50 nl of the SP6 transcripts (\sim 25 ng) were injected per oocyte.

[³⁵S]methionine Labeling

Immature oocytes expressing prolactin were labeled by incubation for 30 min in modified Barth's (88 mM NaCl, 0.83 mM MgSO₄, 1 mM KCl, 0.33 mM Ca(NO₃), 0.41 mM CaCl₂ and 7.5 mM Hepes, pH 7.8) or MR containing [³⁵S]methionine at a concentration of 1 mCi/ml. 30 min after incubation with [³⁵S]methionine, oocytes were transferred to chase medium (at least 200 μ l/oocyte) consisting of Barth's or MR medium with 10 mM non-radioactive methionine. To control for the effectiveness of the chase, we compared the amount of radiolabeled protein synthesized when prolactin RNA was injected into oocytes either before or after the pulse-chase (oocytes were radiolabeled for 30 min with [³⁵S]methionine and chased for 90 min in 10 mM non-radioactive methionine). We found that the amount of radiolabeled prolactin synthesized after the chase was only 17% of the amount of radiolabeled prolactin synthesized before the chase. This experiment demonstrates that the pool of [³⁵S]methionine is effectively diluted by incubation for 90 min in 10 mM non-radioactive methionine. Since embryos do not take up sufficient amounts of [³⁵S]methionine from the incubation medium, we radiolabeled embryos by coinjection of [³⁵S]methionine (0.3 μ Ci) with prolactin RNA into the uncleaved egg or one blastomere at the 8–16 cell stage. In some experiments embryos injected with prolactin RNA during the first cell cycle were radiolabeled by injection of [³⁵S]methionine (0.5 μ Ci) into the blastocoel at midblastula.

Immunoprecipitations

Immunoprecipitations were performed according to Moore and Kelly (1985). Individual or small groups of embryos were lysed in 50 μ l/embryo of NDETS (1% NP-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.1% SDS). Prolactin was immunoprecipitated one time using 1 μ l rabbit antish sheep prolactin antiserum per oocyte or embryo. The immunoprecipitates were eluted from *Staphylococcus aureus* cells by boiling for 3 min in 30 μ l of Laemmli sample buffer.

Acetone Precipitations

Media samples containing prolactin were precipitated by dilution in 4 vol of acetone overnight at -20°C . The precipitates were pelleted by a 15-min centrifugation at 14,000 g and vacuum dried. For PAGE, the acetone precipitates were resuspended in 15 μ l of water, and 15 μ l of 2 \times Laemmli sample buffer.

Indirect Immunofluorescence of VSV G

Immature oocytes were injected with RNA transcripts encoding VSV G and incubated overnight at 20°C . Mature oocytes arrested at second meiotic metaphase were injected with RNA transcripts encoding VSV G and fertilized in vitro as described below. Fertilized eggs were injected with VSV G transcripts during either the first cell cycle or at the 8–16 cell stage. Oocytes or embryos at either the early cleavage divisions or at blastula stage were fixed in 4% paraformaldehyde in 1 \times PBS for 1 h, embedded in O.C.T. compound (Miles Scientific), frozen, cryosectioned (15 μ m), and stored at -20°C . Immunofluorescent staining was carried out as described by Leaf et al. (1990) using a fluorescein-conjugated secondary antibody. Uninjected control embryos (embryos without VSV G RNA) were immunostained with both primary and secondary antibody as described. These uninjected embryos showed no immunofluorescence indicating that the staining protocol was specific for the VSV G protein. Immunofluorescently stained sections were photographed with Ektachrome 400 film (Kodak) on a Zeiss Axiophot Microscope. Black and white photographs of color plates were photographed with T-Max 400 film (Kodak).

Inhibition of Cleavage by Nocodazole

One or two blastomeres of embryos at the 8 cell stage were injected with VSV G RNA. Immediately following injection the embryos were immersed in 1/3 MR containing 10 μ g/ml nocodazole. No new cleavage furrows formed in these embryos indicating that the nocodazole treatment was effective. After 3 h the nocodazole arrested embryos were fixed, sectioned, and immunostained as described above.

Activation of In Vitro Matured Oocytes

Immature oocytes which had been injected with prolactin RNA transcripts and pulse-chase labeled with ³⁵S methionine were induced to mature with progesterone. Three hours after the appearance of the white maturation spot which signals the breakdown of the germinal vesicle, mature oocytes were artificially activated by incubation with the calcium ionophore A23187. A23187 (Sigma Chemical Co.) was diluted to 20 μ M in 30% MR from a 100 \times stock solution prepared in ethanol. After 15 min, the cortical granules exocytosed causing the elevation of the fertilization envelope and the experiment was terminated by the collection of both media and cell fractions.

In Vitro Fertilization of Matured Oocytes

Xenopus oocytes matures in vitro with progesterone will fertilize only after removal of the vitelline envelope that surrounds the oocyte. Normally, this envelope is modified during the passage of the oocyte through the oviduct, but without this modification and the addition of the egg jelly, sperm are unable to penetrate through to the egg plasma membrane. Therefore a technique was developed that gently removes the vitelline envelope using a mild treatment with pepsin and substitutes a soluble extract of the egg jelly for activating the sperm (J. Roberts and J. Gerhart, manuscript in preparation). Using this technique we have been able to follow the embryonic fate of proteins synthesized during the last stages of oogenesis.

