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Location of Golgi membranes with reference to dividing nuclei in syncytial *Drosophila* embryos

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ABSTRACT **The role of cytoskeletal elements in the cellularization of syncytial *Drosophila* embryos is becoming evident; however, the distribution and role of organelles such as the Golgi complex, essential for membrane biogenesis, remain unknown. We have cloned a Golgi-membrane-associated polypeptide, β-COP, from *Drosophila*. Immunocytochemical studies of syncytial *Drosophila* embryos with anti-*Drosophila* β-COP antibody reveal that Golgi membranes are spatially segregated from the rapidly dividing nuclei. In early embryos, the Golgi membranes are located in the embryonic cortex and nuclei are confined to the core. This distribution of Golgi membranes may serve in preparation of the embryonic cortex for the accommodation of nuclei upon their eventual migration to the cortex and in biogenesis of the excessive plasma membrane needed for cellularization of syncytial embryos.**

Embryogenesis in *Drosophila melanogaster* begins with a single zygotic nucleus. This nucleus undergoes eight nearly synchronous mitotic divisions in the embryonic core. Subsequently, a few nuclei move to the posterior pole and, after two more rounds of mitosis, form pole cells. The majority of somatic nuclei migrate to the embryonic surface in cycle 10, undergo four more mitotic cycles, and form >5000 nuclei. The average time required for nuclear division is only 8–12 min for cycles 1–12 (1). During the interphase of cycle 14, membranes form between the nuclei, thus converting a syncytium into a cellularized blastoderm (1).

In mammalian cells, Golgi membranes are usually maintained as a single-copy complex, juxtaposed to the nucleus in the pericentriolar region (2). During mitosis, the Golgi membranes break down into small vesicles that are dispersed throughout the cytoplasm (3, 4). After cytokinesis, the Golgi membranes reassemble from these small vesicular structures and are once again found as a complex juxtaposed to the nucleus in each daughter cell (5, 6). Mitotic vesiculation and reassembly of Golgi membranes is thought to ensure partitioning of the Golgi complex into each daughter cell (7). Hence, in mammalian cells, duplication of Golgi membranes is linked temporally and spatially to the duplication of the nuclei.

The questions we therefore want to address are as follows: How many copies of the Golgi membranes are present at the onset of embryogenesis in *Drosophila?* Do these membranes increase in number with each nuclear duplication event, and like the embryonic nuclei, do Golgi membranes migrate from the core to the cortex in the advancing stages of embryogenesis? To address these questions, we cloned a Golgi-associated protein β-COP (8, 9) from *Drosophila*. *Drosophila* β-COP (dro-β-COP) is 65% identical to its rat homolog (10). Antibody generated in rabbits against bacterially expressed dro-β-COP recognizes a single polypeptide of 108 kDa in extracts from both *Drosophila* embryos and *Drosophila* tissue culture cells. We have used this antibody, by immunofluorescence, to monitor the spatial distribution of Golgi membranes with reference to the rapidly dividing nuclei in early stages of embryogenesis.

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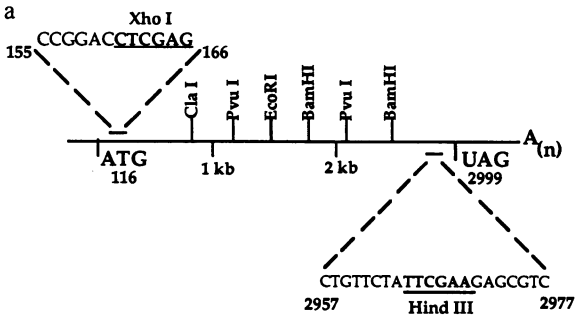
MATERIALS AND METHODS

Isolation and Characterization of β-COP Clones from *D. melanogaster*. Recombinant DNA manipulations were performed using standard procedures (11). Restriction enzymes and other molecular biology reagents were purchased from Boehringer Mannheim, unless otherwise stated.

