Localization of the Lys, Asp, Glu, Leu Tetrapeptide Receptor to the Golgi Complex and the Intermediate Compartment in Mammalian Cells

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Abstract. The carboxyl-terminal Lys-Asp-Glu-Leu (KDEL), or a closely-related sequence, is important for ER localization of both lumenal as well as type II membrane proteins. This sequence functions as a retrieval signal at post-ER compartment(s), but the exact compartment(s) where the retrieval occurs remains unresolved. With an affinity-purified antibody against the carboxyl-terminal sequence of the mammalian KDEL receptor, we have investigated its subcellular localization using immunogold labeling on thawed cryosections of different tissues, such as mouse spermatids and rat pancreas, as well as HeLa, Vero, NRK, and mouse L cells. We show that rabl is an excellent marker of the intermediate compartment, and we use this marker, as well as budding profiles of the mouse hepatitis virus (MHV) in cells infected with this virus, to identify this compartment. Our results demonstrate that the KDEL receptor is concentrated in the intermediate compartment, as well as in the Golgi stack. Lower but significant labeling was detected in the rough ER. In general, only small amounts of the receptor were detected on the *trans* side of the Golgi stack, including the *trans*-Golgi network (TGN) of normal cells and tissues. However, some stress conditions, such as infection with vaccinia virus or vesicular stomatitis virus, as well as 20°C or 43°C treatment, resulted in a significant shift of the distribution towards the *trans*-TGN side of the Golgi stack. This shift could be quantified in HeLa cells stably expressing a TGN marker. No significant labeling was detected in structures distal to the TGN under all conditions tested. After GTPγS treatment of permeabilized cells, the receptor was detected in the β-COP–containing buds/vesicles that accumulate after this treatment, suggesting that these vesicles may transport the receptor between compartments. We propose that retrieval of KDEL-containing proteins occurs at multiple post-ER compartments up to the TGN along the exocytotic pathway, and that within this pathway, the amounts of the receptor in different compartments varies according to physiological conditions.

PROTEIN sorting/targeting along the exocytotic pathway is mediated by various types of targeting/sorting signals (Pelham, 1989; Hong and Tang, 1993). The carboxyl-terminal Lys, Arp, Glu, Leu tetrapeptide (KDEL)[1] (HDEL in yeast) and related sequences have been shown to mediate ER localization of lumenal and membrane proteins that have their COOH terminus within the lumenal side of the membrane (Hardwick et al., 1992; Pelham, 1990; Sweet and Pelham, 1992; Tang et al., 1992*a*). Recent studies argue strongly that KDEL or HDEL sequence functions as a retrieval signal at some post-ER compartment(s) (Dean and Pelham, 1990; Pelham, 1988, 1991; Jackson et al., 1993).

Our understanding of the molecular role of the KDEL sequence has been enhanced significantly by the identification of the yeast ERD2 gene that encodes the HDEL receptor (Lewis et al., 1990; Semenza et al., 1990). Furthermore, the ERD2 gene product is also essential for proper functioning of the Golgi apparatus and the secretory pathway in general (Semenza et al., 1990). Two closely related mammalian

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1. *Abbreviations used in this paper*: COP, coatomer proteins; IC, intermediate compartment; KDEL, Lys, Asp, Glu, Leu tetrapeptide; KDEL-R, KDEL receptor; MAN-I, Golgi mannosidase I; MHV, mouse hepatitis virus; PAC, pancreatic acinar cell; PDI, protein disulfide isomerase; STα-, 6 TGN, *trans*-Golgi network; VSV, vesicular stomatitis virus.

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homologues have been cloned (Lewis and Pelham, 1990; Hsu et al., 1992; Tang et al., 1993). The deduced amino acid sequence predicts that the KDEL receptor is an integral membrane protein having seven hydrophobic regions and a molecular mass of ∼23 kD, which has been confirmed by immunoblotting analysis using a specific antibody (Tang et al., 1993). We have recently proposed that the KDEL receptor (KDEL-R) is an integral membrane protein containing six transmembrane domains with both NH_2 and COOH termini on the cytoplasmic side of the membrane (Singh et al., 1993), although a topology with seven transmembrane domains has also been suggested (Townsley et al., 1993). The fact that this molecule indeed functions as the KDEL receptor has now been established by evidence from genetic (Lewis et al., 1990; Semenza et al., 1990), biochemical (Dean and Pelham, 1990; Wilson et al., 1993), and cell biological (Lewis and Pelham, 1992) approaches. An important question that remains unresolved concerns the ultrastructural localization of the receptor, a question that is the main focus of this paper.

An analysis of the localization of the KDEL receptor is complicated by the difficulty of defining the precise boundary between the rough ER and the beginning of the Golgi complex, which we operationally define as the compartment where the Golgi mannosidase I (Man I) functions. One useful way of defining this boundary in a functional sense is the 15°C block (Saraste and Kuismanen, 1984) that inhibits the entry of newly synthesized membrane proteins from the ER into the Man I compartment (Balch et al., 1986). Within this framework one can now identify two distinct compartments through which proteins must pass before entry into the Golgi complex. The first is the rough ER itself, the site of synthesis, and the second is the organelle that is now generally referred to as the intermediate compartment (IC) (Hauri and Schweizer, 1992; Lippincott-Schwartz, 1993).

The IC has been identified by the use of a number of markers that specifically localize to structures that are distal to the rough ER but proximal to the Man I–Golgi compartment. These markers include p53 (Schweizer et al., 1988, 1991), p58 (Saraste et al., 1987), rab 2 (Chavrier et al., 1990), and rab 1A (Tisdale et al., 1992). We recently described a novel protein, p28, that is also predominantly enriched in the IC (Subramaniam, V. N., G. Griffiths, A. R. B. M. Yussoff, M. Ericsson, and W. Hong, manuscript submitted for publication). The IC has also been shown to be the compartment where coronaviruses bud (Tooze et al., 1988; Griffiths and Rottier, 1992; Krijnse-Locker et al., 1994), as well as the organelle that provides the first membranes during the assembly of vaccinia virus (Sodeik et al., 1993).

There is now a general consensus that the IC is indeed distal to the rough ER but distinct from the Man I compartment, and that the anterograde traffic into the Golgi complex is mediated by vesicles (Balch, 1990; Hauri and Schweizer, 1992; Schekman, 1992). Nevertheless, considerable controversy remains about the nature of the connections between the rough ER and the IC. One model proposes that the IC is physically distinct from the rough ER, with a different lumenal environment, and that traffic between the two organelles would be mediated by vesicular traffic (i.e., two vesicular steps from rough ER to Golgi) (Warren, 1987; Pelham, 1989; Lippincott-Schwartz et al., 1990; Balch, 1990). The alternate view is that the IC is directly continuous with the rough ER, and that only one vesicular transport step would be required from the ER to the Golgi (Hauri and Schweizer, 1992; Griffiths and Rottier, 1992). The latter model would be consistent with the current view in yeast (for a review see Schekman, 1992).

Our recent data argue strongly in favor of the one vesicular step model whereby the IC is a functional domain of the ER (Krijnse-Locker et al., 1994). In the latter study, we used cells infected with mouse hepatitis virus (MHV) as a model system, and we showed that this virus both buds and acquires the first O-linked sugar, *N*-acetyl-galactosamine, on its M membrane protein in the IC. Furthermore, conditions that blocked vesicular transport, such as 14°C in vivo or GTPγS in vitro, had no effect on transport from the rough ER to the IC, although these conditions, as expected, prevented the M protein from acquiring Golgi sugar modifications. Moreover, the compartment where MHV buds was enriched in p58, rab2, as well as in the KDEL protein PDI, which is often considered to be exclusively found in the rough ER from immunofluorescence studies. The latter finding was subsequently strengthened by the localization to the budding compartment of a novel HDEL calcium-binding protein, ERC 55, that was identified in HeLa cells (Weis et al., 1994). In all of our studies using markers of the IC, as well as using PDI and ERC 55, the labeling consistently extended to one cisterna on the *cis* side of the Golgi stack, although we emphasize that because of the three-dimensional complexity of the Golgi stack, this cisterna is not apparent in all sections through the stack. We have proposed therefore that this cisterna is part of the IC, and that the bona fide Golgi complex would start at the next, second cisterna (Krijnse-Locker et al., 1994; Griffiths and Rottier, 1992; see also Lindsey and Ellisman, 1985*a*, 1985*b*).

Preliminary experiments using the immunoperoxidase approach has revealed the presence of the KDEL-R in the ER and in Golgi cisternae (Tang et al., 1993). Because of the limitations of this technique, conclusive and quantitative results were not obtained. In this report, we describe our detailed immunogold labeling experiments using an affinity-purified antibody against the cytoplasmic COOH-terminal sequence of the KDEL-R in thawed cryosections of tissues and cultured cells, and particularly in the mouse L cell model system using MHV and in HeLa cells stably expressing a *trans*-Golgi network (TGN) marker, a construct of the α-2,6-sialyltransferase tagged on its lumenal domain with 10 amino acids from the cytoplasmic domain of the G protein of vesicular stomatitis virus (VSV) (Rabouille, C., F. Hunte, R. Kiekbusch, E. Berger, G. Warren, and T. Nilsson, manuscript submitted for publication). Our results demonstrate that the bulk of the KDEL-R is normally localized to both the Golgi stack and the intermediate compartment, and that increased concentrations reach the TGN when the cells are stressed either by virus infection or by extremes of temperature.

Materials and Methods

Cells and Viruses

Sac(−) cells were grown in Dulbecco's MEM supplemented with 5% fetal calf serum, while HeLa, NRK, and mouse L cells were grown in DME

medium with 10% serum. The MHV A59 was propagated in sac(−) cells and was used to infect L cells for the localization studies, with or without streptolysin O treatment, as described (Krijnse-Locker et al., 1994). The conditions for infecting L cells with VSV-ts045 and for accumulating the G protein at 20°C were as described earlier (Griffiths et al., 1985). For infecting cells with the WR strain of vaccinia virus see Sodeik et al. (1993). The use of the vaccinia recombinant expressing the M protein of MHV is described earlier (Krijnse-Locker et al., 1992). The SA:48 cells are a HeLa cell line stably overexpressing the human α-2,6-sialyltransferase having a 10-amino acid peptide tag (P5D4 epitope) from the cytoplasmic domain of the vesicular stomatitis virus G protein at the lumenal (COOH) terminus (see Rabouille, C., F. Hunte, R. Kieksbusch, E. Berger, G. Warren, and T. Nilsson, manuscript submitted for publication). The characterization of the P5D4 epitope was done according to Soldati and Perriard (1991). For the 20°C "block," the cells were incubated in "air medium" (Matlin and Simons, 1983) for 2 h at 20°C; for the heat shock treatment, they were put in the same medium for 4 h at 43°C. For the treatment of the SA:48 cells with bafilomycin A1 (Bowman et al., 1984; obtained from the Kamiya Medical Co., Thousand Oaks, CA), the cells were allowed to internalize BSA-gold (16 nm) for 3 h (pulse) followed by an 8-h chase in the absence of gold and in the presence of 500 nM bafilomycin A1. The cells were given BSA-gold (5 nm) for 10 min before fixation to label early endosomes. To be sure that the bafilomycin treatment effectively neutralized low pH compartment parallel experiments were done at the light microscopy level using acridine orange (6 μg/ml).

