The Organization of Endoplasmic Reticulum Export Complexes

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Abstract. Export of cargo from the ER occurs through the formation of 60–70-nm COPII-coated vesicular carriers. We have applied serial-thin sectioning and stereology to quantitatively characterize the three-dimensional organization of ER export sites in vivo and in vitro. We find that ER buds in vivo are nonrandomly distributed, being concentrated in regional foci we refer to as export complexes. The basic organization of an export complex can be divided into an active COPII-containing budding zone on a single ER cisterna, which is adjacent to budding zones found on distantly connected ER cisternae. These budding foci surround and face a central cluster of morphologically independent vesicular-tubular elements that contain COPI coats involved in retrograde transport. Vesicles within these export complexes contain concentrated cargo molecules. The structure of vesicular-tubular clusters in export complexes is particularly striking in replicas generated using a quick-freeze, deep-etch approach to visualize for the first time their three-dimensional organization and cargo composition. We conclude that budding from the ER through recruitment of COPII is confined to highly specialized export complexes that topologically restrict anterograde transport to regional foci to facilitate efficient coupling to retrograde recycling by COPI.

Export of protein from the ER is the first step in the vectorial movement of cargo through compartments of the secretory pathway of eukaryotic cells. Pioneering studies by Palade (1975) established that the site of exit from the RER in pancreatic acinar cells is through transitional elements, a region of partly rough, partly smooth tubular ER juxtaposed to the cis face of the Golgi stack. Morphologically, ER-derived vesicles are 60–70 nm in diameter and contain an electron-dense coat when viewed using transmission electron microscopy (Ziegel and Dalton, 1962). This characteristic coat contains COPII components that are assembled in response to the activation of the Sar1 GTPase, a machinery now recognized to be evolutionarily conserved in yeast and mammalian cells (for review see Barlowe, 1995).

While the formation of ER to Golgi carrier vesicles in secretory tissues is largely confined to the transitional region facing the juxtanuclear Golgi apparatus (Palade, 1975), studies in other cell lines have shown that export from the ER can originate from multiple sites that appear randomly distributed throughout the cytoplasm and, in most instances, distant from the Golgi complex. The relationship of these peripheral sites to the transitional region found in secretory cells is unknown, although they are now recognized to consist of clusters of small vesicles and tubular elements (Saraste and Kuismäen, 1984; Schweizer et al., 1990; Saraste and Svensson, 1991; Lotti et al., 1992) referred to as vesicular tubular clusters (VTCs) (Balch et al., 1994). While VTCs are readily detectable at 37°C in vivo (Saraste and Kuismäen, 1984; Saraste and Svensson, 1991) and at 32°C in vitro (Plutner et al., 1992; Pind et al., 1994a), visualization of these structures can be markedly enhanced by incubation of cells at reduced temperature (15°-16°C) (Saraste and Kuismanen, 1984), presumably due to a rate-limiting step in membrane flow through these intermediates. Elements of VTCs lack luminal continuity with the ER (Saraste and Svensson, 1991; Balch et al., 1994; Connolly et al., 1994), although tubular extensions of ER into these structures have been observed (Stinchcombe et al., 1995), particularly in cells infected with certain viruses (Tooze et al., 1984; Krijnse-Locker et al., 1994), reinforcing their close relationship to ER export.

VTCs are dynamic structures with varied morphology in different cell types. In the past, VTCs have been suggested to be the site of O-glycosylation (Tooze et al., 1988), acylation (Rizzolo et al., 1985), and generation of the mannose-6-phosphate signal for lysosomal protein targeting (Pelmham, 1988). Several endogenous proteins serve as useful markers for VTCs. These include the small GTPase Rab2 (Chavrier et al., 1990), the transmembrane protein p58 in rat cells or its human homologue p53 (Schweizer et al., 1988; Saraste and Svensson, 1991), which actively cycle between the ER and VTCs, and the COPI subunit β-COP (Oprins et al., 1993; Pepperkok et al., 1993; Pind et al., 1994).

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1. Abbreviations used in this paper: CGN, cis-Golgi network; GA, glutaraldehyde; NRK, normal rat kidney; RBL, rat basophilic leukemia; rt, room temperature; TEM, transmission EM; VSV-G, vesicular stomatitis virus glycoprotein; VTC, vesicular tubular cluster.
Materials and Methods

Epon Embedding and Serial Sectioning

Cells were fixed in 2.5% glutaraldehyde (GA) in PBS for 1 h at room temperature (rt), scraped in GA, and pelleted at 14,000 g for 10 min. The tight pellet was washed in veronal-acetate buffer (pH 6.0) and stained in 1% buffered OsO₄ for 1 h at rt. After washing in veronal-acetate buffer, pellets were stained in 10% uranyl acetate in methanol for 10 min at rt and Reynolds's lead citrate for 10 min. Budding structures on the ER and VTCs were followed in consecutive sections, and images were overlaid to reconstruct continuity between structures.

Transmission EM and Morphometry

Stereological Parameters

For estimation of the mean cell volume using the second method, NRK and RBL cells were grown on 35-mm tissue-culture dishes (Costar Corp., Cambridge, MA), fixed in the dish in 2.5% GA in PBS for 1 h at rt, and processed for TEM as described above. The cell pellet was cut with a diamond knife on a Reichert ultramicrotome 2E and transferred to a single 2 × 15 mm slot grid (Electron Microscopy Sciences) precoated with a Formvar/carbon film. Sections were counterstained with a saturated solution of uranyl acetate in methanol for 10 min at rt and Reynold's lead citrate for 20 min. The mean height of ~250 cells in a monolayer was measured by the point-counting method (Weibel, 1979). The mean cell volume (Vc.r.) was then determined according to formula:

\[ V_{c.r.} = \frac{S_{c.p.} \times H_c.}{d} \]

where \( S_{c.p.} \) is the mean surface of the cell projection (determined by the phase-contrast light microscopy), \( H_c. \) is the mean height of the cell (determined by TEM).

The mean cell volume was found according to formula (Weibel and Gomez, 1962):

\[ V_{c.r.} = 1.4 \left( S_{c.s.} \right)^{3/2} \]

where \( S_{c.s.} \) is the mean cell surface on thin-section. The surface to volume ratio was found according to formula:

\[ S_{v} = \frac{\Sigma d/\Sigma P \times d}{d} \]

where \( d \) is the number of intersections on a grid, \( P \) is the number of points on the grid, and \( d \) is the distance between the points (Weibel, 1979). Both procedures yielded nearly identical results for RBL cells. The values obtained from the second procedure are reported in Table 1.