An adult *Drosophila* genomic library in EMBL3 (a kind gift from M. Levine, University of California at San Diego) was screened with a 3-kb *Sal* I–*Sac* I fragment from the rat β-COP cDNA (generously provided by Thomas Kreis, University of Geneva, Geneva, Switzerland). The hybridization was performed at 37°C in 6× standard saline citrate (SSC)/0.1% SDS/2× Denhardt's solution/50 mM sodium phosphate, pH 6.8, containing 40% (vol/vol) formamide, followed by washing in 2× SSC/0.1% SDS for 30 min at room temperature with five buffer changes and then for three 20-min periods at 55°C. The 3-kb *Sal* I–*Sac* I probe was labeled with ^{32}P by random priming (11). Two clones hybridizing strongly with the probe were selected for further analysis. One of these clones (β-COP 5) was further examined by restriction mapping and Southern blot analysis. A 4.5-kb *Xho* I restriction fragment was subcloned into pBluescript SK. An internal 1.6-kb *Pst* I fragment was partially sequenced. The sequence showed a very high level of homology with the C-terminal region of the rodent β-COP cDNA and was thus used as a probe in a Northern blot analysis of embryonic RNA and in subsequent cloning procedures.

Northern blot analysis demonstrated the presence of an abundant mRNA in 4- to 8-h *Drosophila* embryos. Two hundred thousand colonies of a cDNA library from 4- to 6-h embryos in plasmid pNB40 (a kind gift from N. Brown and F. Kafatos, Harvard University; refs. 12 and 13) were screened with the 1.6-kb *Pst* I fragment. A 3.2-kb cDNA insert from positive clone pNB405a was sequenced and found to have 65% homology to rat β-COP, thus confirming that it encoded the *Drosophila* counterpart. The 3.2-kb cDNA insert included the full-length coding sequence and was, therefore, subcloned into pBluescript KS (Stratagene) for sequencing. This construct is referred to as pB5a (Fig. 1*a*). Double-stranded DNA sequencing was performed using Sequenase sequencing kit (United States Biochemical) by a combination of subcloned restriction fragments in pBluescript and ExoIII nuclease deletions of pB5a. Synthetic oligonucleotides were used as sequencing primers to obtain overlapping sequences.

Preparation of Fusion Protein and Antiserum. Two oligonucleotide primers were defined to amplify by PCR the segment of pB5a insert lying between them (Fig. 1*a*). The numbers (positions 155–166 and positions 2957–2977) identify the position of each primer relative to the sequence presented in Fig. 1*b*. Mismatches were introduced to create two appropriate restriction sites, *Xho* I (upstream primer) and *Hin*dIII (downstream primer), that did not cut within this insert. DNA amplification reaction mixture (50 μl) consisted