Antibodies

Antibodies against the mammalian KDEL receptor (p23) were raised in rabbits by injecting synthetic peptide (conjugated to keyhole limpet hemocyanin) corresponding to the 21-residue sequence of the COOH terminus (CDFFYLYITKVLKGKKLSLPACOOH) of p23 (Tang et al., 1993). Specific antibody was affinity-purified from positive antisera with the same peptide conjugated to a solid support. Since this COOH terminal sequence is identical between the two closely related mammalian KDEL receptors (Hsu et al., 1992), this antibody will detect both forms of the receptor, and our localization studies here represent a survey of total KDEL receptors. The peptide serum against the COOH-terminal epitope of M protein of MHV, as well as the monoclonal antibody against the NH_2 terminus have been described before (Krijnse-Locker et al., 1994). Monoclonal and polyclonal antibodies against the tail (P5D4 epitope) of the VSV-G protein were kindly provided by Thomas Kreis (Department of Biological Sciences, University of Geneva, Geneva, Switzerland) (Kreis, 1986). In a few experiments using the HeLa cells, a three-step labeling protocol was used whereby the primary antibody was amplified by a pig anti-rabbit antibody that was then detected by protein A gold (see Fig. 4 *A*). The antibody against rabl was made against the purified rablA protein, as described by Saraste, J., and B. Goud (manuscript submitted for publication). This antibody also crossreacts with rablB (Goud, B. and J. Saraste, manuscript submitted for publication). Antibodies against calreticulin (CaBP3), CaBP1, and CaBP2 purified from rat were raised in rabbits. The antigens were purified as described by Nguyen Van et al. (1989, 1993) and Rupp et al. (1994). Antibodies against the KDEL-COOH terminus peptide were raised as described by Vaux et al. (1990). A peptide was synthesized as follows, -K-X-X-X-X-X-K-D-E-L, where X is an equimolar mixture of A, D, H, Q, L, Y, and K. The peptide was coupled with the carbodimide EDC to keyhole limpet hemocyanin and used for immunization of rabbits.

Immunogold Labeling of Thawed Cryosections

Mouse seminiferous tubules, kidney, and liver were fixed with 0.1% glutaraldehyde and 4% formaldehyde in 200 mM Hepes buffer, pH 7.4, for the first 30 min, followed by a subsequent overnight incubation in 4% formaldehyde alone. Pieces of fixed rat liver, pancreas, and kidney that had been fixed in the above mixture were kindly provided by Dr. J. W. Slot (Department of Cell Biology, University of Utrecht, Utrecht, The Netherlands). Mouse L cells were infected with MHV and permeabilized with streptolysin O (SLO) as described previously (Krijnse-Locker et al., 1994). The latter publication also describes the treatment of SLO-permeabilized cells with GTPγS (50 μM). HeLa cells, as well as the SA:48-HeLa cell line (see above), were infected with the WR strain of vaccinia virus, as before (Sodeik et al., 1993). Vero cells were infected with the ts 045 strain of VSV for the 20°C experiments, as described by Griffiths et al. (1985). The cells grown in 6-cm dishes were rinsed with PBS and removed from the monolayer with either proteinase K on ice (20 μg/ml in PBS for 2–3 min) or, in the case of L cells infected with MHV (without SLO), simply by squirting with PBS. A 16% solution of paraformaldehyde was added (final concentration ~4%) to these cell suspensions before centrifugation for 3 min at 1,000 *g*. The supernatant was removed and 4% paraformaldehyde and 0.1% glutaraldehyde in 200 mM Hepes, pH 7.4, was carefully layered on the pellet. The cells were fixed for 4–24 h and centrifuged at 13,000 *g* for 5 min. The SLO-treated cells were fixed for 30 min with 1% glutaraldehyde in 200 mM Hepes, pH 7.4, and scraped with a piece of Teflon. Pieces of these pellets were infused with sucrose, cryo-sectioned, and labeled with immunogold as described by Griffiths (1993). Double labeling was done according to Slot et al. (1991) using a 1% glutaraldehyde step between the two sets of antibodies (see also Griffiths, 1993).

In some experiments using SLO-permeabilized cells, we found a high background labeling using control (irrelevant) antibodies. This was reduced by both diluting antibodies two- to threefold more than normal and by incubating both control (as well as the p23) antibodies in the presence of 0.1% Tween 20 in 2× PBS plus 1% fish skin gelatin (Sigma Immunochemicals, St. Louis, MO).

Quantitation of Immunogold Labeling

For the MHV-infected L cells treated with SLO, the receptor was quantified over the nuclear envelope, rough ER, intermediate compartment (identified by the presence of budding viruses), and Golgi stack. For this, 24 micrographs were taken at a primary magnification of 22,000 of systematically sampled regions of the cell (predominantly the perinuclear regions) that included any of these organelles. The denoting of label per linear trace of membrane was determined using intersections with a series of test lines, as described by Griffiths (1993).

For quantifying the amount the KDEL-R in the *cis* versus the *trans* side of the Golgi stack, we used the HeLa-SA:48 cells, either uninfected or after infection for 9 h with the WR strain of vaccinia virus. In both cases, the cells were treated with SLO before fixation (as above), cryosectioned, and double labeled with anti-KDEL-R and a rabbit antibody against the P4D5 cytoplasmic domain epitope of the VSV-G protein. Golgi stacks cut perpendicularly to the cisternal membranes were systematically sampled and photographed at a primary magnification of 28,000. The labeling for the G protein (indicative of the sialyltransferase, a TGN marker) was used to distinguish the *trans* side of the stack from the *cis*. An arbitrary line was then drawn through the stack to split the stack into *cis* and *trans* sides. The amount of KDEL-R labeling associated with the two sides was then evaluated.

Results

Localization of the KDEL-R in Tissues

We first determined the localization of the KDEL-R in early spermatids of the mouse since the polarity of the Golgi complex, which is involved in secreting material for the developing acrosome, is very clearly defined in this cell (Fawcett, 1986). Moreover, the TGN side of the stack, which is directly adjacent to the developing acrosome, has an extremely high concentration of clathrin (Griffiths et al., 1981). We therefore prepared cryosections of pieces of the seminiferous tubules of the mouse and labeled these with anti-KDEL-R. In these sections, we searched for the early spermatid stages in which the acrosome is beginning to form around the tip of the nucleus of the developing sperm. During these stages, the Golgi complex is extremely pronounced and clearly polarized. The labeling of the Golgi stacks was extensive but essentially all the label was found on membranes at the *cis* side of the stack (Fig. 1). The TGN had extremely low levels of labeling. However, in evaluating many Golgi stacks, it was clear that this low level of TGN labeling was much higher than that seen over the matrix of the nucleus, by definition, background.

We next determined the localization of the KDEL-R in rat exocrine pancreatic acinar cells (PAC), since the morphological aspects of the secretory pathway are well established in these cells (Farquhar and Palade, 1981; Orci et al., 1991;

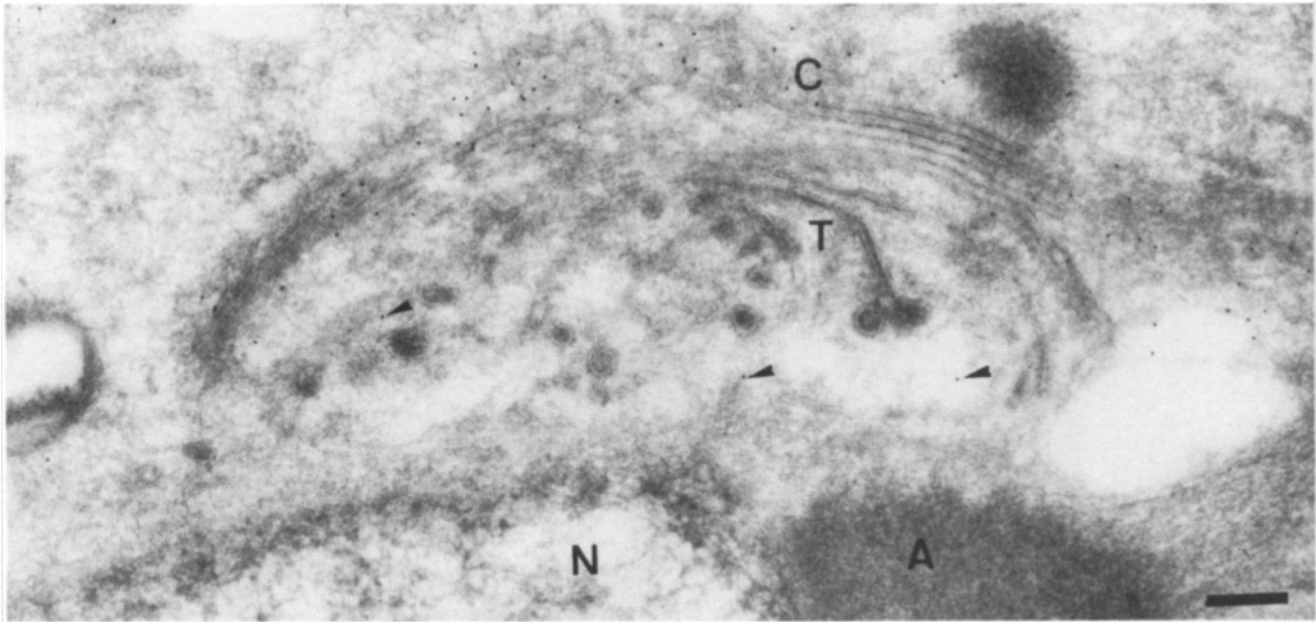


Figure 1. A thawed cryosection of a mouse early spermatid labeled with the anti–KDEL-R. Note the abundance of labeling associated with the cisternae on the *cis* (*C*) side of the Golgi stack. The *trans*/TGN (*T*) elements, which have extensive regions coated with clathrin in these cells (see Griffiths, 1981), have only very low levels of label (*arrowheads*). The elements can also be identified by being directly adjacent to the developing acrosome (*A*), which overlays the nucleus (*N*). Bar, 200 nm.

Oprins et al., 1993). Consistent with the results in spermatids, the bulk of the label for the receptor in the PAC was associated with one side of the Golgi stack, both on cisternal, as well as tubulovesicular structures (not shown). The rough ER was very poorly labeled. Since the condensing vacuoles serve as a *trans* marker in these cells, we could conclude that the bulk of the KDEL-R was associated with the *cis* side of the Golgi stack of PAC. We also examined the labeling with anti KDEL-R on sections of liver and kidney from both rat and mouse. In both tissues, the bulk of the label was on or close to the Golgi stack and, as in the pancreas and spermatids, preferentially concentrated on one side of the stack (results not shown).

Localization of the KDEL-R in Uninfected Cells

We next investigated the localization of the KDEL-R in two commonly used lines, HeLa (Fig. 2) and NRK (not shown). In both cell types a strong labeling was seen on and around the Golgi stack. In the majority of cells, the labeling was associated with one cisterna on one side of the Golgi stack, as well as more peripheral membrane elements, which we assume to be the IC (Fig. 2 *A*). However, we also saw many examples where the Golgi stack was essentially free of labeling, while the peripheral elements labeled significantly (Fig. 2 *B*). Conversely, a few Golgi complexes were seen which had labeling throughout the stack (not shown).

In some experiments, HeLa cells were additionally labeled with BSA gold before fixation to mark the late endocytic structures (16 nm gold) or early endosomes (5 nm gold). As is evident in Fig. 2, *A* and *B*, these organelles were invariably devoid of any label for the KDEL-R.

Localization in L Cells Infected with MHV

We have recently demonstrated that the COOH-terminal 21-residue sequence of the KDEL-R (the peptide epitope) is on the cytoplasmic side of the membrane (Singh et al., 1993) and that some cytosolic factor(s) may specifically mask the epitope (Tang et al., 1994). These observations suggest that some KDEL-R epitopes might not be accessible to the antibody because of masking by either cytosolic factor(s) or, in some structures, because of the high packing of membrane (e.g., the rough ER system in the PAC). To overcome these potential problems, we used an alternative approach, described recently (Krijnse-Locker et al., 1994), to investigate the subcellular localization of the KDEL-R. Mouse L cells, both uninfected as well as after infection with MHV for 6 h, were permeabilized with SLO to remove the bulk of the cytosol and the putative soluble masking factor(s). The cells were then fixed and thawed cryosections were processed for immunogold labeling. This approach not only facilitates the visualization of detailed membrane structures (Krijnse-Locker et al., 1994), but it also allows us to identify the intermediate compartment, marked by the budding profiles of MHV. There was variable labeling throughout the Golgi stack (Fig. 3, *D* and *E*). In most cases, essentially the entire Golgi stack was heavily labeled with gold particles (Fig. 3 *E*). There was also a strong labeling of the IC/MHV budding compartment (Fig. 3, *A*–*C*). A significant amount of gold particles were also detected in the rough ER (Fig. 3, *A* and *D*) and the nuclear envelope (Fig. 3 *E*), although the extent of the labeling was clearly less than that seen over the IC and Golgi stack.