The stereological parameters of nuclei, ER, and Golgi apparatus were determined using the same sections. Randomly taken cell contours were enlarged to a total magnification of 14,000 as described above. Using the point-counting method, we determined the nucleus volume to cell volume ratio and a nucleus surface to nucleus volume ratio as described (Weibel, 1979). Randomly chosen fields of cells containing ER or Golgi membranes were magnified to 31,000 to establish the ER(Golgi) volume to cell cytoplasm volume ratio by the point-counting method (Weibel, 1979).

Some of these images were photolongated to 120,000 to determine the ER(Golgi) surface to volume ratio. The ER(Golgi) surface was found by formula 3. Corrections of bias due to section thickness were done as described (Weibel and Paumgartner, 1978). For the ER volume, the correction factor was 0.51 and 0.65 for the ER surface. Assuming that two-thirds

The Journal of Cell Biology, Volume 135, 1996
of the Golgi complex was composed of cisternae and one-third of tubules, the correction factors for Golgi volume and surface were estimated to be 0.64 and 0.64, respectively.

**Estimation of Number and Density of Total ER-derived Buds.** To estimate the number ER-derived buds, we used two distinct criteria: (a) a bud was considered an elevation on the surface of the ER with a width of 60-80 nm and covered with a characteristic coat on the external leaflet of the membrane; and (b) buds had a direct connection with the ER membrane and were extruded from the membrane by at least 50% of their circumference. The number of ER buds per cell was determined as described (Lucocq et al., 1989). To obtain this value, we multiplied the total number of ER buds detected in thin-sections of ~300 cells by the mean volume of cells and divided that value by the total volume contributed by the ~300 cells. The latter value was found by multiplying the thickness of a section by the total surface area of the ~300 cell sections in which ER buds were counted. By using thin sections corresponding to the average diameter of an ER bud and the above two criteria, we were able to avoid double counting most of the ER buds present on consecutive thin sections. To establish this point independently, we performed a separate experiment in which the total number of ER buds was counted on several stacks of serial thin-sections. By comparing the number of ER buds determined by this reconstruction approach to the number determined by counting of individual sections (indirect method), we found that overestimation by the latter technique was <10-15%. Hence, both methods could be used interchangeably. We also found that the indirect method showed a high reproducibility. In spite of the high degree of ER bud enrichment in local zones, the comparison of estimates of the total number of buds per cell between groups of as few as 35 randomly taken cells with a total surface of 80-100 μm² showed a value of variability less than 10% from one group to another. In addition, in four different experiments, the value of the total number of buds per cell was found to be the same. Because of technical simplicity, we routinely used the indirect method to estimate the average number of buds per cell.

**Estimation of Number and Density of ER Membranes and ER-derived Buds in the Golgi Exclusion Zone.** The Golgi exclusion zone is defined as the Golgi-containing region found in the pericentrosomal region of RBL cells and includes directly adjacent bud-bearing cisternae of the ER. The surface area of the Golgi exclusion zone in each individual section within a stack of 11-20 consecutive sections (referred to as a disector) was determined as described (Sterio, 1984; Lucocq et al., 1989) and determined by the point-counting method (Weibel, 1979), and the mean surface area in each disector was calculated for 12 different cells (range from 30-90 μm²). To find the volume of the Golgi exclusion zone included in the disector, the mean surface area of the Golgi exclusion zone in each disector was multiplied by the thickness of the section and the number of individual sections (35 to 105 μm³ for 12 individual disectors). The total number of ER buds within each disector of a given cell was found by reconstruction from serial sections. The volumetric density of ER buds was calculated by dividing the number of ER buds within each disector by the volume of the disector. The total volume and surface of ER membranes in the Golgi exclusion zone was found by the point-counting method (Weibel, 1979). By dividing the total number of ER buds by the surface within the Golgi exclusion zone, we calculated the density of ER buds in this region.

**Estimation of Number and Density of ER Membranes and ER-derived Buds outside of the Golgi Exclusion Zone.** The density of ER buds outside of the Golgi exclusion zone was found according to the procedure described above for the Golgi exclusion zone.

**Estimation of Number and Density of ER Membranes and ER-derived Buds in Export Complexes.** The local density of buds on individual cisternae associated with export complexes was determined as follows: using a stack of sequential serial sections, we followed one continuous bud-bearing zone of ER membrane that contained at least four buds. The distances between the most distant ER buds in the stack were directly measured in both the plane of the section and the depth of the stack. These two distances were multiplied by one another to get a surface area of the plane of the ER bud-bearing region. The total number of ER buds in such a zone (obtained by reconstruction as described above for the Golgi exclusion zone) was then divided by the area of the bud-bearing zone to determine a local ER bud surface density.

**Exitation of Number of VTCs.** The number of VTCs per cell was determined using the disector method (Sterio, 1984) as described (Lucocq et al., 1989). Sections of 25 random cells were photographed at a calibrated magnification of 7,000 throughout 25-35 consecutive sections in which they were present. Each “end section” was designated the “look-up” section, and all clusters present in the other sections, but not in the look-up section, were counted (Q). Then the volume of disector (V_disector) was determined by multiplying the average surface of individual cells in the stack found by the point-counting method by the depth of the dissector, which is equal to the thickness of the section multiplied by the number of sections in the dissector. The total number of clusters in individual cells (N_disector) was determined by the formula,

\[
N_{\text{disector}} = V_{\text{cr}} \times \frac{1}{Q} \times \frac{1}{V_{\text{disector}}}.
\]

**Total Number of Vesicular Profiles in VTCs.** To determine the number of elements in VTCs, the value reported assumes that all elements found in consecutive sections are discontinuous with one another. This value is likely to be an overestimate, as some of the tubular structures within VTCs may extend across several sections. The number of elements in VTCs was determined by direct counting of individual profiles on each serial section. These were summed throughout all sections through a given VTC to obtain a total value.

**Probability Measurements.** To estimate the probability of a bud having proximity to a second bud in the cell, images of serial sections were photoenlarged to 40,000 to reconstruct a section of the cell in three dimensions. 300 randomly chosen ER buds were taken as the center of reference (referred to as reference buds), and the distance between each individual reference bud and other ER buds in the same and consecutive serial sections encompassing up to 1.3 μm distance above and below the reference bud was measured directly. A series of concentric shells with a volume equal to the volume of the most internal sphere having a 0.2-μm diameter (corresponding to a volume of 0.0042 μm³) was constructed around the reference bud. Subsequently, each measured bud was assigned to a shell with its distance from the reference bud being that of the corresponding outer diameter of each shell. The probability of finding a bud within a particular shell was determined by dividing the number of positive shells by the number of reference buds, and the value was reported as a percentage.