Results

We have previously shown that the constitutive pathway of secretion is specifically inhibited between the *trans*-Golgi and the cell surface during meiotic maturation (Leaf et al., 1990). What is the fate of "stored" vesicles synthesized during oogenesis but not secreted? These vesicles may be turned over or they may be utilized during embryogenesis. It is possible that oocyte secretory vesicles provide a source of new membrane for the embryo during the rapid cleavage divisions. Does the insertion of new membrane at the cleavage furrows account for the polarity of the blastomeres or is polarity dependent on sorting in the secretory pathway? To address these questions we have traced the secretory pathway by following the fate of exogenous proteins encoded by RNA transcripts injected into either oocytes or embryos.

Prolactin in Eggs Activated with the Calcium Ionophore A23187

The first secretory event after constitutive secretion has been blocked at meiotic maturation is the exocytosis of the cortical granules when the egg is activated or fertilized. One mechanism by which secretion from the *trans*-Golgi to the cell surface may become inhibited during meiotic maturation is that all secretory vesicles are shunted into a calcium-dependent regulated pathway. This pathway would then be activated by the increase in intracellular calcium at fertilization that triggers cortical granules exocytosis. We tested whether "stored" prolactin, synthesized during oogenesis but not secreted before meiotic maturation, is released when the cortical granules exocytose.

Oocytes that had been injected with prolactin transcripts

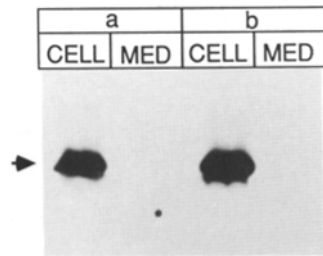


Figure 1. Prolactin is not secreted during cortical granule exocytosis. Immature oocytes were injected with prolactin RNA transcripts, pulse-chase labeled with [³⁵S]methionine and matured with progesterone. 3 h after germinal vesicle breakdown the oocytes were activated by treatment with 20 μM A23187. After 15 min, cell

and media fractions were collected and immunoprecipitated as described in Materials and Methods. Immunoprecipitates were resolved on 10% SDS polyacrylamide gels and autoradiographed for 44 d. The autoradiograph is represented above with prolactin indicated by the arrow. *A* and *B* refer to different batches of activated eggs treated identically.

and pulse-labeled with [³⁵S]methionine were matured in vitro with progesterone and then artificially activated with the calcium ionophore A23187 (Belanger and Scheutz, 1975). Control experiments indicated that >80% of the radiolabeled prolactin was synthesized before the chase in 10 mM unlabeled methionine (see Materials and Methods). 15 min after activation with A23187, when cortical granule exocytosis was complete, the medium and cell fractions were collected. Analysis of the samples by electrophoresis and autoradiography is shown in Fig. 1. None of the "stored" prolactin could be detected in the medium when the cortical granules exocytosed; prolactin was only detected in the cell fraction. Hence, we conclude that in full grown oocytes prolactin is not sorted into secretory vesicles that exocytose in response to an increase in intracellular calcium.

Prolactin in Eggs Matured and Fertilized In Vitro

Since prolactin synthesized by the oocyte was not secreted with the cortical granules we asked whether this pool of prolactin is secreted at all during the cleavage divisions. Oocytes that had been injected with prolactin RNA and pulse labeled with [³⁵S]methionine were matured with progesterone. Hence, the radiolabeled prolactin followed in this experiment was synthesized before meiotic maturation (oogenic prolactin). 3 h after germinal vesicle breakdown, these in vitro matured eggs were fertilized using a protocol developed in this laboratory (J. Roberts and J. C. Gerhart, manuscript in preparation) and briefly described in Materials and Methods.

When the embryos reached the 32 cell stage they were transferred to a Ca²⁺Mg²⁺-free medium to disaggregate the blastomeres. Dissociation of embryos at earlier stages was impractical because the embryos failed to form complete cleavage furrows and leaked cytoplasm into the medium. By dissociating the embryos we were able to collect the total secreted prolactin at regular intervals and determine whether secretion of oogenic prolactin is detectable in embryonic cells. The results of this experiment (Fig. 2) indicate that secretion of oogenic prolactin is detectable at early blastula (stage 7) as determined by the stage of control intact embryos. In this example prolactin secretion appears to increase over time but replicate experiments failed to repeat this trend. Since the eggs were pulse-chase labeled with [³⁵S]methionine during oogenesis, the secreted, radiolabeled prolactin must have been synthesized during oogenesis and stored un-

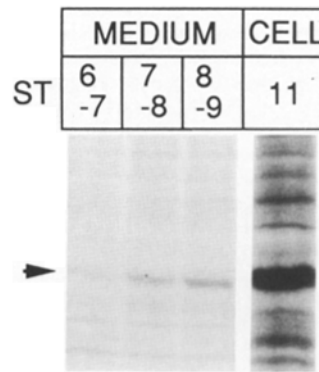


Figure 2. Secretion of "stored" prolactin from a dissociated embryo. Immature oocytes were injected with prolactin RNA transcripts, pulse-chase labeled with [³⁵S]methionine, and matured with progesterone. The matched oocytes were fertilized in vitro as described in Materials and Methods. At st. 6 an embryo was transferred to 2.5 μM EDTA in Ca²⁺Mg²⁺ free 33% MR to dissociate the blastomeres. Media fractions were collected at the stage indicated

above each lane and acetone precipitated. The embryo was homogenized at st. 11 and immunoprecipitated with an anti-prolactin antibody to determine the cell fraction. All samples were then resolved on a 15% SDS polyacrylamide gel and autoradiographed for 44 d. An arrow marks the band containing prolactin on the autoradiograph.

til release during embryogenesis. Hence proteins synthesized by the oocyte can be secreted by the embryo.

