

# Morphological Analysis of Protein Transport from the ER to Golgi Membranes in Digitonin-permeabilized Cells: Role of the P58 Containing Compartment

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**Abstract.** The glycoside digitonin was used to selectively permeabilize the plasma membrane exposing functionally and morphologically intact ER and Golgi compartments. Permeabilized cells efficiently transported vesicular stomatitis virus glycoprotein (VSV-G) through sealed, membrane-bound compartments in an ATP and cytosol dependent fashion. Transport was vectorial. VSV-G protein was first transported to punctate structures which colocalized with p58 (a putative marker for peripheral punctate pre-Golgi intermediates and the *cis*-Golgi network) before delivery to the medial Golgi compartments containing  $\alpha$ -1,2-mannosidase II

and processing of VSV-G to endoglycosidase H resistant forms. Exit from the ER was inhibited by an antibody recognizing the carboxyl-terminus of VSV-G. In contrast, VSV-G protein colocalized with p58 in the absence of  $\text{Ca}^{2+}$  or the presence of an antibody which inhibits the transport component NSF (SEC18). These studies demonstrate that digitonin permeabilized cells can be used to efficiently reconstitute the early secretory pathway *in vitro*, allowing a direct comparison of the morphological and biochemical events involved in vesicular trafficking, and identifying a key role for the p58 containing compartment in ER to Golgi transport.

**T**RANSPORT of protein from the ER to the *cis* face of the Golgi complex is mediated by transport vesicles which are believed to bud from sites found in the transitional ER (Palade, 1975). While the biochemical processes regulating vesicle formation and fusion are largely unknown, recent progress in identifying components of the transport machinery at the genetic and biochemical levels is now providing new insight into this problem (reviewed in Pryer et al., 1992; Mellman and Simons, 1992; Rothman and Orci, 1992).

The steps subsequent to vesicle budding from the ER remain to be rigorously defined. *In vitro* assays which reconstitute the transport of protein from the ER do so through presumptive pre-Golgi intermediates (Beckers et al., 1990; Groesch et al., 1990; Rexach and Schekman, 1991). There is now *in vivo* evidence which suggests that compositionally related peripheral punctate structures and a tubular reticulum located on the *cis* face of the Golgi stack (the *cis*-Golgi network) (CGN)<sup>1</sup> are the initial targets for protein en route to the Golgi stack. Evidence for their role in transport, sorting and recycling stems from three different approaches. Firstly, receptors involved in the retention of resident

KDEL-containing luminal ER proteins appear to circulate between the ER and the CGN (Pelham, 1989; Lewis et al., 1990; Lewis and Pelham, 1992). Secondly, brefeldin A (BFA), a reagent which causes the collapse of the *cis*, *medial*, and *trans* compartments of the Golgi to the ER has been postulated to antagonize the normal balance of anterograde and retrograde flow between early compartments of the secretory pathway (Klausner et al., 1992). Finally, subcellular fractionation and morphological analyses have identified putative marker proteins for these compartments. A 53-kD protein found in Vero cells resides at steady state in distinct punctate compartments found in the peripheral cytoplasm and in a reticulum adjacent to the *cis* face of the Golgi stack (Schweizer et al., 1988). This marker protein has been used to establish a subcellular fractionation procedure to obtain an enriched membrane fraction which lacks properties characteristic of either Golgi or ER membranes (Schweizer et al., 1990; Schweizer et al., 1991). Similarly, a 58-kD protein found in NRK and BHK cells is also found in both peripheral punctate structures and the central CGN at steady state (Saraste and Kuismanen, 1984; Hendricks et al., 1991; Saraste and Svensson, 1991). The circulation of this protein between the ER and Golgi compartments is perturbed by prolonged incubation at reduced temperature (15°-16°C), and by BFA or nocodazole (Saraste and Svensson, 1991). At reduced temperature, protein exported from the ER accumulates in the peripherally disposed p53 or p58

1. *Abbreviations used in this paper:* BFA, brefeldin A; CGN, *cis*-Golgi network; endo H, endo-glycosidase; IF, intermediate filament; ManII,  $\alpha$ -1,2-mannosidase II; MF, microfilament; MT, microtubule; VSV-G, vesicular stomatitis virus glycoprotein.

containing tubular-vesicular structures (Saraste and Kuismanen, 1984; Schweizer et al., 1988; Saraste and Svensson, 1991).