**SEM.** Statistical calculations were performed by determining the SEM for the pooled stereological data for each condition as described in the Results.

**Immunolabeling of Cryosections.** NRK cells grown on 35-mm tissue-culture dishes were infected with vesicular stomatitis virus (strain ts045), postinfected for 4 h at 39.5°C, and permeabilized as described (Plutner et al., 1992). After incubation at the permissive temperature (32°C) as described in the Results, the cells were fixed for 30 min with 3% paraformaldehyde and 0.1% GA in PBS (pH 7.4), washed for 10 min in PBS containing 0.05 M glycine, scraped, mixed with a preheated (40°C) 10% gelatin in PBS, and centrifuged at 15,000 g for 10 min. Cells embedded in gelatin were cooled on ice, and a solid pellet was cut into 1-mm-wide cubes. After overnight cryoprotection by infiltration with a mixture of 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.4) containing 20% polyvinyl pyrrolidone, the cubes were mounted on aluminum nails and frozen in liquid nitrogen. Ultrathin cryosections cut on a Reichert Ultracut E, equipped with a FC-4 cryotatech, were picked up with 2.0 M sucrose-1% BSA in PBS and collected on Formvar/carbon-coated nickel grids. Sections were then quenched in 0.1 M glycine in PBS, incubated for 30 min in 10% FBS-PBS at rt, and for 1-2 h with primary antibodies diluted in 10% FBS-PBS antibody. Excess primary antibody was removed by multiple rinses in 5% FBS-PBS, followed by transfer of the section to a drop of 10% FBS-PBS containing 6 or 10 nm gold–conjugated anti-rabbit antibodies. After a 2-h incubation at rt, the grids were washed in double-distilled water and stained in 2% neutral uranyl acetate (10 min), followed by embedding in 3.2% polyvinyl alcohol/0.2% methyl cellulose, containing 0.2% uranyl acetate. No labeling was observed in controls in which primary antibodies were omitted.

**Immunolabeling of VSV-G Using the Immunodiffusion Approach and Quantitation of VSV-G Concentration in VTCs**

NRK cells were infected with tko45 VSV as described above. After digitonin permeabilization (Plutner et al., 1992) and incubation in vitro as described in the Results, cells were fixed with 0.025% GA/3% paraformaldehyde for 30 min. Cells were washed three times with PBS, then washed with 0.05 M glycine in 10% FBS/PBS for 30 min, and incubated overnight with an anti-VSV-G cytoplasmic tail mAb (P5D4) (Kreis, 1986). Cells were washed twice with FBS/PBS, followed by incubation with rabbit anti-mouse antibodies for 2-2 h. Then the grid was washed with 1% PBS containing 6 or 10 nm gold–conjugated anti-rabbit antibodies. After a 2-h incubation at rt, the grids were washed in double-distilled water and stained in 2% neutral uranyl acetate (10 min), followed by embedding in 3.2% polyvinyl alcohol/0.2% methyl cellulose, containing 0.2% uranyl acetate. No labeling was observed in controls in which primary antibodies were omitted.

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scraped, pelleted, and processed for Epon embedding. For three-dimensional visualization using quick-freeze, deep etch replicas, immunolabeling was performed on glass coverslips.

For quantitation, the Epon-embedded cell pellet was cut as described above. Images (~10,000) were scanned into a computer, and membrane outlines of the ER and VTCs were measured using the program NIH Image, version 5.9. The linear density of gold particles corresponding to VSV-G was determined as described (Balch et al., 1994). For determination of the distribution of vesicles and VTCs, NRK cells grown on glass coverslips were permeabilized and incubated as described in the Results, fixed and labeled for VSV-G using the immunodiffusion protocol, and embedded in Epon on the glass coverslip as described above. After detachment from glass, thin layers of cells embedded in Epon were sandwiched against each other and reembedded in Epon. Vertical sections of cells were prepared and counterstained as described above. ER to Golgi intermediates were identified based on either the presence of gold particles corresponding to VSV-G or on their characteristic morphology as described in the Results.

**Preparation of Quick-Freeze, Deep-Etch Rotary-shadowed Replicas**

After incubation in vitro as indicated in the Results, semi-intact cells grown on 9 × 9 mm glass coverslips were fixed in 2.5% GA in PBS for 1 h, washed in PBS, and divided into small pieces (~3 mm²). Coverslips were rinsed exhaustively in double-distilled water, followed by a rinse with 10% methanol in water, and quick-frozen using a liquid nitrogen-cooled copper block gravity press (Hitek, Benicia, CA). The cells were fractured with a razor blade under liquid nitrogen, freeze dried in a vacuum evaporator (400; Balzers, Inc., Lichtenstein) and replicated with ~2 nm of platinum that was rotary deposited from 24° above the horizontal. The replica was then reinforced with ~140 Å of carbon using an electron gun at an angle of 90° to the horizontal. A drop of 2% colloidion solution was applied on the replica membrane. The coverslips were detached in a 40% solution of hydrofluoric acid, and cells were dissolved in Chlorox. After washing, replicas were transferred to Formvar-coated copper grids, the colloidion film on replicas was dissolved with amylacetate, and images were examined using TEM.

**Results**

**ER-budding Activity Is Enriched within the Vicinity of the Golgi Apparatus**

The basic stereological parameters of the RBL and NRK cell line used in these studies are shown in Table I. To morphometrically evaluate the distribution of export sites, ER-budding structures were identified as an elevation on the surface of the ER with a width of 65–85 nm, extruded from the membrane by at least 50% of their diameter, and covered with a distinctive electron-dense coat (Fig. 1, arrowheads). Budding sites emanate from three locations in the cell including (a) those associated with the nuclear envelope (Fig. 1), (b) those associated with the Golgi apparatus that are analogous in structure to classical ER transitional elements (Palade, 1975; Sesso et al., 1994) (Fig. 2), and (c) those found in more peripheral regions that lack detectable Golgi (Fig. 3).