b

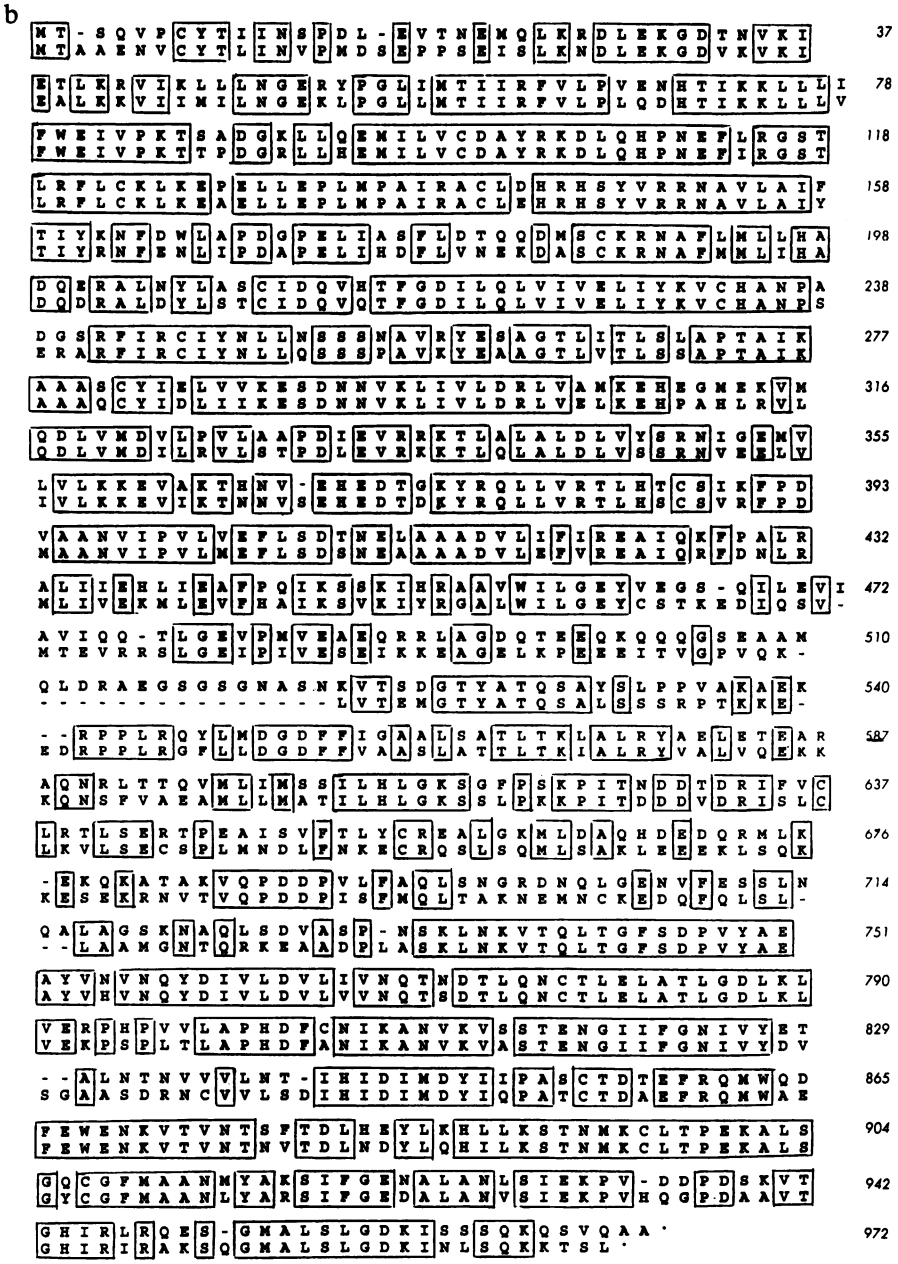


FIG. 1. (*a*) Schematic representation of the pB5a-β-COP cDNA clone encoding the full-length sequence for the *Drosophila* β-COP. The position and the sequence of upstream and downstream oligonucleotides used to insert cDNA into the pGEX-KGSTR expression vector for the production of protein in *E. coli* are shown. (*b*) Nucleotide and deduced amino acid sequence of dro-β-COP cDNA. In the longest open reading frame, amino acids coordinates were measured from the first in-frame methionine predicted to be the translational start codon (see text). Boxed residues are identical residues between *Drosophila* (this study) and rat β-COP (10).

of 1 μg of the pB5a *Not* I–*Hin*dIII insert as template, 50 pmol of each primer, buffer, all four dNTPs, and *Taq* polymerase as recommended by Perkin–Elmer/Cetus, and 30 thermal cycles were run at 60°C. The amplified DNA was digested with *Xho* I and *Hin*dIII and subcloned in *Xho* I/*Hin*dIII cut expression vector pGEX-kG STR. Recombinant protein was expressed in *Escherichia coli* HB101. The production of fusion protein was induced by adding isopropyl β-D-thiogalactoside to 1 mM. After 3 h of growth in the presence of isopropyl β-D-thiogalactoside, bacteria were harvested, resuspended in lysis buffer [25 mM Hepes, pH 7.5/20 mM KCl/2.5 mM EDTA/1% Triton X-100/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/pepstatin (1 μg/ml)/leupeptin (1 μg/ml)], and lysed by three freeze–thaw cycles. Bacterial lysates were clarified by incubation with 0.1 vol of 5 M NaCl for 15 min on ice. After centrifugation, the supernatant was incubated with glutathione-agarose beads (Sigma). The fusion protein was eluted by washing beads with

10 mM reduced glutathione. The eluate was analyzed on a SDS/7.5% polyacrylamide gel (14) and the appropriate band was excised from polyacrylamide gels to prepare antiserum in rabbits (15). The antiserum was further purified by immunoaffinity adsorption. For this, 200 μg of the fusion protein preparation was electrophoresed on a SDS/7.5% polyacrylamide gel, transferred to nitrocellulose, stained with Ponceau S. The nitrocellulose strip containing the fusion protein was excised and first incubated for 1 h in blocking buffer [PBS (1.8 mM NaH_2PO_4/8.4 mM Na_2HPO_4/150 mM KCl) containing 2.5% (vol/vol) fetal bovine serum and 0.1% Tween 20] followed by incubation with the crude antisera. The nitrocellulose strip was then washed for five 3-min periods in PBS and the antibody was eluted by incubation in 0.2 M glycine hydrochloride (pH 2.5). The antibody was neutralized by adding 10× PBS (pH 7.5), dialyzed extensively against PBS, and then stored in small aliquots at −80°C.