To determine whether the "stored" oocyte prolactin is secreted from the external egg surface and/or the cleavage membrane, we collected external medium from intact embryos during blastula and gastrula stages and then gently sheared open the embryos to release the blastocoel fluid without lysing the embryonic cells. The external medium contains proteins secreted from the apical surfaces and the blastocoel fluid contains proteins secreted from basolateral surfaces. The relative fraction of "stored" prolactin secreted across these surfaces is shown in Fig. 3 *A* and summarized comparing the rates of secretion in Table I. As late as gastrulation we found an intracellular pool of "stored" oocyte prolactin that had been neither secreted nor degraded. Interestingly, we found a significant amount of prolactin secreted into the blastocoel cavity but low or undetectable amounts secreted into the external medium. These results suggest that the secretion of "stored" prolactin is polarized such that the protein is only released at the internal basolateral surfaces of cells and not at the apical surfaces of the outer epithelial-like layer of cells.

Prolactin Synthesized by Embryonic Cells

Since "stored" prolactin was only secreted basolaterally in embryos we asked whether embryonic prolactin was similarly restricted from release at the apical surface. Prolactin transcripts were injected into one blastomere at the 8–16 cell stage. The embryos were then labeled by injection of [³⁵S]methionine into the blastocoel of the early blastula. Media and cell fractions were collected and analyzed as described above. In contrast to prolactin synthesized during oogenesis, we found that embryonically synthesized prolactin was secreted at both the apical and basolateral surfaces of the embryo (Table 1, Fig. 3 *C*). The broad range in the rate of prolactin secretion most likely reflects a bias in the partitioning of injected prolactin RNA between the cells of the outer epithelial-like layer (apical secretion) and the deep cells (basolateral secretion). However, we consistently observed that embryonically produced prolactin was secreted at both apical and basolateral surfaces. This is reflected in the aver-

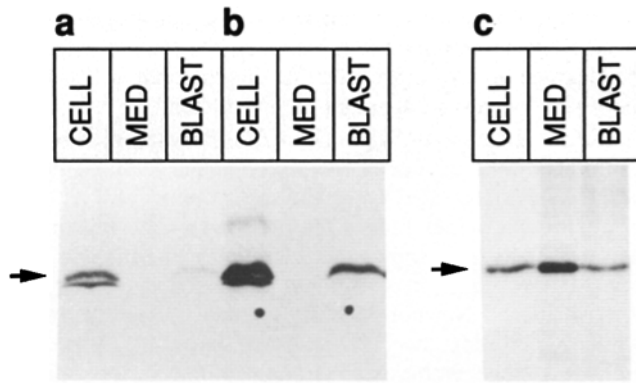


Figure 3. Apical versus basolateral secretion of prolactin in embryos. (*A* and *B*) Prolactin synthesized before fertilization. Prolactin RNA was injected into immature oocytes, the oocytes were pulse-chase labeled with [³⁵S]methionine, matured and fertilized in vitro as described in Materials and Methods. At the blastula stage the embryos were transferred into individual tubes and incubated until (*A*) stage 9 or (*B*) stage 10, early gastrula when the medium surrounding each embryo was collected (*med*) and the embryo was gently sheared to release the contents of the blastocoel (*blast*). Embryos were then homogenized and both cellular (*cell*) and secreted fractions were immunoprecipitated, resolved by SDS PAGE, and autoradiographed. (*C*) Prolactin synthesized after fertilization. Prolactin RNA was injected into one animal hemisphere blastomere of embryos at the 8–16 cell stage. At st. 8, [³⁵S]methionine was injected into the blastocoel, the vitelline envelopes were removed, and three embryos were incubated together in 33% MR. At st. 10, the incubation medium was collected (*med*) and the embryos were sheared open to release the blastocoel fluid (*blast*). The embryos were homogenized and immunoprecipitated and the secreted fractions (*med* and *blast*) were acetone precipitated. Arrows mark the location of the bands containing prolactin.

age 10-fold higher ratio of apical/basolateral rates of secretion in embryogenic relative to oogenic prolactin (Table I). Thus the apical surface of embryonic cells is competent for secretion of embryonically synthesized prolactin and the polarity of prolactin secretion depends upon whether it was synthesized before or after fertilization.

Table I. Polarity of Prolactin Secretion in Embryos

	% Secreted/h		Apical/Basolateral ratio
	Apical	Basolateral	
Oogenic prolactin	0.36 (<i>n</i> = 11)* range = 0.3–0.7	5.1 (<i>n</i> = 7) range = 1.7–9.3	0.07
Embryogenic prolactin	3.5 (<i>n</i> = 31) range = 0.8–9.8	5.2 (<i>n</i> = 12) range = 4.4–6.0	0.67

Values are given as averages for the percent of total radiolabeled prolactin secreted per hour. Below the average is the range of values from which the average was determined. Percent secreted per hour was calculated by dividing the percent secreted apically ([external medium/(external medium + blastocoel fluid + cell)] × 100) or basolaterally ([blastocoel fluid/(external medium + blastocoel fluid + cell)] × 100) by the time over which the sample was collected. All secreted fractions were collected between the blastula and gastrula stages. The amount of prolactin in each fraction was determined by laser densitometric scanning of autoradiographs of samples resolved by SDS PAGE. Experimental procedures are as described in Materials and Methods and Figure legend 3. The *n* value refers to the number of embryos. In several experiments examining the secretion of embryogenic prolactin, fractions from individual embryos were pooled before analysis.