Buds found at the tip of tubular projections from the surface of the ER had an average diameter of 78 ± 6 nm. Although tubular projections were generally shorter than 150 nm (Fig. 1, section 5: arrowheads), they could be as long as 350 nm based on reconstruction from serial-thin sections. Buds were covered with an ~8–10-nm-thick electron-dense coat. On grazing sections and at high magnification, the coat of ER buds possessed a lattice-like appearance due to a semi-regular array of 4–5-nm elongated particles (Fig. 1, inset). These coats are similar to those observed in pancreatic acinar cells (Merisko et al., 1986). Based on analysis of random sections through ~300 cells (see Materials and Methods), the average number of buds in a cell was found to be 250 ± 10. 45% of budding profiles were found on ER tubules located in the vicinity of Golgi complexes, whereas 55% were located in regions without noticeable juxtaposition to Golgi. 11% of total buds were observed emerging from the nuclear envelope. Thus, it is apparent that a substantial level of membrane exiting the ER appears to do so from sites distant from Golgi elements.

The overall average density of total ER buds based on the cross-sectional volume of the cytoplasm was found to be 0.5 buds per µm³, or 0.14 buds per µm² of total ER surface. However, the average density of ER-budding profiles found within the pericentrosomal area containing the Golgi apparatus (referred to as the Golgi exclusion zone) was five- to sevenfold higher (3.3 buds per µm³ of cytoplasm or 0.8 buds per µm² of ER surface) than the average value found on the total ER membrane. Outside of the Golgi region, the overall mean density was 1.5–3-fold lower (0.22 buds per µm² cytoplasm or 0.04 buds per µm² of ER surface) than the average values found for the cell. The markedly increased budding density around the Golgi apparatus is consistent with the highly focused export activity observed from ER transitional elements present in the Golgi region of pancreatic acinar cells (Palade, 1975).

**Table I. Basic Morphometric Parameters of NRK and RBL Cells**

| Azimuths | NRK | RBL |
|---------|-----|-----|
| Volume (µm³) | 1,205 ± 43 | 780 ± 38 |
| Surface (µm²) | 3,868 ± 127 | 665 ± 27 |

**Figure 1.** Export sites adjacent to the nuclear envelope of RBL cells. Six consecutive serial sections through a VTC adjacent to the nuclear envelope. ER buds (arrowheads) emerging from the nuclear envelope (section 1) and parallel ER membrane (stars in section 2–6) are facing VTCs. (Inset) Higher magnification view of two ER buds. The slice of the section presented in the inset encompasses either only the coat (right), or both the coat and the membrane and the luminal part of a bud (left). Individual 4–5-nm electron-dense particles arranged in a semiregular pattern (arrows). Notice the same appearance of the coat under lower magnification of serial sections that contain a honeycombed appearance consisting of a semiregular array of electron-dense particles (arrows). Adjacent to buds, we observe a typical pleomorphic element (large asterisk in section 5) within a VTC that has numerous tubular projections (small asterisks in sections 4 and 6), indicative of its fenestrated structure. Tubules in the fenestrated elements of the VTC possess a dark dense coat (arrows in sections 4 and 6) typical of those found on Golgi compartments and are readily distinguishable from the alveolate coat associated with COP II buds emanating from the ER. Bar, 0.1 µm.
Figure 2. Export complexes adjacent to the pericentrosomal Golgi region of RBL cells. 15 consecutive serial sections through three (a, b, and c) Golgi-adjacent export complexes (encircled by dotted lines) are shown with ER buds (arrowheads). Export complex a contains 13 ER-derived buds, export complex b contains 11 buds, and export complex c contains seven buds. Note the characteristic cup-shaped appearance of ER bud-bearing zones especially evident for export complex b.

**ER-budding Activity Is Also Enriched in Peripheral Export Complexes**

To develop a detailed understanding of the topological organization of ER export throughout the cytoplasm, we carried out a morphological reconstruction of those sites that were not adjacent to Golgi elements, referred to as peripheral sites. An analysis of peripheral sites allows us to discriminate the structure of ER-derived intermediates...
from that of the fenestrated cis-Golgi network (CGN), which is always associated with ER buds near the Golgi. ER buds on peripheral sites (Fig. 3) typically emanated from short stretches of ER membrane. These regions were separated by long distances from other similar budding foci. Frequently, ER buds found on different cisternae were closely juxtaposed and faced each other. These features are more evident in Fig. 4 (A and B), which presents an overlapping reconstruction of serial sections of the peripheral site shown in Fig. 3. Budding profiles (blue) protrude from the ER (green) into a central region containing a collection of vesicles and tubular elements comprising VTCs (red). This typical organization of peripheral sites is also characteristic of budding sites associated with the nuclear envelope (Fig. 1) and budding sites adjacent to the Golgi stack (Fig. 2). The close topological relationship between ER buds and distinct VTCs suggests that these structures function as a compact morphological unit that we now refer to in its entirety as an export complex.

Morphometric analysis of peripheral export complexes

Figure 3. Export sites in peripheral regions of the cell cytoplasm that are distant from Golgi stacks. 10 consecutive serial sections through a peripheral export site of RBL cells are shown with ER buds (arrowheads). ER strands bearing nine buds partially surround the VTC. Tubular elements of the VTC possess a Golgi-like uniform, dense, thick coat (arrows in sections 7 and 8). Bar, 0.1 μm.
revealed that these sites typically contained two to six buds emanating from the ER, although this could approach a value of 20 for some exceptional, larger clusters (Fig. 5 A, closed circles). These sites had an average number of buds per site of $4.4 \pm 0.3$ (Table II), a value that was slightly smaller than the average number of buds per site found in export complexes adjacent to Golgi ($6.1 \pm 0.4$ buds per site; Table II). By including only ER-budding profiles facing VTCs, we were able to estimate a “local” bud density in these export complexes. On average, this value was $17.2 \pm 2.5$ buds per $\mu m^2$ of ER surface, which, on a relative scale, is 125 times higher than the average bud density found on the total ER surface ($0.14$ buds per $\mu m^3$) and approximately five times higher than the bud density found on the surface of the ER within the Golgi exclusion zone.

**Buds Are Formed Nonrandomly along the ER Surface**

The apparent nonrandom distribution of ER buds led us to quantitatively estimate the probability of a given bud having proximity to a second bud in the cell. For this purpose, we constructed a series of concentric shells of equal volume that radiate outward from the center of randomly chosen buds. The radius of the first internal shell was arbitrarily assigned a value of $0.1 \mu m$ to encompass the entire tip of a budding structure. Each increment in the diameter of successive shells extending outward from the first shell progressively decreased in dimension to encompass the same volume in three-dimensional space. Given the average number of buds in a cell ($\sim 250$), the volume of cell cytoplasm ($560 \mu m^3$), and the volume of a shell ($4.2 \times 10^{-3} \mu m^3$), if buds assumed a strictly random distribution, then the probability of encountering another bud in a given concentric shell would remain equal with a value of $0.18\%$ (Fig. 6, diamonds). However, if buds were confined to regional foci, the probability of encountering a second bud would be high in the first series of concentric shells, and then fall off very rapidly with increasing distance in three-dimensional space.