SDS/PAGE and Immunoblot Analysis. Whole-cell lysates were made from S2 *Drosophila* cells by incubating cell pellets in PBS (pH 7.5) containing 1% Triton-X 100 for 15 min on ice. The lysates were centrifuged at 3000 rpm in an Eppendorf microcentrifuge for 10 min to remove nuclei and the supernatant (S2 cell extract) was mixed with an equal volume of 2× SDS/PAGE sample buffer (14). The samples were boiled for 2 min and then analyzed by SDS/PAGE. Post nuclear supernatants (PNSs) from *Drosophila* embryos were made as follows. *Drosophila* embryos were collected, washed, dechorionated, and devitellinized as described below. Embryos were then homogenized using a Yamato Science (Tokyo) LSC homogenizer (16). The embryonic extracts were centrifuged for 10 min at 3000 rpm to remove nuclei and the supernatant (PNS) was then analyzed by SDS/PAGE.

S2 cell extracts (25–50 μg) and *Drosophila* PNS (25–50 μg) were subjected to SDS/PAGE, and the proteins were then transferred to nitrocellulose filters and examined by Western blot analysis. The nitrocellulose was incubated with blocking buffer [TBS (25 mM Tris·HCl/140 mM NaCl/3 mM KCl, pH 7.4/2.5% fetal bovine serum/0.1% Tween 20] for 1 h at room temperature, followed by sequential incubation with anti-dro-β-COP antibody (1:200 dilution in blocking buffer), three 5-min washes in blocking buffer, goat anti-rabbit antibody conjugated to alkaline phosphatase (Bio-Rad, 1:3000 dilution in blocking buffer) for 1 h at room temperature, three 5-min washes in blocking buffer, and then three washes in 0.15 M Tris (pH 9.6). The blot was developed by the alkaline phosphatase developing system as described by the suppliers (Bio-Rad).

Immunofluorescence on *Drosophila* Tissue Culture Cells and Embryos. *Drosophila* S2 cells were attached to poly(L-lysine) (1 μg/ml)-coated glass coverslips and then fixed with methanol at −20°C for 10 min. Cells were then incubated in blocking buffer for 1 h at room temperature. The cells were incubated with anti-dro-β-COP antibody (diluted 1:200 in blocking buffer) for 1 h at room temperature, followed by three 5-min washes with PBS, incubation with goat anti-rabbit IgG conjugated to rhodamine (Jackson ImmunoResearch) for 1 h at room temperature, and then three 5-min washes with PBS. The cells were then incubated with the bisbenzimide fluorochrome H33342 (Calbiochem) for 3 min. The cells were then washed in PBS, mounted on glass slides with phenyldiamine (10 μg/ml) in 90% glycerol, and visualized with a Nikon microscope through a ×100 oil immersion lens.

Embryos were dechorionated, fixed, devitellinized, stored in methanol, and rehydrated by standard procedures (17). They were then treated with at least two changes of 10% (wt/vol) bovine serum albumin in PM buffer (10 mM potassium phosphate, pH 6.8/15 mM NaCl/45 mM KCl/2 mM $MgCl_2$) for a total of 24 h at 4°C. Embryos were then washed twice with PMN buffer (PM buffer containing 0.5 M NaCl) and incubated for 2 h with affinity-purified anti-dro-β-COP antibody (1:200 dilution in PMN) at room temperature. After washing over a 2- to 3-h period at room temperature with at least five buffer changes, the embryos were incubated with a mixture of fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:500 dilution in PMN) and propidium iodide (Sigma; 1:100 dilution) for 2 h at room temperature or overnight at 4°C, then washed with five changes of PMN for 2 h at room temperature and washed for three 5-min periods in PBS, and mounted on glass slides with 2% (wt/vol) *n*-propyl gallate in 70% glycerol. β-COP-positive membranes and nuclei were observed in the same optical planes by confocal microscopy (Bio-Rad MRC 600) equipped with a krypton/argon laser attached to a Zeiss Axiovert 35M microscope. Digital images were converted to the PICT file format and combined into RGB composite images using Adobe photoshop (Adobe Systems, Mountain View, CA) and printed with a Tektronix phaser IISD dye sublimation printer. Developmental stages were determined by counting the number of propidium iodide-stained nuclei and their location within the embryo (1).