To document the localization of the KDEL-R more clearly, the immunogold labeling of the KDEL-R in MHV-infected mouse L cells was quantitated as described in the Materials and Methods (Table I). The specific density of the label (gold per micrometer of membrane) was highest in the IC/MHV budding compartment. The Golgi stack had slightly less but comparable amounts of gold particles. The gold density of

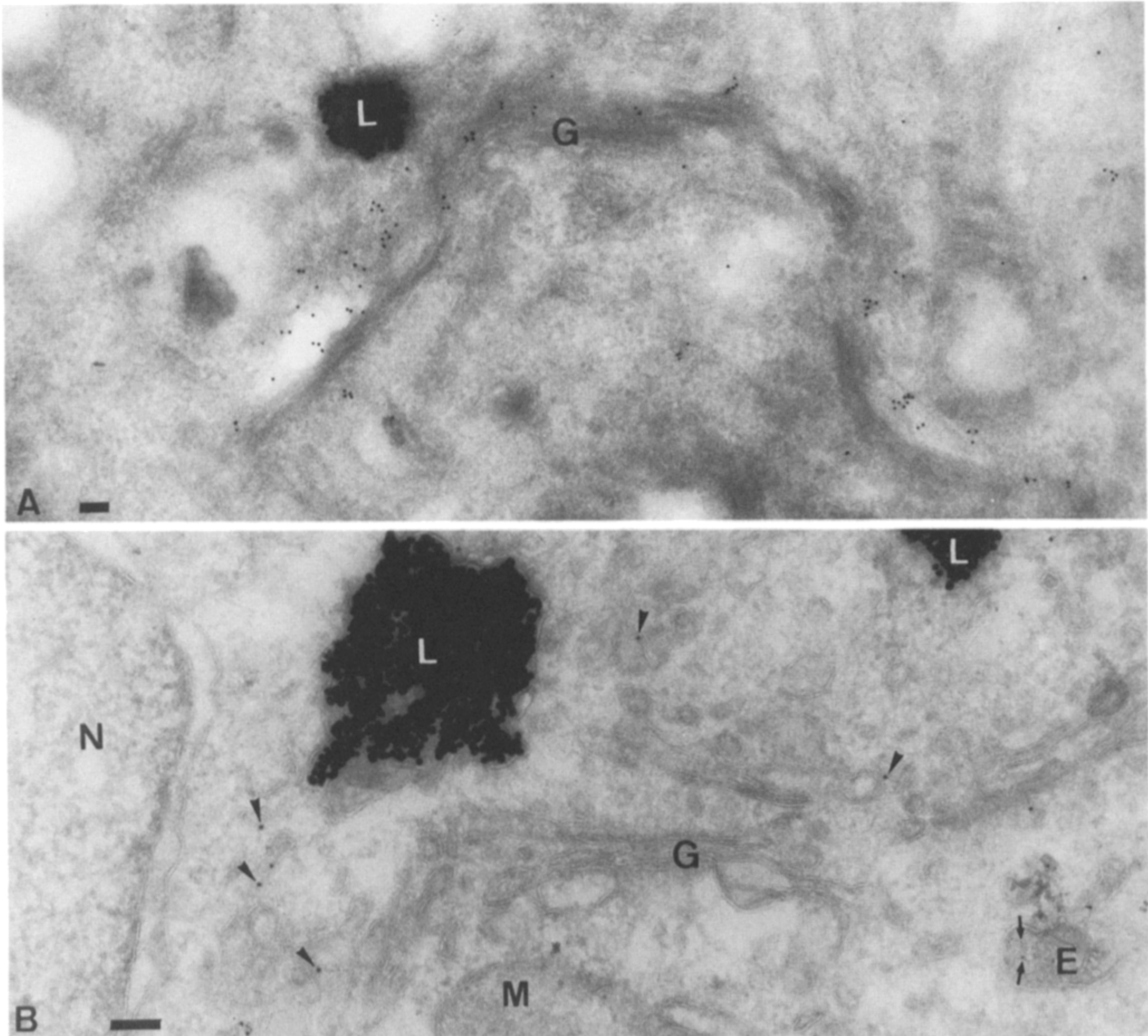


Figure 2. Labeling of HeLa cells for the KDEL-R. These cells were allowed to internalize two different sizes of BSA-gold particles before fixation. First, a 16-nm particle was chased overnight into late endosomes and lysosomes (*L*), and second, a 4-nm particle (*arrows*) was internalized for 5 min into early endosomes (*E*). The membranes of the endocytic compartments are invariably devoid of label for the KDEL-R. (*A* and *B*) Two typical but different labeling patterns. In *A*, the label is predominantly associated with cisternae on one side of the Golgi stack (*G*), whereas in *B*, the stack is essentially free of label, while the peripheral elements label (*arrowheads*). In *A*, the clustering of the label is caused by the use of an intermediate pig anti-rabbit antibody step that amplifies the signal from the bound antibody. *M*, mitochondrium; *N*, nucleus. Bars, 100 nm.

the IC and the Golgi stack is severalfold higher than the average of the rough ER system (rough ER and nuclear envelope). No label was detected on the plasma membrane in this sampling analysis. These quantitative results further illustrate that the receptor is highly enriched in both the IC and the Golgi stack with significant amounts in the rough ER system.

We also labeled sections of SLO-treated, uninfected L cells. The labeling pattern was qualitatively similar to that seen after infection (see below, Fig. 7). When L cells either with or without infection were not treated with SLO, the labeling pattern was similar but there was significantly less labeling than seen in the presence of SLO (not shown). We assume that this is caused by a decrease in accessibility of the antibody to the antigen in the fixed cells that are not treated with SLO.

Establishment of rab1 as an Intermediate Compartment Marker

In MHV-infected L cells, there is considerable amounts of the KDEL-R in the IC, defined as the membrane structures where the virus assembles. To document this point more clearly in uninfected cells, we used an affinity-purified antibody against rab1 since preliminary experiments showed that this antibody was an excellent marker of the IC.

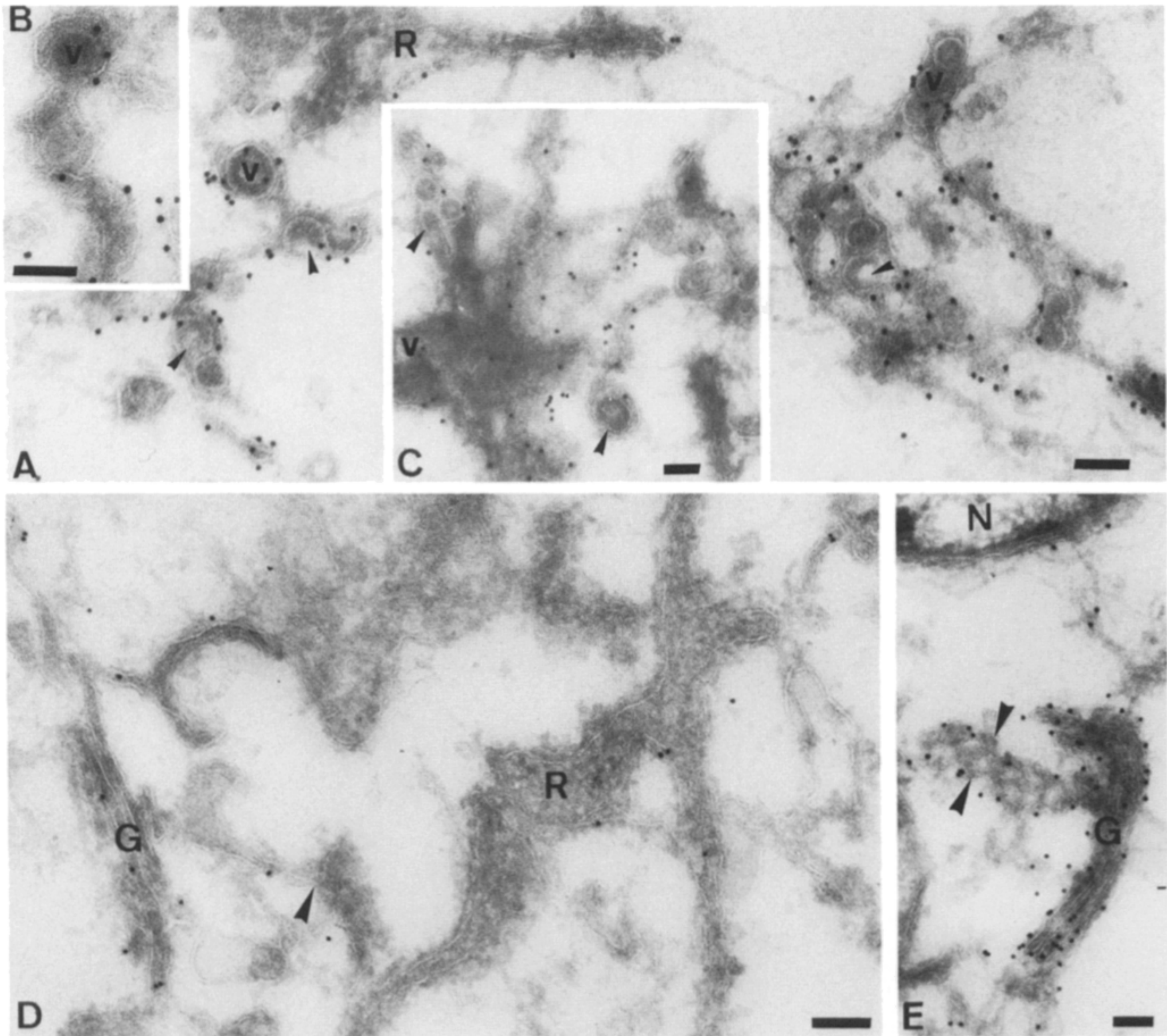


Figure 3. Labeling of SLO-permeabilized, MHV-infected mouse L cells with anti-KDEL-R. Note the abundant labeling of the IC, identified by the presence of virions (*V*), many of which are in the process of budding (*small arrowheads*). The labeling of membranes of the compartment around the virions is especially clear in *B* and *C*. In most (*E*), but not all cases (*D*), the Golgi stack (*G*) is also strongly labeled. There is also detectable labeling of the RER (*R*) in *A* and *D*, as well as the nuclear envelope in *E*. *N*, nucleus. In *D*, the large arrowhead points to a possible continuity between the IC and the RER. In *E*, the large arrowheads denote labeling of a fenestrated cisterna on one (presumably the *cis*) side of the Golgi stack. Bars, 100 nm.

Table I. Quantitation of the KDEL Receptor Labeling of L Cells Infected with MHV and Treated with SLO

Compartment	Gold/μm membrane*
Nuclear envelope	0.8 ± 0.2
Rough ER	1.6 ± 0.2
Intermediate compartment	
(budding MHV)	5.3 ± 0.9
Golgi stack	4.2 ± 0.7
Plasma membrane	0

*These data represent the average of 24 micrographs for each structure. Numbers give the mean and the standard error of the means.