* Since the amount of prolactin secreted apically was undetectable in nine of these embryos an estimate for the lowest detectable rate of secretion (0.3%/h) was used to calculate the average percent secreted and the range.

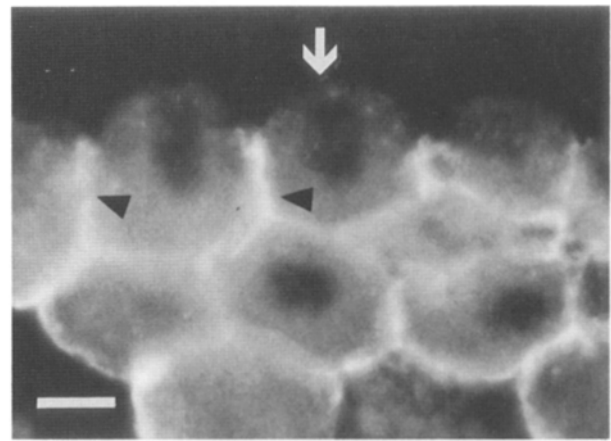


Figure 4. Expression of VSV G is polarized in the epithelial-like cells of the blastula. Micrograph of a section through a fluorescent immunostained blastula stage embryo. VSV G RNA was injected into one animal hemisphere blastomere of 8 cell embryos. At stage 8, embryos were fixed, sectioned and immunostained as described in Materials and Methods. VSV G is found only in the basolateral surfaces of the blastomeres (*black wedges*), the outer apical surface does not stain (*white arrow*). Controls (embryos not injected with VSV G RNA) did not show any immunofluorescent staining. Bar, 30 μm.

Polarized Insertion of VSV G in Embryonic Membranes

The pattern of prolactin secretion described above suggests that there are pathways for both apical and basolateral transport in embryos. Therefore, we asked whether sorting signals that direct specific proteins to either apical or basolateral surfaces in epithelial cells are recognized by the secretory pathway in embryonic cells. To address this question we examined the localization of a basolateral marker, VSV G, the coat glycoprotein of vesicular stomatitis virus. VSV G has been shown to insert specifically into the basolateral surfaces of MDCK cells, a differentiated kidney epithelial cell line (Rodriguez-Boulan and Pendergast, 1980; Gottlieb et al., 1986; reviewed in Simons and Fuller, 1985).

We injected transcripts encoding VSV G into one blastomere of an 8- or 16-cell embryo to determine if embryonic cells sort VSV G basolaterally. At the late blastula stage, the embryos were fixed, cryosectioned, and immunostained using a fluoresceinated secondary antibody. The pattern of staining (Fig. 4) indicates that VSV G is specifically inserted into the basolateral surfaces of cells and does not appear on the external, apical surface. In contrast to embryonically synthesized prolactin, VSV G is apparently inserted only at the basolateral surfaces of embryos. Hence the embryonic cells appear to recognize the same basolateral sorting signal that directs VSV G to the basolateral membranes in MDCK cells.

Do embryonic cells also sort proteins apically? One well-characterized apical sorting signal is the glycosylphosphatidylinositol (GPI) modification of the COOH terminus. Proteins with GPI lipid tails are exclusively sorted to the apical surface of MDCK cells (Lisanti et al., 1988, 1989; Brown et al., 1989; reviewed in Ferguson and Williams, 1988). We chose the fusion protein GThy-1 which is sorted specifically to the apical surface of MDCK cells as an apical marker

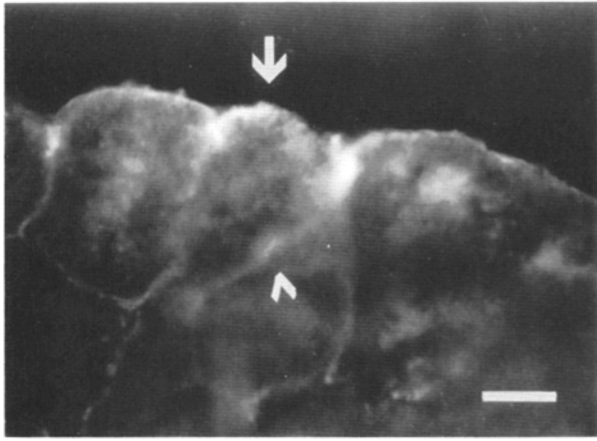


Figure 5. Nonpolarized expression of GThy-1 in the epithelial-like cells of the blastula. Section of a fluorescent immunostained embryo that had GThy-1 RNA injected into one animal hemisphere blastomere at the 8 cell stage. At mid-blastula (stage 8), embryos were fixed, sectioned, and immunostained as described in Materials and Methods. GThy-1 was found in both the basolateral (interior) and apical (exterior) membranes. Apical membrane is marked by the arrow, basolateral membrane is marked by the arrowhead. Controls (embryos not injected with GThy-1 RNA) did not show any immunofluorescent staining. Bar, 30 μm .

(Brown et al., 1989). GThy-1 consists of the extracellular domain of VSV G fused with the COOH terminus of Thy 1 which contains a GPI attachment site. Embryos that were injected with GThy-1 transcripts, fixed and sectioned as described above, were stained with the same antibodies used to stain VSV G. In contrast to GThy-1 localization in MDCK cells, we found that GThy-1 appears on both the apical and basolateral surfaces of the embryonic epithelial cells (Fig. 5). This result indicates that vesicles containing GThy-1 insert into the apical membrane derived from the oocyte. However, GThy-1 appears in both basolateral and apical membranes, possibly as a consequence of the greater volume of membrane traffic to the cleavage membranes (see Discussion).