Ovaries dissected from *D. melanogaster* (18) were further dissociated in PMN buffer, rinsed three times in the same buffer, and fixed for 10 min at room temperature in PBS containing 4% (wt/vol) paraformaldehyde. They were then washed three times with PBS, and the follicle cells, chorion, and vitelline membranes were removed from the fixed egg chambers (16). The egg chambers were rinsed three times with PMT buffer (PM buffer containing 0.05% Triton X-100), incubated with PBS containing 1% Triton X-100 for 2 h at room temperature, and then rinsed three times with PMT. They were then incubated with immunopurified anti-dro-β-COP antibody (10 μg/ml in PMT) for 2 h at room temperature. The egg chambers were then washed for 1 h with PMT with at least five buffer changes, followed by incubation with fluorescein-conjugated goat anti-rabbit antibody (1:100 dilution in PMT) for 2 h at room temperature. They were washed with PMT for 1 h with at least five changes, mounted, and visualized by confocal microscopy as described above.

RESULTS AND DISCUSSION

The Golgi membranes of *Drosophila* have not been well characterized, although their presence during oogenesis (19) and in fully cellularized embryos (20) has been reported before. In particular, a detailed description of their organization and distribution during embryogenesis is lacking. To address these questions, we cloned and generated an antibody against a Golgi-membrane-associated protein, β-COP, from *Drosophila* embryos.

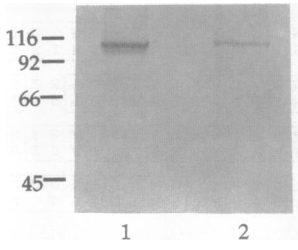


FIG. 2. Immunoblot analysis of dro-β-COP in *Drosophila* tissue culture cells (lane 1) and PNSs (lane 2) from embryos. S2 cell extracts (25–50 μg) and *Drosophila* PNS (25–50 μg) were analyzed by SDS/PAGE in 7.5% gels followed by Western blot analysis with affinity-purified anti-dro-β-COP antibody. The blot was developed by the alkaline phosphatase developing system as described by the suppliers (Bio-Rad). Standard molecular mass markers were as follows: 116 kDa, β-galactosidase; 92 kDa, phosphorylase B; 66 kDa, bovine serum albumin; 46 kDa, ovalbumin.