A number of studies have established that the GTP-binding protein rab1 or its yeast homologue, YPT-1, is involved in ER to Golgi transport (Schekman, 1992; Tisdale et al., 1992). In a recent paper, Pind et al. (1994) showed by immunoelectron microscopy that rab1 is enriched in the tubular-vesicular structures where the G protein of VSV is enriched when ER to Golgi transport is blocked. By immunofluorescence microscopy these structures colocalized with p58, the marker of the IC. Whereas the well-established markers of the IC such as p58/p53 or rab2 are often in relatively low levels for immunogold labeling, when we tested an affinity-purified antibody against rab1, we noticed that this marker appeared to

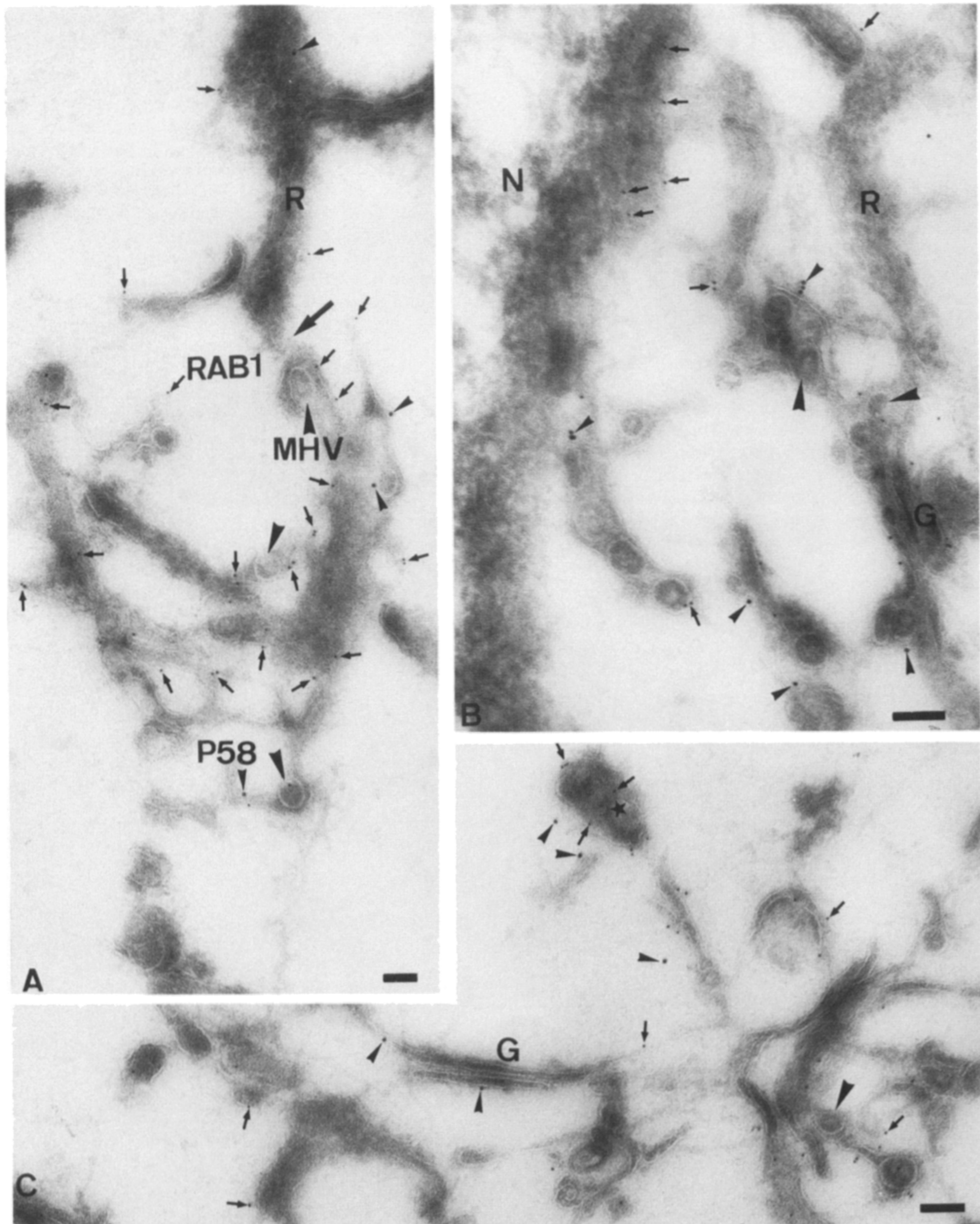


Figure 4. Cryosections of MHV-infected L cells treated with SLO. The sections were double labeled for rabl (*small arrows*, 5 nm gold) and p58 (*small arrowheads*, 10 nm gold). Note the extensive labeling for rabl on the membranes associated with the budding virions (*large arrowheads*). This labeling extends to parts of the nuclear envelope (*B*), as well as to one or two Golgi cisternae (*C*). p58 labels the same structures, albeit with less intensity. The rough ER (*R*) is relatively poorly labeled for both rabl and p58. A possible continuity between the RER and the IC is indicated in *A* (*large arrow*). The asterisk in *C* shows an electron-dense "vesicular domain" of the IC. Bars, 100 nm.

be much more abundant in the IC than the previously used markers.

We first asked whether rabl colocalized with p58 and with the membranes where MHV bud in L cells infected with this virus and permeabilized with SLO. As shown in Fig. 4, there was extensive labeling for rabl in all the membrane structures that we had previously identified as having a role in the budding of the virus, including parts of the nuclear envelope (Fig. 4 *B*). The labeling for rabl colocalized with p58, but it was much more extensive. There were detectable but significantly lower levels of labeling for both markers over the rough ER (Fig. 4, *A* and *B*). The rabl labeling also extended to one or two cisternae on the *cis* side of the Golgi stack, which also contained budding virion profiles. Rabl and p58 also colocalized in uninfected L cells treated with SLO, and the amount of labeling for both markers was very similar to the levels seen in infected cells (Fig. 5 *A*). Fig. 5 *A* documents the extensive network of interconnected membrane structures that extend considerable distances away from recognizable Golgi stacks. This network comprises many different morphological structures. The large arrow in Fig. 5 *A* points to a continuity between an apparently tubular structure with a larger, more electron-dense domain of the IC. Vesicular buds that have the morphological features expected of COP coats are also apparent on the IC (Fig. 5 *A*). In Fig. 5 *B*, the extent of rabl labeling of Golgi associated cisterna(e) is apparent.

Colocalization of the KDEL-R with KDEL Ligands and rabl

In our previous study, we showed that the KDEL-containing protein disulfide isomerase (PDI) labeled not only the rough ER, but also the IC, extending to one cisterna on one side of the Golgi stack (Krijnse-Locker et al., 1994). As shown in Fig. 6, the PDI labeling also colocalized with rabl in an uninfected L cell treated with SLO, both extending to one Golgi cisterna. This point was documented further by using antibodies against a spectrum of KDEL proteins (Nguyen Van et al., 1989; Peter et al., 1992), including the calcium binding proteins CaBPl, CaBP2, and CaBP3 (calreticulin), as well as a generic anti-KDEL peptide antibody. The localization of these proteins in infected (not shown) and uninfected cells (Fig. 7) was indistinguishable from that for PDI with a strong labeling of the rough ER, nuclear envelope, and the IC, extending in some sections to one, and occasionally two Golgi cisternae (not shown; see below). Fig. 7 shows representative examples of the double labeling of KDEL-R and either the anti-KDEL peptide antibody (Fig. 7, *A* and *B*) or anti CaBPl (Fig. 7 *C*) in uninfected cells permeabilized with SLO. Whereas the Golgi stack is, for the most part, unlabeled for the KDEL proteins, significant levels of labeling are usually found in one and occasionally two cisternae, as well as in typical IC membranes on one side of the stack (Fig. 7, *A–C*). These membrane structures also la-

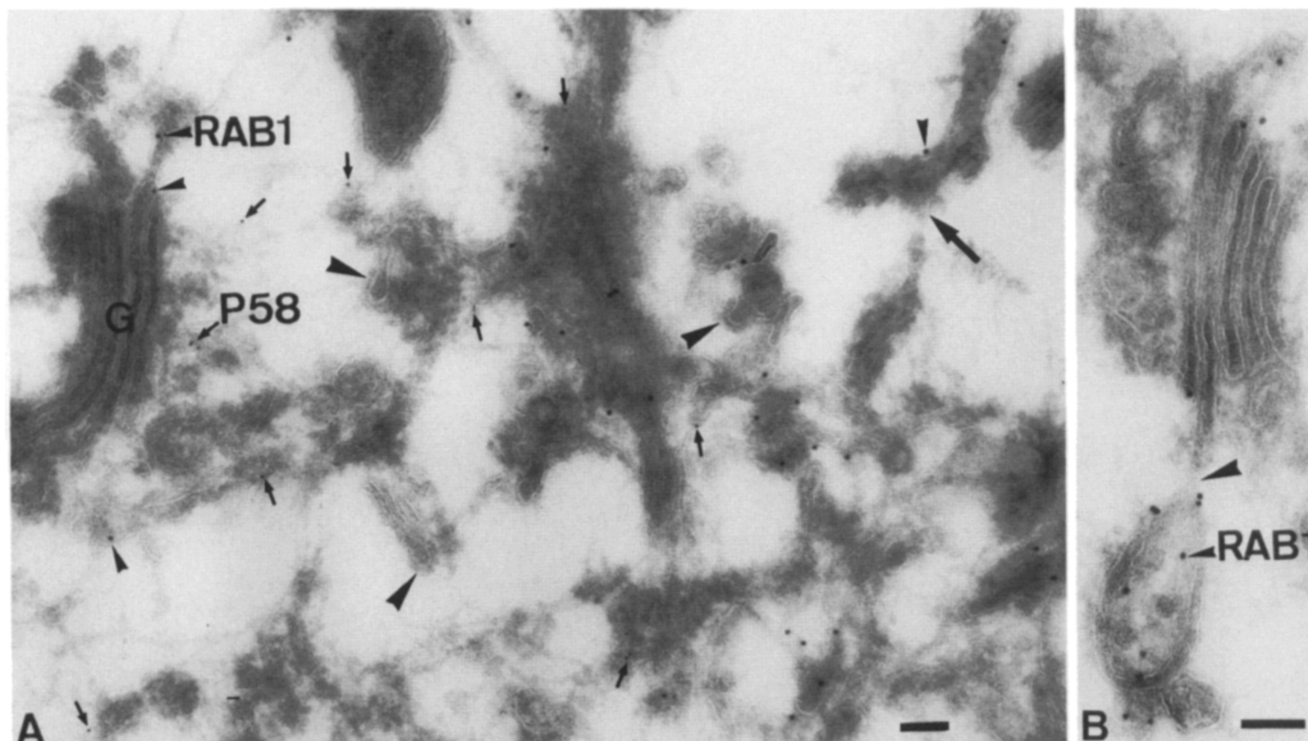


Figure 5. Cryosections of an uninfected L cell permeabilized with SLO. (*A*) Double labeling for rabl (*small arrowheads*, 10 nm gold) and p58 (*arrows*, 5 nm gold). Note the extensive nature of the IC extending to one side of the Golgi stack (*G*). The large arrow shows a continuity between a narrow, presumably tubular domain with a larger, more electron-dense vesicular profile of the IC. The large arrowheads show coated buds that are most likely to be COP. (*B*) Single labeled for rabl, a continuity between the stack and a curved, cisternal element labeled for rabl is shown. Bars, 100 nm.