To begin to understand the role of intermediate compartments in ER to Golgi transport at the molecular level, we have used the selective properties of the glycoside digitonin to efficiently permeabilize the plasma membrane. In this study we document the requirement for NSF and Ca<sup>2+</sup> in the vectorial flow of the vesicular stomatitis virus glycoprotein (VSV-G) protein from the ER via p58 containing compartments to the Man II containing medial-Golgi membranes.

## Materials and Methods

### Materials

<sup>35</sup>S-translabel (>1,000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Cytosol used in transport assays was prepared from uninfected CHO wild-type cells or rat liver as described previously (Beckers et al., 1987; Davidson et al., 1992). Endoglycosidases D and H were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). A polyclonal serum recognizing  $\alpha$ -1,2-mannosidase II (anti-Man II) was obtained from M. Farquhar (University of California, San Diego, CA). mAbs recognizing the cytoplasmic tail (P5D4) or a luminal domain of VSV-G protein (8G5) were kindly provided by K. Howell (University of Denver, Denver, CO) and B. Wattenberg (Upjohn, Kalamazoo, MI), respectively. A polyclonal reagent recognizing p58 was as described previously (Saraste and Svensson, 1991). An anti-RER (MERG) antibody used to determine the distribution of total ER membranes was provided by D. Meyer (University of California, Los Angeles, CA). Anti-NSF (clone 4A6) was provided by J. Rothman (Sloan Kettering Institute, New York, NY). Polyclonal reagents recognizing vimentin and tubulin were obtained from Sigma Chemical Co. (St. Louis, MO). Phalloidin was obtained from Molecular Probes (Eugene, OR). Digitonin was obtained from Calbiochem-Behring Corp. (La Jolla, CA).

### Morphological Analysis of Transport

To follow the morphological maturation of VSV-G protein from the ER to the Golgi, NRK cells were plated to confluency on 12-mm round coverslips (Bellco, Vineland, NJ) 1–2 d before use. Cells were infected at 32°C for 45 min and postinfected at 40°C for 3 h. Cells were then rapidly transferred to ice and washed immediately with ice-cold KHM (110 mM KOAc, 20 mM Hepes, pH 7.2, 2 mM MgOAc). Multiple slips were then digitonin permeabilized at 25–40  $\mu$ g/ml in KHM for 5 min on ice using a 20 mg/ml stock in DMSO kept frozen in small aliquots at –20°C. Coverslips were transferred to individual 16-mm wells in KHM and incubated 20 min to enhance cytosol dependence. Before transport, the KHM buffer was aspirated and an assay cocktail described below was added to each well (final volume of 200  $\mu$ l). Coverslips were incubated at 32°C, reactions terminated by transfer to ice and washing with ice cold KHM, and the cells immediately fixed by adding 4% formaldehyde in PBS for 15 min at room temperature.

### Indirect Immunofluorescence

Fixed cells were blocked for 10 min with 5% goat serum in PBS (PBS/Goat). For most experiments, fixed cells were stained with a primary antibody directed to the carboxyl-terminal cytoplasmic tail of VSV-G (P5D4) in the absence of any other permeabilizing reagent protein for 20 min in PBS/Goat. In cases where the luminal specific reagent was used (8G5), cell membranes were permeabilized with 0.1% saponin before incubation with antibody. Before the addition of the second primary reagent (to detect luminal ER, intermediate and Golgi marker proteins) cells were permeabilized with 0.1% saponin for 20 min in PBS/Goat. Cells were subsequently washed and the primary antibody tagged with Texas red goat anti-mouse IgG (Molecular Probes, Eugene, OR) at 1:250 and/or FITC goat anti-mouse gamma chain (Calbiochem-Behring Corp.) at 1:100. Rabbit antisera were detected with either a FITC goat anti-rabbit IgG (Organon-Technica-Cappel, West Chester, PA) or Texas red goat anti-rabbit IgG (Molecular Probes). Coverslips were mounted in Moviol (Calbiochem-Behring Corp.) and viewed using an Axiocvert microscope at 630 $\times$  (Carl Zeiss, Oberkochen, Germany). Confocal microscopy was performed using a con-