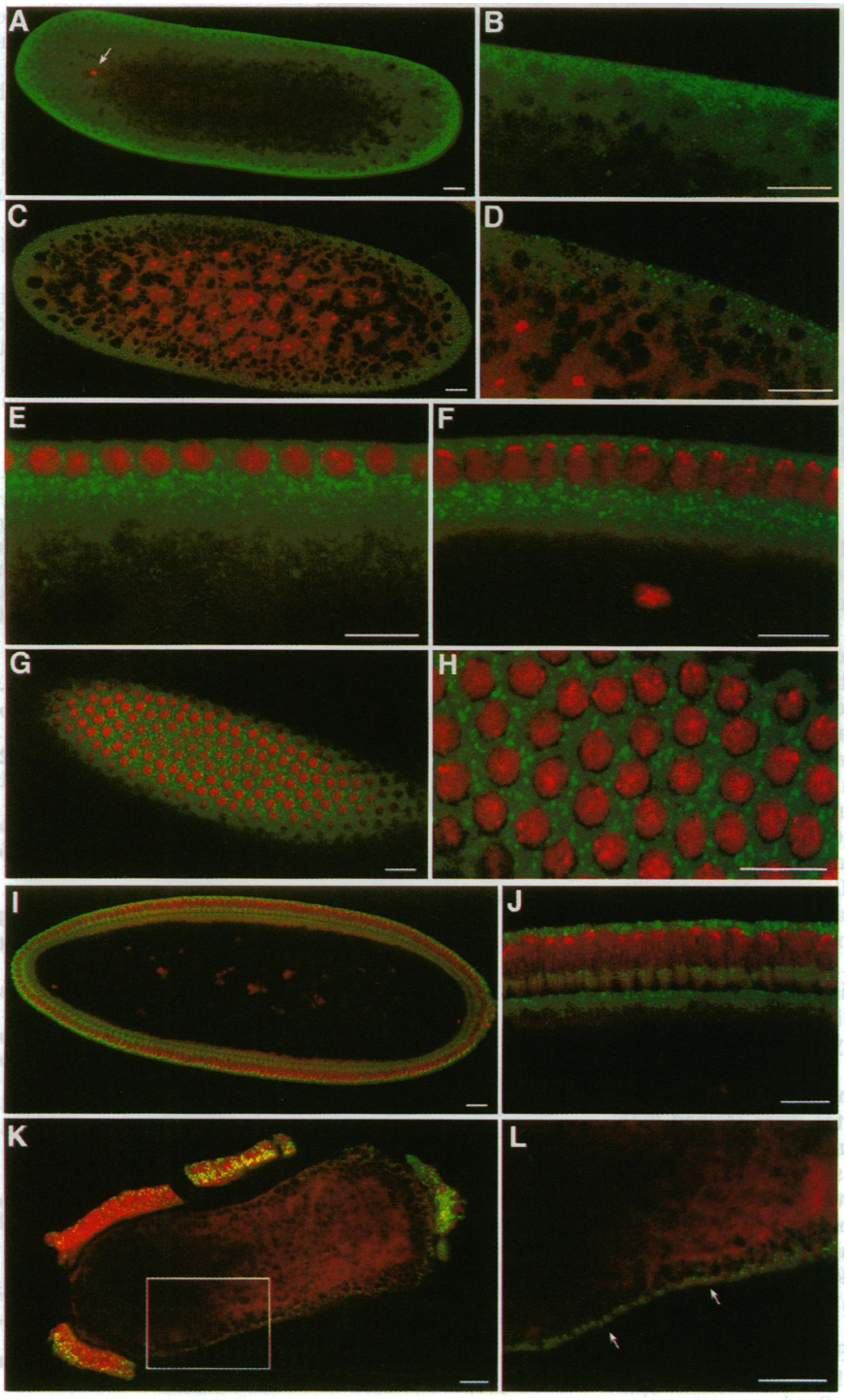


FIG. 3. Distribution of β-COP-containing Golgi membranes in early embryogenesis and oocytes. *D. melanogaster* (Canton-S) embryos (*A–J*) and a nearly mature oocyte (*K* and *L*) were double-stained with antibody to dro-β-COP (green) and a nucleic acid-specific dye, propidium iodide (red). *B*, *D*, *H*, *J*, and *L* are enlarged portions of *A*, *C*, *G*, *I*, and *K*, respectively. (Bars = 10 μm.)

Dro-β-COP Is 65% Identical to Its Rodent Counterpart. Several lines of evidence indicate that we have cloned the *Drosophila* homolog of β-COP. Northern blot analysis of poly(A)$^+$ mRNA from early *Drosophila* embryos indicates the presence of a single transcript of 3.5 kb (data not shown). The sequence of the isolated cDNA reveals an open reading frame of 2883 bp starting at the first ATG codon at position 116. A stop codon is present in the 5′ untranslated region 63 bp upstream of this first ATG. The nucleotide sequence surrounding this ATG is compatible with the consensus sequence for the translational initiation signal in *Drosophila* (21) and is shown in Fig. 1*b*. The cDNA isolated predicts a protein with an estimated molecular mass of 108 kDa (Fig. 1*b*), consistent with the protein recognized by antibody prepared against the cognate cDNA (Fig. 2). Potential protein sequence similarities between dro-β-COP and other known proteins were analyzed by searching the GenBank (LL.NAM) data library using the FAST88 algorithm. The dro-β-COP is 65.7% identical to the rodent counterpart (10) (Fig. 1*b*).

In situ hybridization of polytene chromosomes revealed that dro-β-COP gene maps on the X chromosome at position 17a, band 9-10 (data not shown). It is interesting to note that this region is saturated with early zygotic lethals (22). Isolation of mutants defective in β-COP should provide further insights into the functional significance of this polypeptide.