Insertion of VSV G into the Cleavage Membrane after Fertilization

Although we have shown above that VSV G is inserted specifically into the basolateral surfaces of the blastula stage embryos, this does not resolve when the embryonic cells first distinguish between apical and basolateral surfaces. To address this we injected VSV G transcripts into oocytes after meiotic maturation, incubated for several hours, and then fertilized *in vitro* as described previously for prolactin. Since the RNA was injected after the onset of meiotic maturation, no VSV G was inserted into the plasma membrane of the oocyte. After two cleavage divisions the embryos were fixed, sectioned, and immunostained. The results of this experiment reveal that VSV G is preferentially inserted into the cleavage furrow membrane and is excluded from the outer oocyte-derived membrane (Fig. 6). We cannot determine how much of the VSV G protein we detected by immunostaining was synthesized before or after fertilization, but since the distribution is polarized both oocyte and newly synthesized VSV G appear to be targeted to the cleavage furrow

membrane. Examination of a newly forming cleavage furrow reveals intense immunofluorescence at the leading edge of the cleavage furrow, suggesting that this region may be a primary site for vesicle aggregation and fusion.

The localization of VSV G in the cleavage membrane could be a consequence of the high demand for new membrane during cleavage. Does the insertion of VSV G into cleavage furrow membranes require active cell division? It has previously been shown that secretion is inhibited in embryos arrested in M-phase by the expression of cytosstatic factor (Kanki and Newport, 1991). To induce cleavage arrest without inhibiting the cell cycle oscillator, we incubated eight-cell stage embryos in 10 $\mu\text{g/ml}$ nocodazole which blocked further cell division. In embryonic cells nocodazole blocks nuclear and cytoplasmic division but does not inhibit the cell cycle oscillator since MPF activity continues to cycle with normal periodicity (Gerhart et al., 1984; Kimelman et al., 1987). It has been proposed that nocodazole blocks cleavage by depolymerizing the astral microtubules which interact with the actin cortex to initiate furrow formation (Rappaport, 1986). Transcripts encoding VSV G were injected into one or two blastomeres and the embryos were immediately immersed in media containing nocodazole. Hence, we only followed the behavior of newly synthesized membrane. Since the embryos stopped cleaving in nocodazole we concluded that the drug treatment was effective. After incubating the embryos for 3 h they were fixed and processed as described above. As evident in Fig. 7, insertion of newly synthesized VSV G into the plasma membrane was not blocked by nocodazole-induced inhibition of cleavage. Nocodazole treatment also did not affect the polarity of insertion of VSV G. Newly synthesized VSV G only appeared in the preexisting cleavage membranes destined to become the basolateral surfaces of the embryonic epithelium. Thus, like VSV G in polarized MDCK cells (van Zeijl and Matlin, 1990) newly synthesized VSV G in embryos is sorted basolaterally in the absence of cell division.

Surface Expression of VSV G and GThy-1 in Oocytes

Since the oocyte membrane will become the future apical membrane of the embryo, it is possible that the apical character of this membrane is present as early as oogenesis. To determine if the oocyte plasma membrane functions as an apical membrane, transcripts for either VSV G or GThy-1, our basolateral and apical markers, respectively, were injected into the immature stage 6 oocytes. After a 24-h incubation the oocytes were fixed, sectioned, and stained as described in Materials and Methods. In Fig. 8 (A and B) it is evident that both VSV G and GThy-1 are inserted into the oocyte membrane. Also, VSV G (Fig. 8, C and D) and GThy-1 are found in the plasma membrane of both the animal or vegetal hemispheres. Hence, surface expression of these proteins is constitutive in oocytes and membrane traffic is not polarized with respect to the insertion of apically or basolaterally targeted proteins.

Discussion

Biogenesis of Cell Polarity at First Cleavage

In the frog embryo, the segregation of internal and external surfaces apparently begins at first cleavage. Tight junction-