The results of such an analysis are shown in Fig. 6 where we have plotted the probability of encountering a bud relative to its location in sequential concentric shells of equal volume (Fig. 6, open circles), or relative to the diameter of the outermost surface of a given shell in which a bud is found (Fig. 6, closed circles). It is clear that the distribution did not follow that predicted for a strictly random budding event. The probability of detecting a second bud within the first 50 consecutive shells having up to a $0.6-\mu m$ outer shell diameter markedly exceeded that of a random distribution (Fig. 6). After a plateau at a value similar to that calculated for a random distribution (up to an outer diameter of $0.8 \mu m$ or 100 shells), the probability fell dramatically within the three-dimensional space defined by the outermost shell examined ($\sim 1.2\mu m$). We conclude that budding is not a random event along the surface of the ER, but rather is remarkably restricted to regional hot spots of budding activity associated with export complexes.

**Structural Organization of Export Complexes In Vivo**

As illustrated in Fig. 4, ER-budding profiles predomi-
the absence of cytosol failed to generate detectable VTCs (not shown). This result is consistent with the fact that pre-existing VTCs are unstable during permeabilization (Aridor et al., 1995) and that cytosol contains essential soluble components of the COPII machinery required for the export of cargo from the ER (Barlowe et al., 1994; Kuge et al., 1994). VTCs generated in vitro in the presence of cytosol are nearly identical to those observed in vivo. We have previously shown that they are composed of a compact network of tubules and vesicles that lack direct luminal connections to ER membranes and frequently label positively for β-COP using immunoelectron microscopy (Balch et al., 1994; Pind et al., 1994a). However, ER-budding profiles, like those observed adjacent to VTCs in vivo (Figs. 1–3, arrowheads), were rarely observed in vitro. Since previous studies used mild fixation conditions in conjunction with...
with an immunodiffusion procedure to label VSV-G in VTCs (Balch et al., 1994), we reasoned that ER buds may be labile structures. We therefore applied more stringent fixation and embedding conditions to preserve ultrastructural details (see Materials and Methods). Under these conditions, ER buds were observed that had a characteristic coat resembling those found in vivo and were only detected adjacent to VTCs (not shown), suggesting that even in semi-intact cells, budding is restricted to specialized regions of the ER.

To examine the formation of buds and their relationship to VTCs in vitro, we made use of the nonhydrolyzable analog of GTP, GTPγS, which permanently activates Sar1 and other GTPases, leading to stable coat assembly and accumulation of buds. Interestingly, we observed for the first time using strong fixation conditions that nascent budding profiles generated in the presence of GTPγS had not only a cluster appearance (Pind et al., 1994a), but also frequently had a distinctive “beaded necklace” appearance with each vesicle being a bead (Fig. 7, B and C). The vesicles were nearly identical in size but lacked luminal continuity. These strings of vesicles were similar to the shorter necklaces sometimes observed in vivo under normal incubation conditions (Fig. 7 A). These necklaces were confined to only a small fraction of the total ER surface. By analyzing sections through >600 individual necklaces, we have never detected them to have more than one connection to the ER membrane, supporting the possibility that each string grows from a local area of budding activity. These observations suggest that budding continues

Table II. Morphometric Parameters of Golgi-adjacent and Peripheral VTCs

|                     | Golgi-adjacent | Peripheral | Total       |
|---------------------|----------------|------------|-------------|
| Average number per VTC: |                |            |             |
| ER buds             | 6.0 ± 0.45     | 4.4 ± 0.3  | 5.1 ± 0.3   |
| Vesicular elements  | 35 ± 3         | 30 ± 3     | 32 ± 3      |
| Average diameter of VTC (μm) | 0.40 ± 0.006 | 0.39 ± 0.006 | 0.39 ± 0.006 |

* Morphometric parameters were determined as described in Materials and Methods. A total of 116 Golgi-adjacent and 163 peripheral reconstructions of export complexes was used in generating the data shown.

Figure 5. Quantitative analysis of the composition of export complexes. (A) Quantitative analysis of the number of ER buds associated with an individual cluster in Golgi-adjacent (open circles) and peripheral (closed circles) export complexes was determined from serial sections as described in the Materials and Methods. (B and C) Quantitative analysis of the size of VTCs according to their diameter (B) or number of vesicular elements associated with a VTC (C) was determined by reconstruction of serial sections from Golgi-adjacent (open circles) and peripheral (closed circles) structures as described in the Materials and Methods. Data were collected from complete reconstructions of 116 Golgi-adjacent and 164 Golgi-peripheral export complexes in RBL cells.

Figure 6. Probability of a given bud having proximity to a second bud in the cell. Randomly chosen ER buds present in RBL cells were assigned as the center of reference, and distances between it and any other buds present in 30 consecutive serial sections were determined by building a series of concentric shells with a constant volume of 0.0042 μm³, corresponding to the volume of the first internal shell having a diameter 0.2 μm (to encompass a single bud) as described in the Materials and Methods. The probability was determined by counting the number of buds detected in each shell relative to the total number of buds detected (percentage of total). This value is plotted as shell number in which a second bud was found (open circles) or relative to the outermost diameter of a particular shell (closed circles). The calculated probability of a second bud having a completely random distribution in the cell (0.18%) is presented for comparison (diamonds).
Figure 7. Morphological features of ER-derived buds observed in vivo and in vitro. (A) Buds protruding from the ER of RBL cells in vivo (arrowhead) show a necklace-like appearance with two to three incompletely pinched-off vesicles remaining attached to the single bud protruding from the ER. (B) Semi-intact NRK cells were incubated in vitro in the presence of cytosol and ATP as described in the Materials and Methods. Note that buds (arrowheads) can be readily detected protruding from ER-like elements. (C) ER buds and vesicles (arrowheads) formed in vitro in the presence of GTP\(\gamma\)S show a zig-zag appearance with occasional singular branches (arrows). (D and E) ER buds accumulated in the presence of the GTP-restricted Sar1[H79G] mutant can form grape-like groups (D) or a highly branched network (E). (Arrows) Single branches. Bar, 0.05 \(\mu\)m.

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In the absence of GTP hydrolysis, but that the vesicles fail to complete separation from one another. Upon immunolabeling, we found accumulated vesicles to be substantially enriched in VSV-G (Pind et al., 1994a) and components of both COPI (\(\beta\)-COP) (Pind et al., 1994a; Griffiths et al., 1995b) and COPII coats (Sec13 and Sec 23) (Fig. 8, A–C).