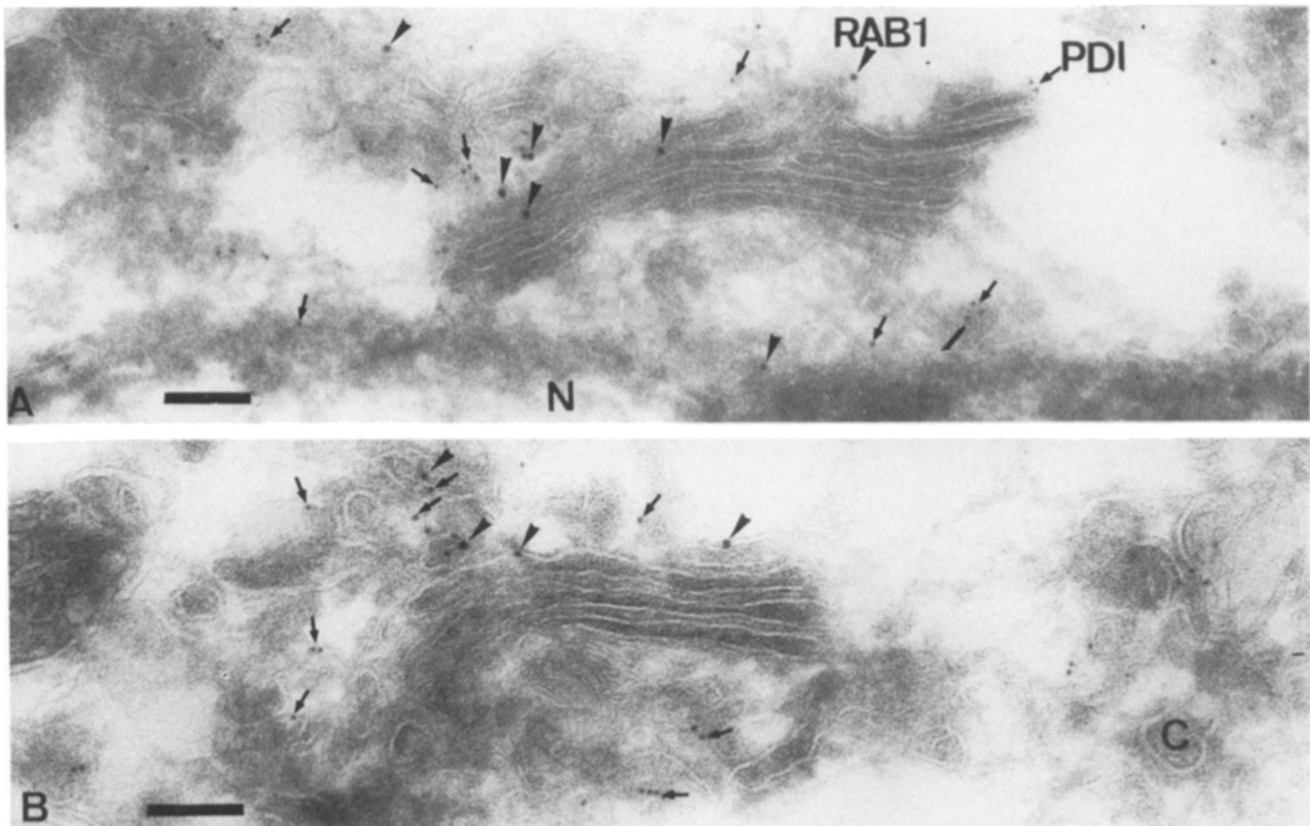


Figure 6. Colocalization of rabl and PDI in an SLO-treated, uninfected L cell. Rabl (*arrowheads*, 10 nm gold) colocalizes with PDI (*arrows*, 5 nm gold) in membrane structures close to the *cis* side of the Golgi stack, including one cisterna. *N*, nucleus; *C*, putative clathrin bud. Bars, 100 nm.

beled significantly for the KDEL-R (Fig. 7, *A–C*). However, the labeling of the latter often extends throughout the Golgi stack, although on average, the bulk was concentrated on the *cis* side of the stack, as for the other cell types examined. From this analysis, we conclude that the distribution of KDEL ligands and the KDEL-R significantly overlap in IC structures, including cisternal domains on one side of the Golgi stack.

The extent of colocalization of the KDEL-R with rabl was next analyzed by double labeling of cryosections of SLO-permeabilized, uninfected L cells. As is evident in Fig. 8, there was extensive colocalization of these two proteins, a phenomenon most prominently seen on one side of the Golgi stack and in the IC, evident as membrane structures extending from the stack. The KDEL-R also tended to label the whole stack (Fig. 8, *A–C*), albeit with a high variability from one stack to the next, as before. The three-dimensional complexity of the Golgi complex is especially evident in Fig. 8 *D*, where a cisterna labeled for both rabl and the KDEL-R appears to wrap around two unlabeled cisternae. This double labeling analysis is consistent with the results of the MHV-infected cells, with both sets of experiments arguing strongly for a significant concentration of the KDEL-R in the IC and in the Golgi complex.

Labeling of the KDEL-R in the TGN of Cultured Cells: Effect of Virus Infection, Temperature, and pH

The data from tissue sections, and especially the early spermatids, argue that the levels of the KDEL-R in the TGN are extremely low when compared to the amounts seen on the *cis* side of the stack. The same impression was obtained in NRK and HeLa cells. In contrast, in the L cells infected with MHV (and to a lesser extent also in uninfected L cells), it seemed that there was more labeling over the whole Golgi stack. However, we were not confident that we were visualising the TGN under this condition. We therefore used two well-characterized model systems where the TGN is more distinct.

The first system was VSV-infected cells that have been left at 20°C for 2 h. Under this condition, the bulk of the G protein accumulates predominantly in the TGN, which enlarges considerably while the Golgi stack shrinks (Griffiths et al., 1985, 1989). In addition to the TGN, significant amounts of G protein may also accumulate throughout the Golgi stack. Here, we used mouse L cells that were infected with a ts045 strain of VSV at 37° for 1 h, switched to 39° to accumulate the G protein in pre-Golgi structures, and then put at 20°C for 2 h in the presence of cycloheximide (Griffiths et al., 1985). When sections of these preparations were double-labeled for the G protein (using antibodies against either the lumenal or cytoplasmic domains) and the KDEL-R, there was extensive colocalization of both markers (Fig. 9). In many parts of these cells, the distinctive features of the TGN could be easily recognized, and some of these regions had relatively high levels of labeling for the KDEL-R (Fig. 9).

The second model we tested was vaccinia virus infection.

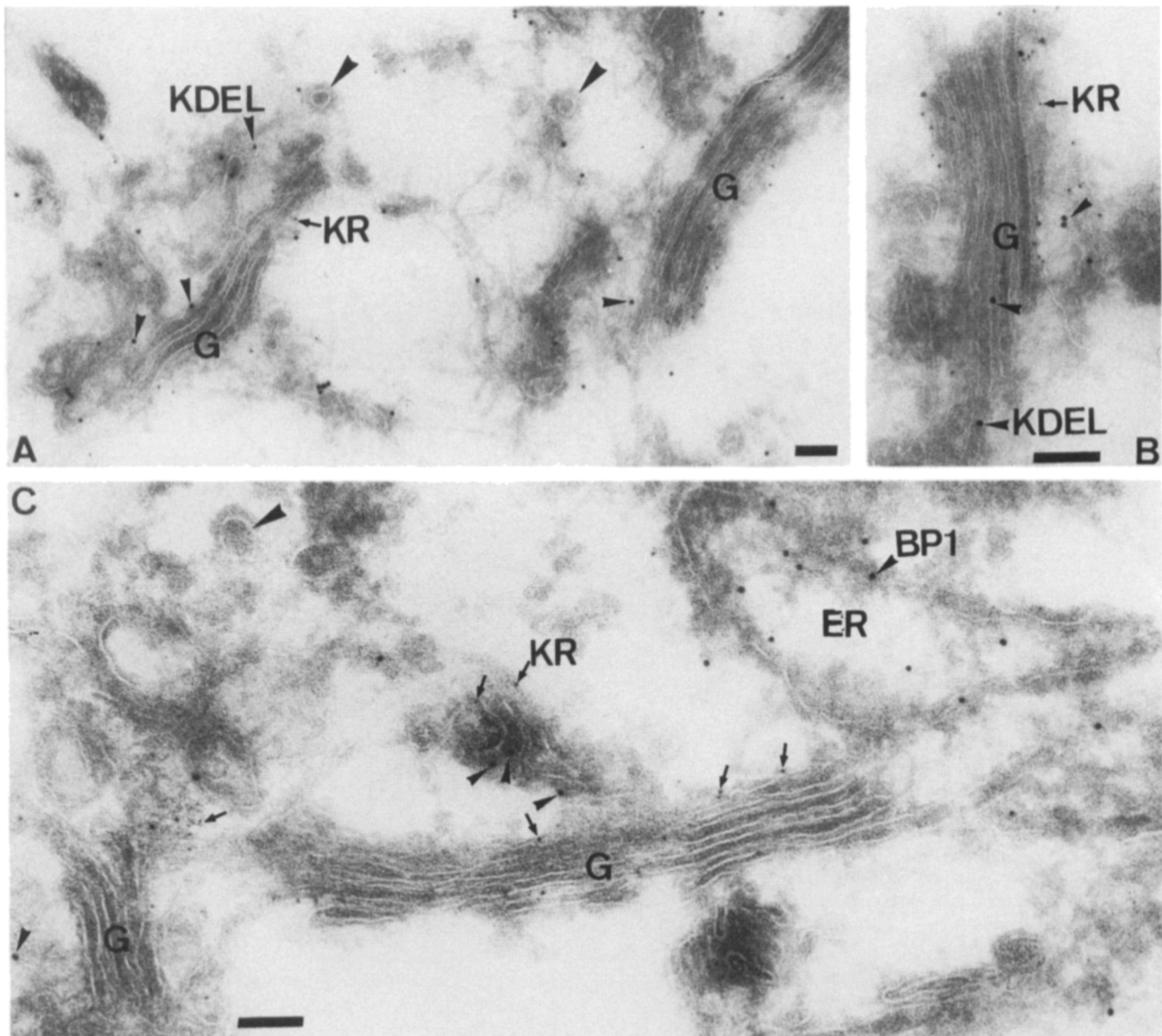


Figure 7. Partial colocalization of KDEL proteins (*arrowheads*, 10 nm gold) with the KDEL-R (*KR*) (*arrows*, 5 nm gold) in SLO-treated, uninfected L cells. (*A* and *B*) An anti-KDEL peptide (*KDEL*) is used while *C* shows labeling for CaBPl (*BPI*). While the rough ER is strongly labeled for all KDEL proteins (shown for CaBPl in *C*), it has only very low levels of labeling for the KDEL-R (no labeling evident in *C*). In structures directly adjacent to the stack, including the first cisterna, there is extensive colocalization of both the KDEL ligand and the KDEL-R. The latter also extends into more central regions of the stack. The arrowheads indicate putative COP buds. Bars, 100 nm.

Whereas the early stages of the virus acquire their membranes from the IC to form the intracellular mature virus (IMV), at a late stage in the infection, the IMV becomes enwrapped by a cisterna that originates from the TGN (Schmelz et al., 1994). In sections of cells infected for >8 h with vaccinia, these wrapping membranes can easily be identified. The use of a vaccinia recombinant expressing the M protein of MHV gave us a second marker for the TGN since, after a cycloheximide chase, the bulk of this protein in this system is localized to the *trans*-Golgi/TGN under these conditions (Krijnse-Locker et al., 1992). Sections were made of L cells infected for 8 h with the M protein recombinant vaccinia virus. These were double labeled for the KDEL-R and for the M protein. The two proteins extensively colocalized and this colocalization extended to the membranes enwrapping the virions (not shown; see below).

Thus, in two different viral systems, the KDEL-R appeared to be found in extremely high amounts in the TGN. This result was clearly at variance with the very low levels detected on the *trans* side of the stack, both in the tissues and the uninfected HeLa and NRK cells. Since the KDEL-R labeled the whole stack in the MHV-infected L cells, these data collectively suggested that, after viral infection, the distribution of the KDEL-R shifts from the *cis* side towards the TGN.