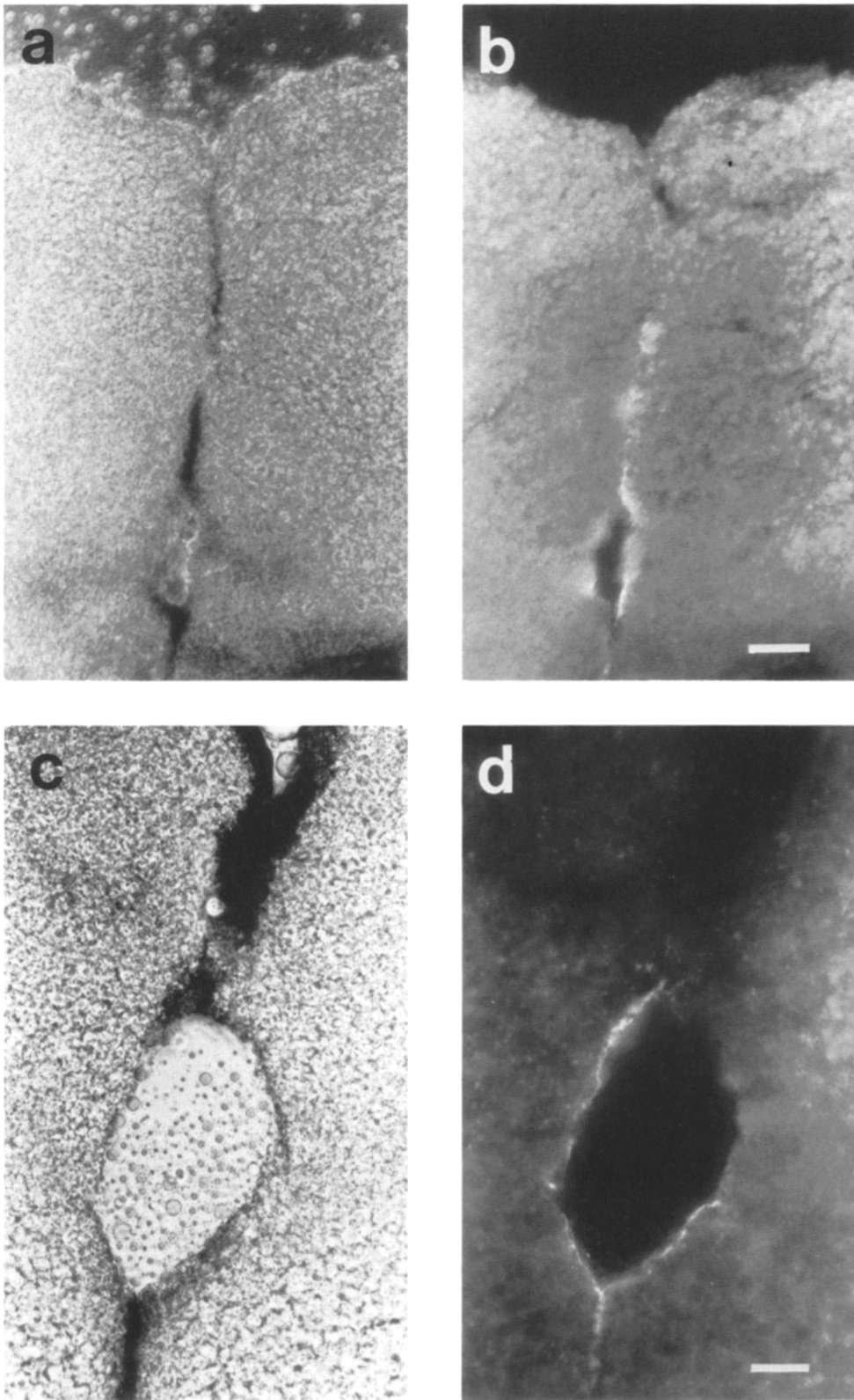


Figure 6. Expression of VSV G in the cleavage furrow of a 2-4 cell embryo. Shown are phase (A and C) and the corresponding fluorescent (B and D) micrographs of immunostained sections of embryos in the midst of the second cleavage division. VSV G RNA was injected into mature oocytes that were then fertilized in vitro as described in Materials and Methods. At the beginning of the second cleavage, embryos were fixed, sectioned, and immunostained as described previously. The staining is strongest in the leading edge of the cleavage furrow. Bars: (A and B) 45 μm ; (C and D) 30 μm .

like complexes have been identified in electron micrographs and there is little mixing of the new cleavage membrane with the old egg membrane (Bluemink, 1971; Sanders and Zalik, 1972; Byers and Armstrong, 1986). The electrical properties of the cleavage and egg membranes also diverge in the

early cleavage divisions (Kline et al., 1983). The early establishment of epithelial-like barriers in the frog embryo may be an adaptation to external development—the embryo needs to defend itself against a low ionic strength environment (pond water) from the time it is laid and fertilized.

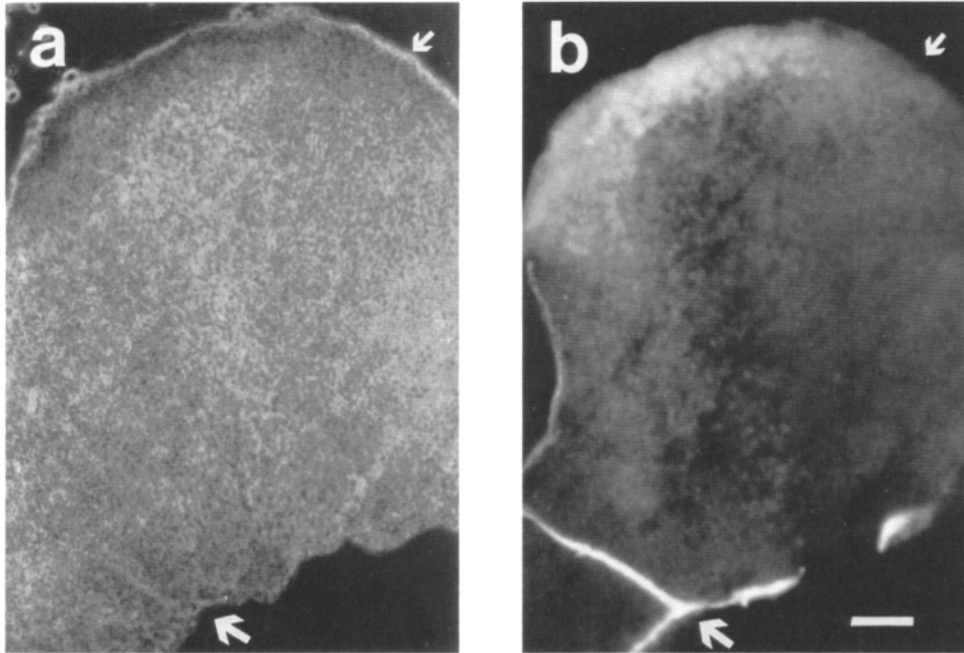


Figure 7. VSV G in the cleavage membranes of embryos treated with nocodazole. (A) Phase micrograph of a section of an embryo which had been injected with VSV G RNA and immersed in a 10 $\mu\text{g/ml}$ solution of nocodazole at the 8 cell stage which resulted in the inhibition of further cell division. 3 h after injection these cleavage-arrested embryos were fixed and immunostained as described previously. The membrane staining reflects the insertion of newly synthesized protein since VSV G RNA was injected just before the inhibition of cleavage. The small arrow points to the apical outer surface and the large arrow indicates the preexisting cleavage membrane (basolateral). (B) Corresponding fluorescent micrograph shows staining for VSV G in the cleavage membrane but not at the apical surface. Bar, 60 μm .