To establish that necklaces formed in response to a specific block in COPII coat disassembly, we examined the effect of an activated, GTP-restricted mutant of Sar1, Sar1[H79G], which promotes vesicle accumulation in vivo and in vitro (Aridor et al., 1995; Kuge et al., 1994). Examination using strong fixation conditions revealed a striking similarity to GTP\(\gamma\)S-formed structures. Vesicles were detected as both clusters (Fig. 7 D) or as necklaces (Fig. 8 E) emerging from restricted regions of the ER. As expected, coats were enriched in the COPII components Sec13p

Figure 8. ER buds formed in vitro in the presence GTP\(\gamma\)S or the Sar1-GTP-restricted mutant contain components of COPII coats. Semi-intact NRK cells were incubated in vitro, and cryosectioning and immunolabeling of cell pellets were as described in Materials and Methods. Clusters of vesicles (arrows) accumulated in the presence of GTP\(\gamma\)S (A, B, and C) or Sar1[H79G] (D and E) contain Sec13p (arrowheads in A, C, and D) and Sec23p (arrowheads in B, and E) as shown by the distribution of gold particles using specific antibodies. Note that the cisternal portion of the Golgi apparatus with the trans face labeled in C and F remains unlabeled by Sec13p- and Sec23p-specific antibodies, whereas clusters (arrows) that are closely adjacent to the Golgi complex contain both Sec13p and Sec23p (C and F). Bar, 0.05 \(\mu\)m.
To examine the role of COPII in VSV-G concentration and the appearance of VTCs in export complexes, we used semi-intact cells infected with a temperature-sensitive form of VSV-G whose transport is blocked at the restrictive temperature (39.5°C) (Laëuf, 1974; Plutner et al., 1992). Transfer of cells to the permissive temperature (32°C) results in the migration of a synchronous wave of ts045 VSV-G from the ER to VTCs and subsequent Golgi compartments (Plutner et al., 1992; Balch et al., 1994). After incubation at 32°C in vitro, cells were fixed and stained with an antibody specific for the cytoplasmic tail of VSV-G using the immunodiffusion protocol (Balch et al., 1994). As shown in Table III, incubation in the presence of wild-type Sar1 had little effect on ER export as judged by the abundance of VTCs detectable in vitro. In contrast, the guanosine diphosphate (GDP)-restricted form of Sar1 (Sar1[T39N]) drastically reduced the formation of vesicles and VTCs (Table III), demonstrating the essential need to activate Sar1 to promote membrane flow from the ER through export complexes. In contrast, incubation in the presence of the GTP-restricted mutant (Sar1[H79G]) caused a dramatic accumulation of vesicles in clusters and an approximate twofold increase in the apparent number of clusters per section that could be detected (Table III).

When we determined the density of VSV-G in clusters formed in the presence of the Sar1-GTP restricted mutant, it was approximately five- to sixfold higher than that found in the ER before incubation (Table III). This fold-concentration was identical to that observed in VTCs present in control incubations lacking inhibitors (Table III). The five- to sixfold increase in the density of VSV-G in vesicles that accumulate in the presence of the mutant demonstrates that VSV-G is concentrated during packaging into COPII-coated vesicles.

**Visualization of ER Export by a Three-dimensional Technique Confirms the Organization of Export Complexes Reconstructed from Serial Thin-Section**

To develop a three-dimensional view of export complexes, we applied for the first time a modification of the quick-freeze, deep-etch methodologies used previously to visualize vesicles budding from the plasma membrane (Heuser, 1980) and Golgi compartments (Weidman et al., 1993). The approach is particularly applicable to semi-intact cells where the cytosol can be readily washed away to reveal structural features of the ER membrane surface.

After incubation in vitro in the presence of ATP and cytosol, semi-intact cells were fixed, rapidly frozen, and fractured to expose internal membranes. After etching and replication, intracellular organelles are rendered visible as three-dimensional structures. The surface of the ER was readily distinguishable from other subcellular compartments by the presence of ribosomes or, in the case of the nuclear envelope, additionally, nuclear pores. Adjacent to the surface of the ER, we frequently observed compact structures of similar size and apparent vesicular-tubular composition to VTCs observed in thin-sections (Fig. 9 A). These structures were completely absent in incubations that lacked cytosol or ATP. They varied in diameter, but were generally ~0.3-0.5 μm across and ranged from a circular to a more oblong shape under normal incubation conditions. Assuming that the distinctive ~80-nm surface undulations correspond to vesicle profiles (Fig. 9 A) and that a cluster can be represented as a sphere with a similar range of diameters, we estimate that VTCs detected in replicas could contain 50-110 individual elements. This value is compatible with the number of vesicular profiles determined by reconstruction from serial thin-sections.

To identify whether the above structures formed in the presence of cytosol and ATP contained ER-derived cargo proteins such as VSV-G, NRK cells were infected with ts045 VSV at the restrictive temperature. After permeabilization and incubation in vitro at 32°C, cells were immuno-labeled for VSV-G using the immunodiffusion protocol (Balch et al., 1994) and replicas were prepared. While a combination of prolonged incubation using mild fixation conditions to label VSV-G and the presence of prominent “gold shadows” from the etching and replication reduces the ability of the technique to reveal surface features of these immunogold-labeled clusters, the distribution of VSV-G reveals the striking role of COPII in concentrative export. Before incubation of semi-intact cells at 32°C, gold was distributed on the surface of the ER (Fig. 10 A, arrowheads) and nuclear envelope (Fig. 10 B, arrowheads) was distributed in an apparent random manner throughout the ER cisternal network (Plutner et al., 1992; Balch et al., 1994). The surface density of VSV-G in these ER membranes was 32 ± 6 gold particles per μm². In contrast, incubation for 45 min in the presence of cytosol and ATP led to a dramatic change in the distribution of VSV-G, rearranging gold particles to a limited number of VTCs that were well isolated from each other (Fig. 10 C). The density of gold over VTCs projected as a flat surface parallel to the ER membrane (referred to as a planar projection) was ~850 ± 250 gold particles per μm², a value markedly higher than that observed in the plane of the ER membrane before incubation.