Organization of Golgi Membranes in *Drosophila* Embryos. Before the first zygotic nuclear division (1), the β-COP-positive membranes, seen as punctate structures, are confined in the embryonic cortex (Fig. 3 *A* and *B*). This cortical distribution of β-COP-positive membranes is maintained

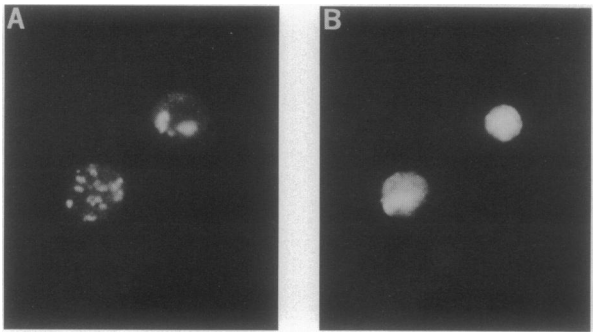


FIG. 4. Subcellular localization of dro-β-COP in *Drosophila* tissue culture cells. *Drosophila* S2 tissue culture cells were fixed with methanol at −20°C for 10 min. The cells were then incubated with anti-dro-β-COP antibody followed by goat anti-rabbit IgG conjugated to rhodamine (*A*). The nuclear DNA is labeled with the bisbenzimide fluorochrome H33342 (*B*).

through stage 6 whereas the majority of nuclei remain concentrated in the core (Fig. 3 *C* and *D*). These findings indicate that, in the early syncytial embryo, Golgi membranes are located in the cortex whereas the rapidly dividing nuclei are contained in the core.

In cycles 10 and 11, a majority of the nuclei migrate to the cortex and are found immediately below the embryonic surface (Fig. 3*E*). As their number increases by division, they become oblong, more closely packed, and separated from the embryonic surface by a cytoplasmic margin (Fig. 3*F*). The presence of Golgi membranes between hexagonally arranged nuclei is readily observed in tangential optical sections through the embryonic surface (Fig. 3 *G* and *H*). In stage 14, when the embryo is fully cellularized, an unstained plane that represents the basal borders of the cells separates the cellularized cortex from the rest of the embryo, also known as the vitelloplasm (Fig. 3 *I* and *J*). Golgi membranes are present in a thin vitelloplasmic layer below this plane and within the cells. The significance of vitelloplasmic Golgi membranes is unknown at this time. Within the cells, Golgi membranes appear to be more abundant in the cytoplasmic region between the nucleus and the embryonic surface (Fig. 3*J*).

We examined whether the distribution of Golgi membranes is maternally derived. For this, oocytes were stained with anti-dro-β-COP antibody. As shown in Fig. 3 *K* and *L*, in a near mature oocyte, Golgi membranes are restricted to the cortex, essentially as seen in the fertilized egg. Thus, the mechanism by which the Golgi membranes are confined to the embryonic cortex is established during the oocyte development and is, therefore, maternally derived. It is important to note that the immunostaining with anti-dro-β-COP antibody represents both the soluble and the Golgi-membrane-associated β-COP. In early embryos (Fig. 3 *A* and *B*), a large proportion of β-COP appears randomly diffused but, in the later stages of embryogenesis (Fig. 3 *C–J*), a larger proportion of β-COP is associated with the membranes. Whether this is due to an increase in the number of Golgi membranes or an increase in the affinity of β-COP for Golgi membranes in the latter stages of embryogenesis, to our knowledge, is currently not known.

Previous electron microscopic studies of early *Drosophila* embryos have shown that Golgi membranes are composed of clusters of tubulo-vesicular structures (19, 20). We have examined ultrathin sections of *Drosophila* embryos in pre- (cycle 6)- and mid (cycle 14)-cellularization stages by electron microscopy and found numerous such clusters in the embryonic cortex (data not shown). These clusters correlate well, in distribution and number, with the β-COP-positive punctate structures observed in optical sections. In *Drosophila* tissue culture cells (Fig. 4), the β-COP-containing Golgi membranes are evident as discrete structures distributed in the cytoplasm. Therefore, the cytoplasmic distribution of Golgi membranes in *Drosophila* tissue culture cells and cellularized embryos is similar to that reported for *Saccharomyces cerevisiae* (23, 24) and distinct from mammalian cells, which usually contain a single Golgi complex in the pericentriolar region (2, 25, 26).

Genetic manipulation of *Drosophila*, combined with the availability of Golgi-specific antibodies, should provide further opportunities to investigate the mechanisms regulating the intracellular location of Golgi membranes in *Drosophila* embryos and the role of these membranes in the origin of the extra plasma membrane needed for cellularization.

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