Recently a cadherin-like protein, CLP or U-cadherin, has been identified in *Xenopus* eggs that is expressed on the basolateral surfaces of the blastula stage embryo (Choi et al., 1990; Ginsberg et al., 1991; Angres et al., 1991). A mAb specific to U-cadherin inhibited cell adhesion in aggregation assays of early embryonic blastomeres indicating that this cadherin is involved in promoting cell-cell contacts in early embryos (Angres et al., 1991). In differentiated fibroblasts, the expression of another cadherin, uvomorulin, is sufficient to induce the polarized distribution of Na^+K^+ ATPase and the reorganization of the membrane cytoskeleton (McNeill et al., 1990). Since U-cadherin in *Xenopus* embryos is expressed exclusively in cleavage membranes starting with the first cleavage division it may be one of the molecules important for implementing the epithelial-like polarity of blastomeres.

The generation of cell polarity at first cleavage requires a mechanism that differs from that of differentiated cells because in the unicellular egg there are no cell-cell or cell-substratum contacts to define different membrane domains. Instead, only the insertion of new membrane at the site of the cleavage furrow distinguishes the second membrane domain. A plausible mechanism for establishing cell polarity in the early embryo could be the insertion of "stored" oocyte vesicles exclusively into the cleavage membranes. In this scenario, a subset of proteins synthesized during oogenesis would be segregated into secretory vesicles that are "stored" because they can only fuse to form new basolateral membrane at the cleavage furrows and cannot fuse with the original oocyte membrane. Included in these "stored" vesicles would be proteins like the cadherins that form cell-cell contacts and promote the segregation of membrane domains. Hence "stored" oocyte vesicles would not only be a source of membrane for the cleavage divisions but also would con-

tain proteins that specify the basolateral domain of the blastomeres.

In support of this model, we found that a secretory protein (prolactin) synthesized before fertilization was released specifically into the cleavage membranes and not into the original egg membrane. "Stored" oocyte vesicles may be specified to insert only into basolaterally oriented cleavage furrow membranes. The sorting mechanism in oocytes for these "stored" vesicles may differ significantly from sorting in embryos. Embryonically synthesized prolactin is packaged in vesicles that can insert at either the cleavage furrow membrane or the apical surface of the embryo whereas in the oocyte prolactin is packaged into vesicles that after meiotic maturation will insert only into the embryonic cleavage membrane. Hence, "stored" vesicles in the oocyte behave like basolateral vesicles in the embryo. There appear to be three classes of vesicles made during oogenesis: (1) cortical granules, a regulated-type vesicle released across the surface of the egg by a calcium stimulus at fertilization; (2) constitutive vesicles, released throughout oogenesis; and (3) "stored" vesicles that are only competent for fusion with the basolateral cleavage membranes formed after fertilization.

Maintenance of Cell Polarity

If cleavage establishes the polarity of the membranes by producing new basolateral surfaces, how is this polarity maintained in the superficial cells that comprise the epithelial-like layer of the blastula and gastrula? Polarized traffic of secretory vesicles into specific membrane domains has been proposed as a mechanism for maintaining polarity in epithelial cells (Matlin and Simons, 1984; Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990).