We next used replicas to follow the effects of GTPyS and the Sar1-GTP restricted mutant on budding and concentration of VSV-G. As shown in Fig. 10 (B and C), incu-
Figure 9. Structure of VTCs adjacent to the ER surface as seen by the quick-freeze, deep-etch replication technique. NRK cells were permeabilized and incubated at 32°C for 45 min in the presence of ATP and cytosol (A), or additionally supplemented with 10 μM GTPγS (B and C) or 1 μM Sar1[H79G] (D). Clusters viewed as replicas are present on the ER membrane (B and C) or the nuclear envelope (A and D) as indicated by the presence of ribosomes (numerous, small projections on ER surface) (A–D) and/or nuclear pores (not shown), respectively. GTPγS accumulated vesicle clusters collapsed onto the surface of the ER during etching usually exhibit a necklace-like morphology with a zig-zag appearance (B and C). Sar1[H79G] accumulated vesicles appeared as necklace-like structures (not shown) or as compact vesicular clusters (D). Note that the cluster in D formed in the presence of the Sar1[H79G] mutant has a more vesicular appearance of surface projections than the control cluster shown in A, which appears partially tubular in composition. We have noted a frequent association of VTCs with microtubule-like filaments (arrow in A). Bar, 0.1 μm.

Discussion

We have provided the first quantitative, stereological description of the three-dimensional organization of cellular structures involved in transport of cargo from the ER to the Golgi apparatus. Export complexes have a hierarchical organization that can be conceptually divided into three tiers (Fig. 11 A). The first tier (Fig. 11 A, dotted box) consists of closely adjacent buds on a single ER cisterna. Each can give rise to an individual string or group of ER-derived buds containing COPII coats. These budding foci were limited to specific regions of the ER in vivo, suggesting the existence of a defined number of export sites in the living cell. The second tier (Fig. 11 A, cylindrical region outlined by dashed lines) comes from the observation that buds on one cisterna were often found in close proximity to budding profiles emanating from ER cisternae derived from distantly connected regions of the ER. The third tier of organization encompassing the entire export complex (Fig. 11 A, solid box) includes ER-derived buds that face into a region housing a central VTC. Tubular elements within VTCs contain distinctive COPI coats and are luminally discontinuous with the ER. While professional secretory
Figure 10. VSV-G is concentrated in ER-derived vesicles and VTCs. NRK cells infected with ts045 VSV at the restrictive temperature (39.5°C) to retain VSV-G in the ER were permeabilized with digitonin (Plutner et al., 1992) and either fixed immediately (A and B) or incubated for 45 min at the permissive temperature (32°C) in the presence of cytosol and ATP (C), or additionally supplemented with 1 μM Sarl[H79G] (E). After incubation, cells were fixed and VSV-G labeled with 10 nm (A–C, and E) or 6 nm (D) gold particles using the immunodiffusion protocol as described in the Materials and Methods. Cells were either prepared for thin-section TEM (D) or for quick-freeze, deep-etch replication (A–C, and E). At the restrictive temperature, VSV-G was uniformly distributed within the ER membrane (A) or the nuclear envelope (B). After a shift to the permissive temperature (B–E), VSV-G was concentrated in newly formed 80-nm vesicles associated with export complexes. (Arrowheads) Location of 6 nm (D) or 10 nm (A–C, and E) gold particles corresponding to the distribution of VSV-G. Due to the use of an immunodiffusion protocol before preparation of replicas and the high density of label, extended shadows from the gold particles partially obscure membrane outlines. Bar, 0.1 μm.

cells such as those found in the pancreas confine export predominately to a single transitional region juxtaposed to the cis face of the Golgi apparatus (Palade, 1975). the two different cell lines used in the present study were found to have export complexes distributed throughout the cytoplasm. Our studies provide evidence that budding from the ER occurs in areas of intense morphological specialization. Each level of organization is discussed in detail below.

Tiers I and II Define the Distribution of ER-budding Profiles

While there is an apparent random distribution of export complexes in the cytoplasm, we found a very high degree of organization in the distribution of ER buds in the cell. We observed not only a high local density on the same stretch of ER membrane (Fig. 11, tier I, dotted boxes), but found distantly connected ER bud-bearing zones encircling the same VTC (Fig. 11, tier II, dashed cylindrical region), suggestive of a regional specialization of the COPII export machinery. Consistent with this interpretation, buds and vesicles accumulated in semi-intact cells in the presence of GTPyS or the Sarl[H79G] mutant strongly labeled with antibodies specific for mammalian homologues of the yeast components Sec13p and Sec23p.

Both the GTPγS and the GTP-restricted Sarl[H79G] mutant led to the formation in vitro of ER-derived vesicle clusters and strings of vesicles with a necklace-like appearance. Vesicles accumulated as necklaces had no obvious continuity between the lumen of individual vesicles, suggesting that membrane fission had gone to completion. Moreover, in both thin-section and replicas, clusters that formed in the presence of GTPyS or the Sarl GTP-restricted mutant clearly had a more vesicular surface appearance than those formed in the absence of inhibitors. The fact that separation of vesicles appears to be blocked in the absence of GTP hydrolysis raises the distinct possibility that the function of Sarl is normally required for this event. Given the fact that uncoating occurs rapidly after budding (Aridor et al., 1995), coat disassembly could be associated with release of vesicles from necklaces.

The morphological effect of GTPyS on the budding from the ER is very different from its effect on Golgi membranes incubated under identical conditions. In the latter case, buds appearing in replicas are isolated, single
structures and are uniformly distributed throughout the entire Golgi surface (Weidman et al., 1993). The regional confinement of budding in ER membranes to necklaces or clusters therefore also supports our interpretation that export occurs from areas of luminal and/or membrane specialization. Since budding from the ER frequently occurs from the tips of short, coated tubules emanating from ER cisternae, clusters and necklaces could be derived from either sequential or synchronous fission of these tubular elements.

In contrast with the fact that export complexes observed in vivo appear to be completely surrounded by budding profiles from topologically distant ER cisternae (Fig. 11 A, tier II), semi-intact cells lacked this feature (Fig. 11 B). Thus, permeabilization destroys confinement of several sites to one area and allows them to form in a more random fashion. We have previously noted that ER-derived vesicles and downstream compartments are not released from semi-intact cells during incubation in vitro at 32°C (Beckers et al., 1987). In contrast, assays that reconstitute vesicle budding from semi-intact yeast cells (Baker et al., 1988) release free 60-nm COPII-coated vesicular carriers (Barlowe et al., 1994). The inability of mammalian semi-intact cells to release vesicles suggests that vesicles are tethered to a scaffold of unknown composition. In yeast, either vesicles are not linked to such a scaffold, or this aspect has not been successfully reconstituted. In either event, while the striking degree of morphological specialization observed in mammalian cells may contribute to the overall efficiency of budding and transport in the early secretory pathway, it is apparently not essential.