To unequivocally identify the TGN in an uninfected cultured cell, we used a HeLa cell (SA:48) stably expressing the cDNA encoding the human α-2,6-sialyl transferase tagged

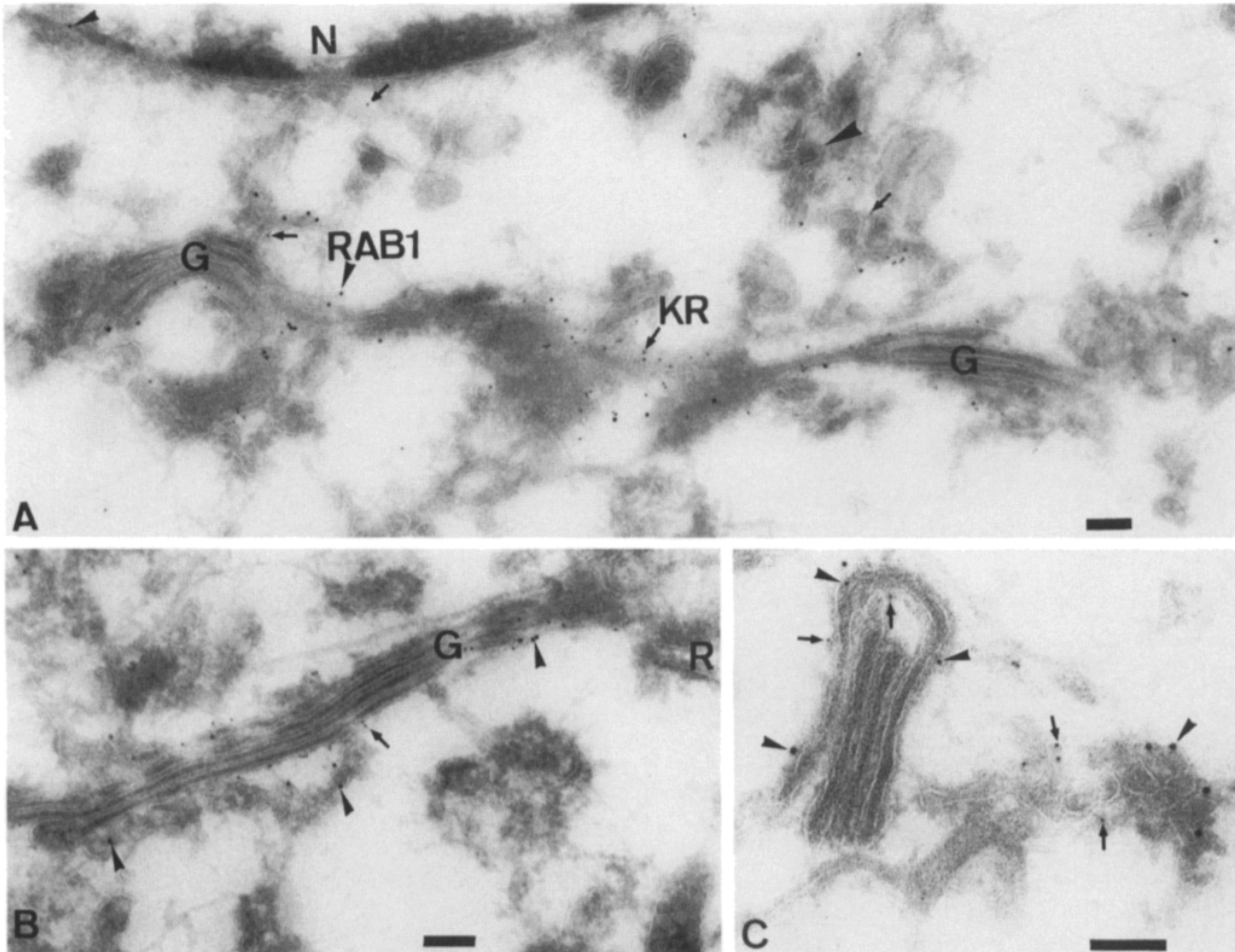


Figure 8. Colocalization of the KDEL-R (*KR*; 5 nm gold) with rabl (10 nm gold) in SLO-permeabilized, uninfected L cells. Note that both markers codistribute over extensive regions adjacent to the Golgi stack (*G*), and this colocalization extends also to one (or two) cisternae of the stack. In *D*, note the bending of one cisterna, labeled for both markers, around the central cisternae. *N*, nucleus. Bars, 100 nm.

on the lumenal side with the P5D4 epitope (Kreis, 1986) from the cytoplasmic domain of the VSV-G protein. The sialyl transferase is known to be localized to the TGN (Roth et al., 1985), and this construct is efficiently targeted to the TGN by all criteria tested (Rabouille, C., F. Hunte, R. Kieksbusch, E. Berger, G. Warren, and T. Nilsson, manuscript in preparation). When sections of these cells were double labeled with the anti-KDEL-R and a polyclonal antibody against the G protein tail epitope, the bulk of the KDEL-R did not colocalize with the structures enriched in the sialyltransferase construct (Fig. 10 *A*). However, a small, but significant amount of labeling did colocalize (not shown; see Table II). In contrast, in SA:48 cells infected with the vaccinia virus recombinant expressing the M protein of MHV, there was significantly higher labeling for the KDEL-R in the *trans* cisternae/TGN (Fig. 10, *B* and *C*).

The quantitative evaluation of these data from SLO-treated cells indicate that, whereas only 17% of the KDEL-R is on the *trans* side of the stack in the uninfected cells, after vaccinia infection, the bulk (62%) was now shifted to the *trans* side (Table II).

To test whether the observed shift of the KDEL receptor towards the *trans* side of the stack was a specific response to viral infections, or rather a general response to cellular stress, we cultured uninfected SA:48 cells under two different extremes of temperature, 20°C (2 h) or heat shock, 43°C (4 h). These cells were then prepared for immunocytochemistry and double labeled as before for the KDEL-R and the G protein tag. A quantitative analysis of these experiments gave the following results. After the 20°C incubation, 56% of the Golgi stack-associated labeling for the KDEL-R was detected on the *trans* side, while after the heat shock treatment, the corresponding value was 46%. These results suggest that the shift of the KDEL receptor towards the TGN side of the stack may be a general response to conditions of cellular stress.

A recent paper from the Pelham group has provided in vitro data showing that binding of KDEL ligands to permeabilized Golgi membranes is highest at acidic pH (optimal pH ~5) (Wilson et al., 1993). We therefore asked whether the localization of the KDEL receptor was affected by bafilomycin A1, a specific inhibitor of the vacuolar proton ATPases (Altendorf et al., 1989). For this, the SA:48 HeLa cells were filled with 16-nm gold-BSA (to mark late endosomes and

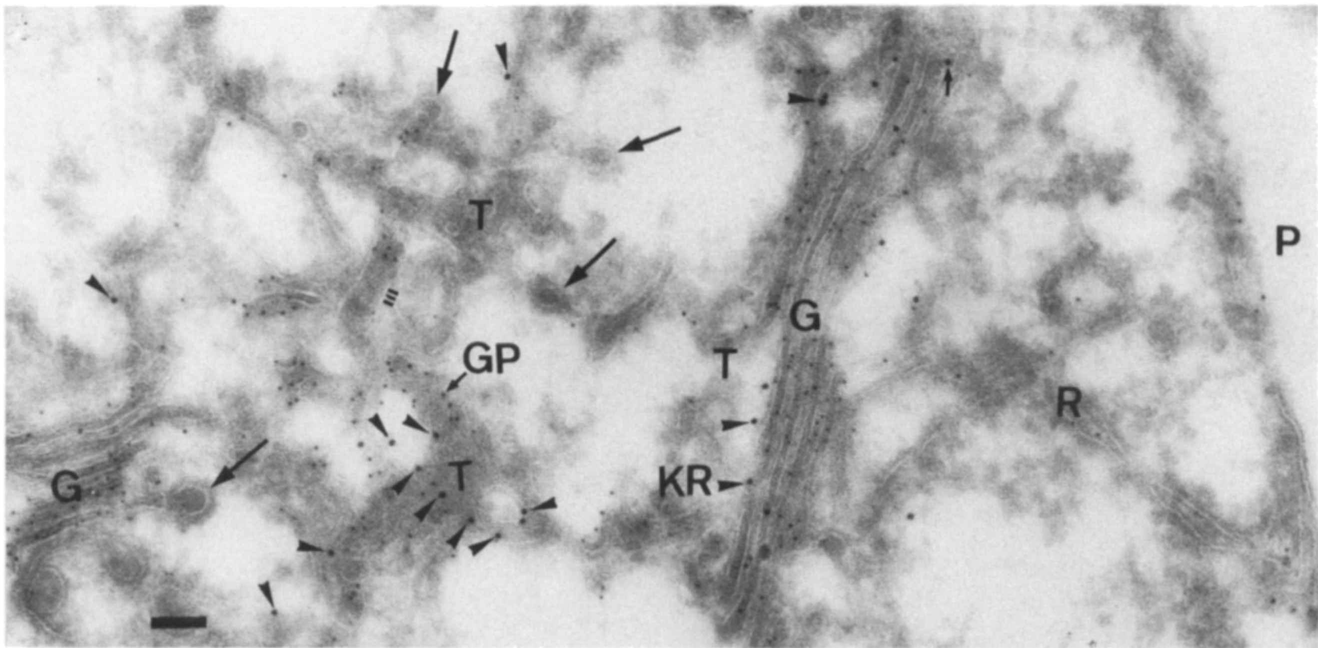


Figure 9. Double labeling of SLO treated L cells infected with the ts 045 VSV and switched to 20°C (plus cycloheximide) using anti-KDEL-R (*KR*; *arrowheads*, 10 nm gold) and anti-G protein spike (*GP*; *small arrows*, 5 nm gold). Note the extensive TGN elements (*T*) that label significantly for both the G protein and for the KDEL-R. The close packing of G protein into morphologically distinct quasicrystalline assays in the TGN at 20°C has been previously documented (see Griffiths et al., 1985, 1989). These assays (*parallel bars*), as well as the numerous buds that accumulate (*large arrows*), are diagnostic features of the TGN under this condition. The G protein labeling also extends throughout the stack although it is often lower on the *cis* side (note the stack on the lower left part of *A*). Some residual G is also present in the rough ER (*R*), and a small amount evidently leaked to the plasma membrane (*P*). Bars, 100 nm.

lysosomes, after an overnight chase) and subsequently with 4-nm gold-BSA for 5 min (early endosomes) before fixation. For the last 8 h before fixation, the cells were treated with 500 nM bafilomycin A1. This treatment effectively neutralized low pH compartments since by light microscopy we observed the loss of acridine orange staining of endosomes/lysosomes (results not shown). The endocytic compartment-marked, bafilomycin-treated, SA:48 cells were then double-labeled with anti G tail (6 nm gold) and anti-KDEL-R (10 nm gold). Under this condition, there was no significant labeling of any endocytic structure (not shown). When we quantitated the KDEL-R labeling over the Golgi stack, as above, there was no change in the distribution of the receptor (with 18% of the receptor on the *trans* side of the stack and 82% on the *cis*). These data argue that bafilomycin has no effect on the distribution of the KDEL-R.

Localization of the KDEL-R to β-COP Buds/Vesicles

The nonclathrin COP vesicles are widely believed to facilitate transport between the ER and the Golgi complex, as well as intra-Golgi transport (Balch, 1990; Rothman and Orci, 1992). When permeabilized cells or in vitro Golgi fractions are treated with GTPγS, a large increase can be seen in the number of COP-containing buds and/or vesicles (Rothman and Orci, 1992; Duden et al., 1991). In our recent study, we could show that after GTPγS treatment of SLO-permeabilized mouse L cells, many of the β-COP-containing vesicular buds were continuous with the membranes of the IC (Krijnse-Locker et al., 1994). We therefore postulated that these COP vesicles are responsible for the first vesicular transport step in the biosynthetic pathway, a step that mediates transport from the IC to the Golgi compartment, where Man I is expected to be localized.