GThy-1, which is targeted to the apical surface of MDCK

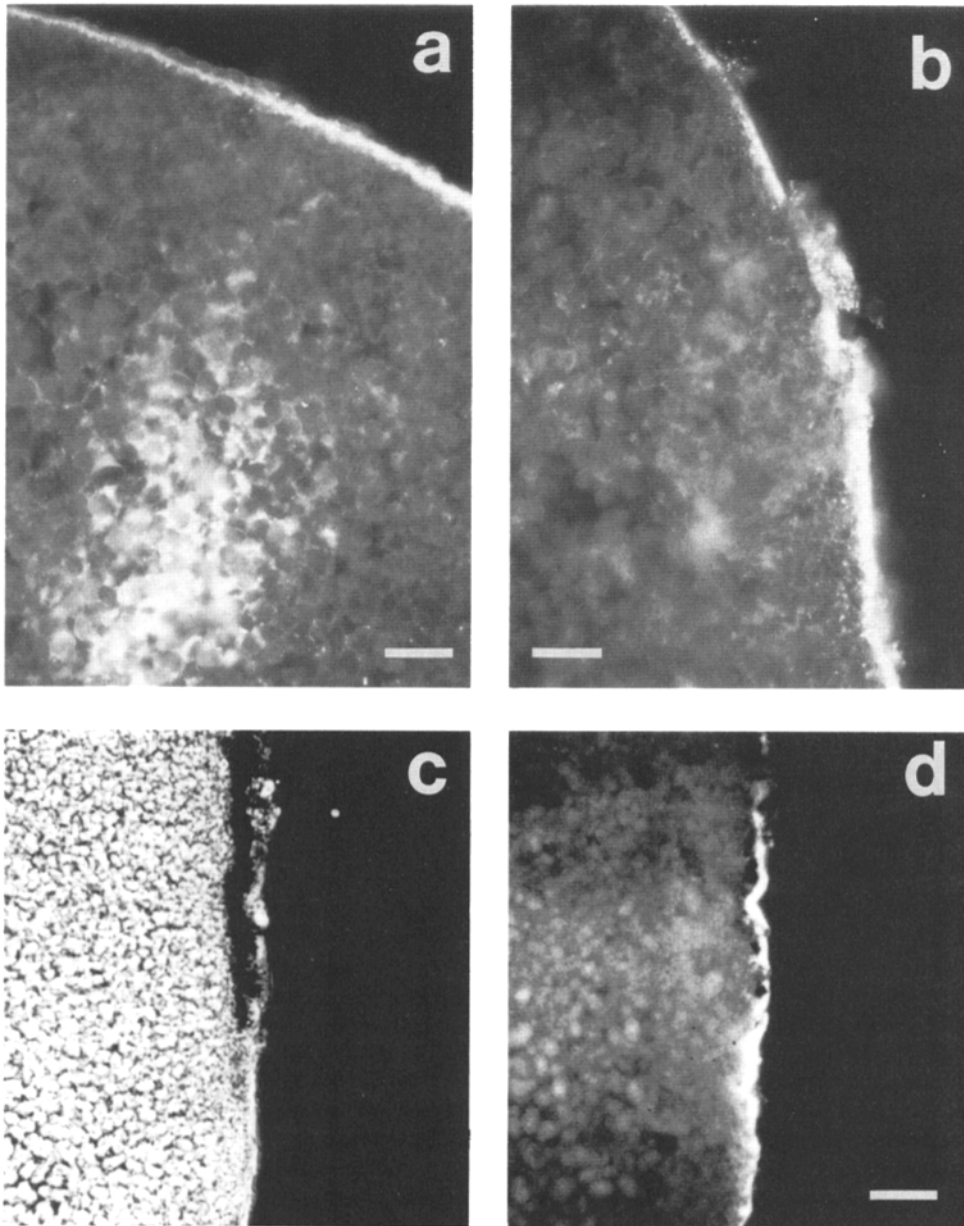


Figure 8. VSV G and GThy-1 expressed on the surface of st. 6 oocytes. *A* and *B* are immunofluorescent micrographs of sections from oocytes injected with either VSV G RNA (*A*) or GThy-1 RNA (*B*). 24 h after the injection of RNA the oocytes were fixed, sectioned, and immunostained as described in Materials and Methods. The staining indicates that VSV G and GThy-1 are expressed both internally and at the cell surface. *C* and *D* illustrate the expression of VSV G in both animal and vegetal hemisphere membranes. *C* is a phase-contrast micrograph of a section through an oocyte that spans the interface between the animal and vegetal halves. The animal membrane is pigmented (*upper half*). *D* is the corresponding fluorescent micrograph with staining for VSV G apparent in both the animal (pigmented) and vegetal (unpigmented) oocyte membrane. Similar results were obtained for GThy-1 (not shown). Bars: 30 μ m.

cells (Brown et al. 1989), does not show a polarized distribution in the superficial epithelial-like cells of the blastula. GThy-1 appears on both the external apical surfaces and the internal basolateral faces of these embryonic cells. One explanation for this discrepancy relative to MDCK cells is that the demand for new membrane during the rapid cleavage divisions overwhelms the sorting of GThy-1 to the apical surface. For example, suppose that membrane traffic to the cleavage furrow is 100-fold greater than to the egg surface. In this case, even if the apical sorting of GThy-1 is 99% efficient (1% of GThy-1 leaks into basolateral vesicles), as much GThy-1 will accumulate in the basolateral domain as in the apical domain. It would be interesting to determine if GThy-1 becomes apically restricted after the period of rapid cleavage.

In contrast, our experiments with VSV G (a basolateral protein in the MDCK epithelial cell line) indicate that this protein accumulates exclusively in basolateral membranes.

In blastula stage embryos the VSV G protein appears only in membranes that are contiguous with the blastocoel. These surfaces are analogous to the basolateral surfaces of epithelial cells in that they polarly secrete extracellular matrix proteins, contain Na^+K^+ ATPase, and are sealed from the external surface by tight junctions (Kalt, 1971; Slack and Warner, 1973; Morrill et al., 1975; Peracchia, 1984; Keller and Winklbauer, 1990).

Although cleavage may dominate membrane traffic in the early embryo it is not the only mechanism directing vesicles into the basolaterally oriented membranes. In embryos that were incubated in nocodazole and then injected with VSV G RNA we found that VSV G protein accumulated exclusively in the preexisting cleavage membranes. This result demonstrates that even in the absence of cleavage, the blastomeres are capable of processing membrane proteins, transporting them to the cell surface, and maintaining the same polarity in membrane traffic. Hence basolateral sorting does not re-

quire cell division or intact microtubules. In these aspects of basolateral sorting of newly synthesized proteins, embryonic cells are similar to differentiated polarized epithelial cell lines such as MDCK and Caco 2 (van Zeijl and Matlin, 1990; Gilbert et al., 1991).

Evidence that the internal surfaces of the blastomeres have a different composition than the outer surface was first given by Holtfreter (1943, 1944, 1948). He recognized that the properties of these two surfaces, especially differences in adhesiveness and permeability, have important roles in morphogenesis both for the formation of the blastocoel and the invagination of cells during gastrulation. In these studies, Holtfreter also observed that these differences are present from the earliest cleavage divisions (Holtfreter, 1943). Using foreign proteins encoded by injected RNA transcripts we have been able to trace the processes by which these differences in membrane composition are established and can now explore the mechanisms which regulate the polarity of cells in the earliest epithelial layer of the embryo.

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