We have previously suggested that export from the ER is accompanied by concentration of VSV-G (Balch et al., 1994). This point was the subject of a recent debate (Balch and Farquhar, 1995; Griffiths et al., 1995a). We have now applied an independent approach using quick-freeze, deep-etch methodologies in conjunction with immunolabeling of VSV-G to generate three-dimensional replicas that allow us to directly assess the concentration of VSV-G in ER-derived vesicles. We found, on average, a value of ~800 gold particles per μm² in planar projection of clusters accumulated in either the absence or presence of GTPγS, or in the presence of the activated Sar1-GTP restricted mutant. The inclusion of the inhibitors prevents further rounds of vesicle budding, ensuring that we are examining concentration associated with export from the ER. A planar projection, however, is not a good approximation of the total surface area available on clusters for antibody binding. Since only the external surface of vesicles found on the perimeter of clusters is available for antibody binding. Since only the external surface of vesicles found on the perimeter of clusters is available for antibody binding (Balch et al., 1994; Pind et al., 1994a) (see Fig. 11 D), a more reasonable estimate of VSV-G density can be determined if we assume that the antibody has access to the outer-half of a shell of 80-nm vesicles that occupy the perimeter of a 0.4-μm sphere (the size of a typical VTC). Compared with the surface area of the planar projection (~0.13 μm²), the surface area of such a population of vesicles corresponds to a value of ~0.7 μm². This is an increase of approximately fivefold over the planar projection. Therefore the actual surface density of VSV-G in clusters observed in replicas is 800 gold particles per μm² divided by 5 or 160 gold particles per μm². This value, when compared to the average surface density of VSV-G before incubation in vitro (32 gold particles per μm²), sug-

Figure 11. Diagram summarizing the three tiers of organization of ER export complexes in vivo (A) and in vitro (B). (A) An individual ER cisterna contains a collection of closely opposed buds that define a local transitional region (light zone with buds on the ER). This specialized region is dominated by the presence of COPII coats and is referred to as tier 1 (box outlined with dotted line). Tier II (cylindrical region outlined by dashed lines) includes buds on distantly connected ER strands that face a central VTC consisting of a collection of distinct vesicular-tubular elements that have COPI coats. Tier III includes the entire export complex and is outlined by the box with the solid line that encompasses both ER buds and a central VTC. The possible elevated concentration of COPII and COPI coat components within the local cytoplasm of export complexes is depicted by small dots and lines. (B) In semi-intact cells, there appears to be a more limited number of ER buds associated with the local transitional region defined by tier 1 (box outlined with dotted lines). The tier II level of organization is completely missing in semi-intact cells, as the association of distantly connected ER strands appears to be lost during cell permeabilization. However, tier III (box outlined with solid line) is maintained, highlighting the juxtaposition of VTCs to buds on one ER strand.
suggests that VSV-G is concentrated five- to sixfold during budding from the ER. The fold-concentration detected here is very consistent with that observed previously using serial thin-sections (Balch et al., 1994; Find et al., 1994a) or in the present studies in the presence of the Sar1-GTP restricted mutant (Table III). These results confirm the validity of our previous technical advancements (Balch et al., 1994; Balch and Farquhar, 1995) and now firmly establish that cargo is concentrated during ER export (Balch et al., 1994).

**The Third Tier: Budding Sites Surround VTCs**

A third tier of organization of export sites was found in the striking relationship between flanking ER-connected budding profiles and VTCs to form the functional morphological unit we refer to as export complexes (Fig. 11A, area enclosed by box with solid line). Images reconstructed from conventional TEM and those observed in replicas yielded identical results. The distinctive morphological characteristics of the export complexes reconstructed in the present studies are consistent with previous qualitative morphological descriptions (Saraste and Kuismanen, 1984; Schweizer et al., 1988, 1990; Saraste and Svensson, 1991) and a recent study in which a HRP-tagged reporter protein was used to characterize the organization of the ER/Golgi region using immunocytochemistry (Stinchcombe et al., 1995). The replicas were particularly striking in that they allowed us to visualize for first time the overall compact composition of VTCs and their localization to specific foci found on the ER surface.

The overall topological organization of export complexes fits well with the proposed function of the ER in the sorting and concentration of cargo during budding via COPII coats, and the subsequent coupled recycling of proteins from VTCs via COPI coats (Aridor et al., 1995). The close association of these two sorting stations (ER and VTCs) may provide for increased efficiency in ER to Golgi transport and/or promote more rapid exchange of coats within the confines of the complex where a higher local coat concentration may be found. Consistent with this proposal, incubation of pancreatic acinar cells in the absence of ATP (Merisko et al., 1986) or in the presence of brefeldin A (Hendricks et al., 1993; Ori et al., 1993b) leads to the accumulation of COPI and COPII-containing aggregates in transitional regions. The basic morphological organization of export complexes may be more or less extensive in different cell types depending on the relative rates of formation and consumption of vesicles and tubules comprising central VTCs. The extensively fenestrated network found at the cis face of the Golgi stack, referred to as the CSN (Mellman and Simon, 1992), is also considered to be a site of membrane recycling. It may be an enlarged variation of the more compact VTCs, reflecting the intensity of vesicular traffic in this region of the cell.

In general, our studies have provided insight into the fundamental morphological organization of the first steps in the secretory pathway that promote the movement of cargo from the ER to the Golgi complex. There has been considerable controversy regarding the morphological organization of this stage of secretory pathway, given the complexity of the pre-Golgi region and the frequent use of reduced temperature to augment the visibility of intermediates (VTCs). Our ability to provide a description of the three-dimensional organization of export complexes at sites distant from the Golgi apparatus under normal incubation conditions now illustrates their basic organization in living cells and their essential role in ER to Golgi transport.

We thank Dr. G. Palade, Dr. M.G. Farquhar, and Michael McCaffery for their many helpful comments concerning the EM.

This work was supported by grants from the National Institutes of Health (GM 42336; CA 586689) (to W.E. Balch), and postdoctoral fellowships from The Human Frontier Science Program Organization, Muscular Dystrophy Association (to T. Rowe), and the Cystic Fibrosis Foundation (to S. Bannykh). This study made extensive use of Core B (Immunoelectron Microscopy) in CA 586689.

Received for publication 22 April 1996 and in revised form 11 June 1996.

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The Journal of Cell Biology, Volume 135, 1996
Bannykh et al., Organization of ER Export Complexes