Since the KDEL receptor would be expected to be continuously trafficking between the IC and the Golgi complex we asked whether this transport step might also be mediated by COP vesicles. We therefore prepared L cells that were either not infected or infected with MHV for 6 h and permeabilized with SLO in the presence of 50 μM GTPγS to facilitate the identification of these COP buds/vesicles. These cells were sectioned and double labeled with antibodies against the KDEL-R and β-COP. After GTPγS treatment, there was extensive proliferation of buds/vesicles that labeled with anti-β-COP in single labeling studies (not shown). When the sections of this preparation were double-labeled with anti-β-COP and anti-KDEL-R, there was extensive colocalization of the two markers (Fig. 11, *A–C*). In MHV-infected cells, many of these buds were continuous with the IC, defined as membrane profiles into which the MHV assemble (Fig. 11 *D*; see Krijnse-Locker et al., 1994). Thus, a significant fraction of the COP buds containing detectable amounts of KDEL-R labeling appear to be derived from the IC. The absence of appropriate (early) Golgi markers made it difficult, as in our previous study, to identify the COP buds/vesicles that originate from Golgi compartment(s).

To document this result more clearly, we carried out a quantitative analysis. For this, the GTPγS-treated cells were double labeled with anti-KDEL-R and anti-β-COP. Using the β-COP labeling to identify the buds/vesicles, the number of COP vesicles that gave at least one gold particle for the KDEL-R were counted (total 200 buds/vesicles). This anal-

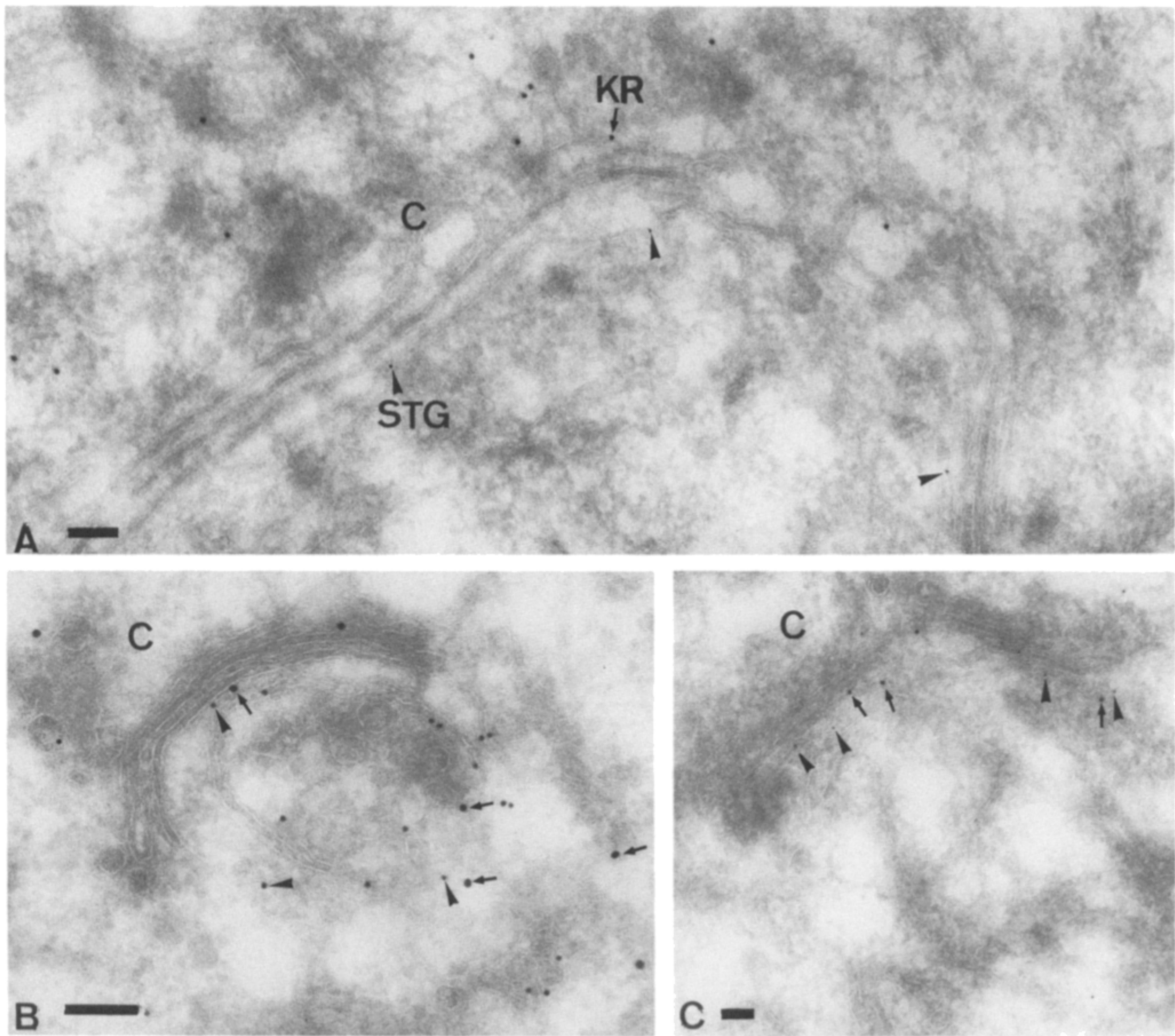


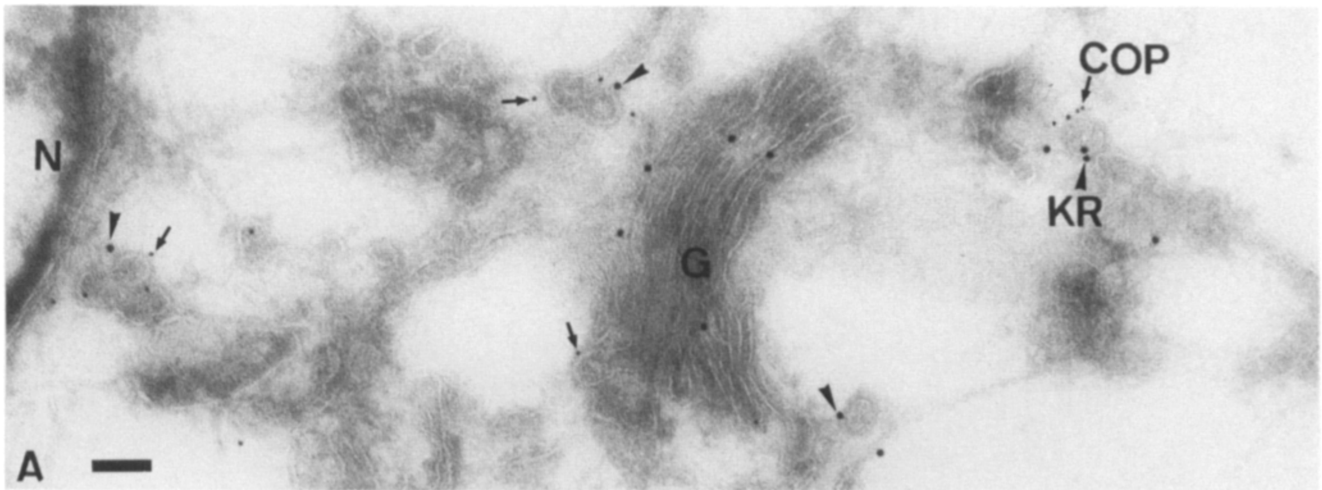
Figure 10. Labeling of HeLa SA:48 cells with anti-KDEL-R (*KR*; *small arrows*, 10 nm gold) and the anti-G tail, which detects the sialyl transferase-G hybrid protein (*STG*; *arrowheads*, small gold). *A* is from uninfected cells, while *B* and *C* are from vaccinia-infected cells. In *B* the KDEL-R is labeled with 15 nm gold and the G is labeled with 10 nm gold, while in *C* it is KDEL-R (10 nm) and G protein (5 nm). The bulk of the KDEL-R labeling is on the *cis* side of the Golgi (*C*) stack in uninfected cells, but the distribution shifts towards the TGN after vaccinia infection. Bars, 100 nm.

*Table II. Quantitation of the KDEL Receptor in SLO-treated HeLa SA:48 Cells Expressing the G Protein–Sialyl Transferase Hybrid Molecule**

	Total Gold particles		Percent of KDEL-R on *trans* side of stack
	cis side‡	*trans* side‡	
Uninfected cells (*n* = 28; Golgi stacks)	151	30	16.6
Vaccinia infected (*n* = 22; Golgi stacks)	44	72	62.0

*The cryosections were double labeled with anti-KDEL-R (10 nm gold) and a rabbit anti-G protein tail (P4; 5 nm gold).
‡The *cis* and *trans* sides were distinguished using the 5-nm gold labeling, and an imaginary line was used to define the middle of the stack.

ysis showed that 24% of these vesicles were labeled for the KDEL-R, whereas on average, only 4% of the COP-coated buds were labeled using two irrelevant antibodies against vaccinia virus proteins. We conclude that a significant amount of the KDEL receptor can be detected in COP buds/vesicles after the GTPγS treatment. Using double labeling studies, we also found a significant labeling for rabl on these COP/bud vesicles (results not shown). The latter result is consistent with the recent data of Peter et al. (1993) and Pind et al. (1994). In the absence of the GTPγS, there were far fewer COP buds/vesicles, which made it much more difficult to evaluate whether significant amounts of the KDEL-R were also present in these structures under normal conditions.


COP
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KR
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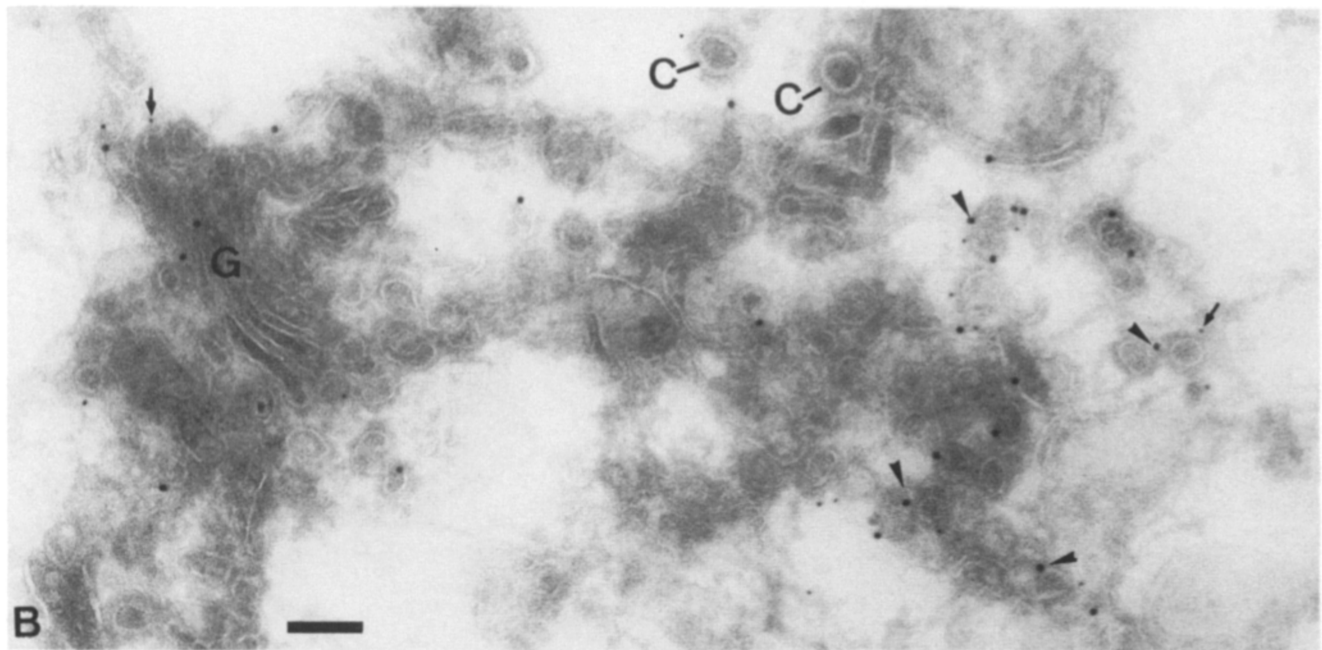
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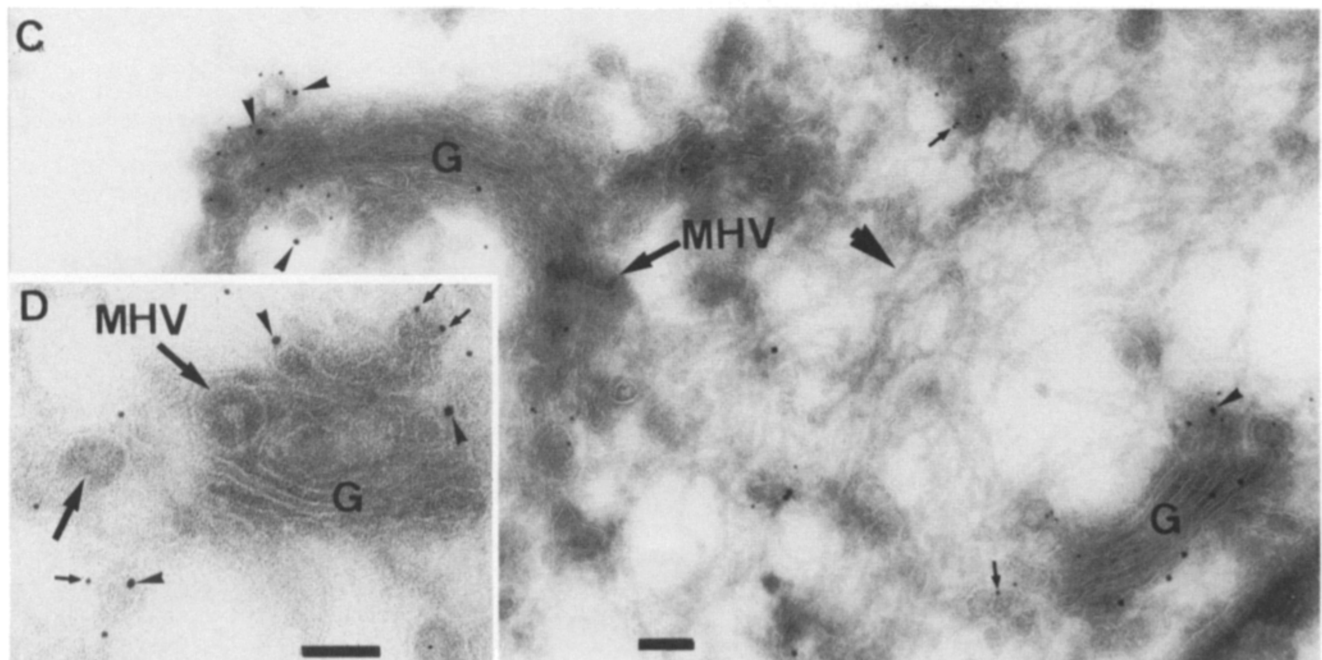
C
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Discussion

The Distinction between the Intermediate Compartment and the Golgi Complex

We have recently presented evidence that the intermediate compartment between the rough ER and the Golgi stack is essentially a specialized subdomain (or domains) of the ER from which COP vesicles destined for the Golgi complex originate (Krijnse-Locker et al., 1994). We operationally define the first of the Golgi compartments as that where Man I functions. This notion would be consistent with the fact that this is the first N-linked oligosaccharide modification seen after release of the 15°C block (which defines the ER to Golgi boundary) (Balch, 1989). In the present paper, we provide additional support for our model. First, we show that rabl is an excellent marker for the IC since it precisely colocalizes with both p58 (which is a less abundant marker) in both uninfected cells and in MHV-infected cells. In many cases, the labeling for rabl extends to the first (and occasionally two) cisternae of the Golgi stack. Further, we provide additional evidence that KDEL proteins are also enriched in the IC. In most cases, these KDEL proteins are only in very low amounts in central parts of the Golgi stack. It should again be emphasized that a more definitive definition of the boundary between the IC and the bona fide Golgi complex awaits the availability of high titer antibodies against early Golgi proteins. A consequence of this problem is that it is difficult at present to estimate how much of the rabl is found in the Golgi compartments distal to the IC.

The Localization of the KDEL-R

In every cell type we investigated in this study, there was a high concentration of the KDEL-R on the cisternae localized to the *cis* aspect of the Golgi stack. Under normal, steady-state conditions, our data argue that only low levels of the receptor are found in the *trans* cisternae and the TGN. Under no condition did we detect any significant amounts of this protein in any compartment distal to the TGN.

In many, but not all of the cell types we examined, the labeling extended to membrane elements peripheral to the Golgi stack. The bulk of this labeling was localized to the IC, which we could convincingly identify in the SLO-permeabilized, MHV-infected L cells by the presence of budding virions. In these cells, the concentration of the receptor in the IC, as estimated by quantitation of the immunogold labeling, was similar to that observed in the Golgi stack. In these cells, we could also detect a significant (albeit fivefold lower than the IC/Golgi) labeling of the rough ER and a still lower level in the nuclear envelope. In these SLO-treated cells, the accessibility for the cytoplasmic epitopes of the KDEL-R for the antibody was clearly enhanced on the sections. This was evident from a comparison of the labeling of permeabilized with nonpermeabilized cells. Therefore, in the absence of the permeabilization step to remove cytosolic components, the anti-KDEL-R may have significantly less accessibility to its antigen on the section. This point should be considered in evaluating the absence of detectable labeling in the rough ER of cells such as the pancreatic acinar cells.

We were initially surprised in this study to see such high levels of labeling for the KDEL-R in the Golgi stack and in the IC, levels which approached those seen for viral spike proteins in the Golgi complex of infected cells. However, a recent study by Wilson et al. (1993) estimated from the number of KDEL binding sites available in alkali-treated rat liver Golgi fractions that the KDEL receptor would constitute ~1.3% of the mass of total Golgi membranes. These data, in conjunction with our results, indicate that the receptor is a highly abundant protein of both the Golgi complex and the IC.

The validity of using the MHV budding compartment as a marker of the IC was supported by our analysis of rabl. This marker colocalized with the MHV budding compartment and with p58 in both infected and uninfected cells. Whereas the density of immunogold labeling seen for rab2, p58/p53 is usually relatively low, the affinity-purified antibody against rabl gave unexpectedly strong labeling of the extensive peri-Golgi membrane structures. It also facilitated our appreciation of the heterogeneous nature of the IC since it labeled many different kinds of morphological entities within what we consider to be the IC network in uninfected cells. In the absence of such a marker, many of the electron-dense elements (Figs. 5 *A* and 7 *A*) might easily be mistaken for parts of the endocytic pathway. The use of this marker established beyond doubt that, also in uninfected cells, considerable amounts of the KDEL-R can be localized to the IC at steady state.

Shift of the KDEL-R to the TGN under Conditions of Stress

While the levels of the KDEL-R in the TGN of all cell types we examined under normal conditions were relatively low, our data suggest that when the cells are infected with viruses or treated at low or high temperatures, the protein shifts its distribution such that much higher levels are found in the TGN. These observations suggest that the shift of the KDEL-R towards the TGN may be a general response of the cell towards conditions of stress. It should be noted that, for all of the virally infected cells we used, host protein synthesis is effectively switched off. In these cases, the redistribution of the KDEL-R must be independent of host protein synthesis. Further studies will be necessary to determine whether this is a general response of cells to situations of stress, as well as to work out the functional significance of such a shift in distribution.

Although the KDEL-R is normally present at lower levels in the TGN as compared to the IC and the Golgi stack, its

Figure 11. Sections of uninfected (*A* and *B*) or MHV-infected (*C* and *D*) L cells treated with SLO followed by GTPγS and double labeled with anti-KDEL-R (*KR*; 10 nm gold) and anti-β-COP (*arrows*, 5 nm gold). The COP buds/vesicles that label for the KDEL-R are indicated by arrowheads. *C* denotes putative clathrin coated buds/vesicles. In *C* and *D*, the structures where the assembling virions are found (*large arrows*, MHV) are continuous with buds/vesicles that label for β-COP and for the KDEL-R. The large arrowhead in *C* indicates the network of intermediate filaments that often become visible after SLO extraction. Bars, 100 nm.

existence in the TGN may play a functional role. The TGN is the major site where the endocytic pathway converges with the exocytic pathway (Griffiths and Simons, 1986; Duncan and Kornfeld, 1988; Neefjes et al., 1988; Green and Kelly, 1990), and recent studies have shown that the effects of some toxins, which enter the cell via the endocytotic pathway, depend on their COOH-terminal, KDEL-like sequence (Chaudhary et al., 1990; Pastan et al., 1992 and references therein; Pelham et al., 1992 and references therein). Indeed, the cytotoxic activity of some toxins could be enhanced by having the KDEL sequence on the COOH terminus (Seetharam et al., 1991). Furthermore, a KDEL-containing ER glycoprotein, calreticulin ($CaBP_3$), acquires significant amounts of galactose, a *trans*-Golgi modification, in rat liver (Peter et al., 1992). These observations, in conjunction with the localization of some KDEL-R to the TGN, suggest that KDEL receptor-mediated retrieval from this compartment may be involved in the transport of these toxins to the ER, where translocation to the cytosol has been proposed to occur (Pastan et al., 1992; Pelham et al., 1992). A recent study by Sandvig et al. (1992) has now directly demonstrated that endocytosed Shiga toxin could be transported to the ER, most likely via the TGN and the Golgi stack.

The Role of COP Vesicles in KDEL Receptor Trafficking

There is now compelling evidence that COP vesicles mediate both ER to Golgi, as well as intra-Golgi traffic (Pepperkok et al., 1993; Peter et al., 1993; Rothman and Orci, 1992). In permeabilized cells, the budding and/or fusion of these vesicles can be arrested with GTPγS, a treatment that leads to a considerable increase in the frequency of COP vesicles (Krijnse-Locker et al., 1994). A significant finding in the present study was the presence of easily detectable levels of the KDEL-R in these GTPγS-arrested COP buds and/or vesicles. Using the MHV (as well as rabl: see Fig. 5 *A*) to identify the IC, we can conclude that at least some of these buds/vesicles originate from the IC, and that they are probably involved in anterograde traffic from the IC into the Golgi complex (presumably the Man I compartment). The simplest interpretation is that the "empty" receptor follows the normal vesicular traffic route from the ER (IC) to the Golgi. Whether any of the COP vesicles may also be involved in the retrograde trafficking pathway can only be investigated when a suitable system becomes available for following this retrograde transport step in more detail.

Retrieval of KDEL-containing Proteins at Multiple Post-ER Compartments

The finding that a significant amount of the KDEL receptor is present in both the IC and the Golgi stack means that the receptor is concentrated in at least two distinct functional compartments that are connected by vesicular transport, at least in the anterograde direction. This observation is significant since it is consistent with the generally accepted model for KDEL receptor function. Accordingly, the receptor would bind ligand in a downstream compartment and recycle it back to an upstream organelle that, by definition, must have a different lumenal environment necessary for the ligand to dissociate (Munro and Pelham, 1987; Pelham, 1988, 1989). A recent study has shown that the binding of KDEL ligands to Golgi membranes in vitro is enhanced at relatively low pH (~5) (Wilson et al., 1993). Although this suggests that pH may be an important factor for binding in vivo, this notion is hard to reconcile with other lines of evidence that argue in favor of a neutral pH in the pre-TGN compartment(s) of the Golgi complex (Griffiths and Simons, 1986; Anderson and Orci, 1988). In this study, bafilomycin A1 had no effect on the distribution of the receptor. While the precise role of pH in the binding of KDEL ligands to the receptor in the cell remains to be elucidated, our data argue that the "downstream" organelle where the KDEL-R binds its ligands is the Golgi complex, while the upstream (dissociation) organelle would be the IC. The number of compartments comprising the Golgi is still controversial (see Mellman and Simons, 1992). However, the finding of low but variable labeling for the KDEL-R in the TGN would necessitate, at a minimum, one additional recycling step from the TGN back to earlier compartments.

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