focal laser scanning microscope (model 600; Bio-Rad Laboratories, Cambridge, MA).

### Incubation Conditions and Analysis of Transport

To measure ER to Golgi transport, digitonin permeabilized NRK cells were incubated in vitro using a transport cocktail containing, in a final total volume of either 40  $\mu$ l (suspension cells; see below) or 200  $\mu$ l (coverslips) (final concentration): 25 mM Hepes-KOH, pH 7.2, 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl<sub>2</sub>, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 50–100  $\mu$ g cytosol/40- $\mu$ l incubation volume, and in the case of suspension cells, 5  $\mu$ l (25–30  $\mu$ g of protein; 1–2  $\times$  10<sup>5</sup> cells) of permeabilized cells. Transport was initiated by transfer to 32°C.

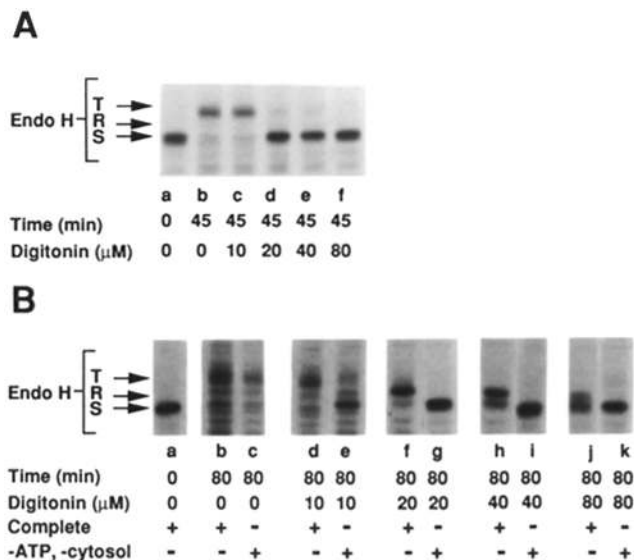
To follow transport using suspension cells for biochemical analysis of transport based on oligosaccharide processing, two 10-cm dishes containing confluent NRK cells were infected for 45 min at 32°C and postinfected for 3.5–4 h at 32°C. Cells were radiolabeled at 40°C as described previously (Beckers et al., 1987). Cells were rinsed once with PBS at 40°C. Then, 1.5 ml of PBS containing 2.5 mg/ml trypsin and 0.25 M unlabeled methionine was added and the cells incubated for 2 min at 40°C. Subsequently, the medium was removed and cells quickly shifted to ice where cold KHM containing 100  $\mu$ g/ml soybean trypsin inhibitor (Sigma Chemical Co.) was added. Cells were gently released from the plate by trituration, pelleted, resuspended in 2 ml of 25–40  $\mu$ g/ml of digitonin in KHM, and incubated on ice for 5 min. To terminate permeabilization and remove excess detergent, the medium was diluted to 12 ml, spun and the cells resuspended in 12 ml of buffer containing 50 mM Hepes, pH 7.2 and 90 mM KOAc. After 10–20 min the cells were pelleted and resuspended in a final volume of 200–400  $\mu$ l. 5  $\mu$ l of cells were portioned into 40- $\mu$ l reaction cocktail as described above and supplemented with various reagents as indicated. After termination of transport by transfer to ice, the membranes were pelleted by a brief (15 s) centrifugation in a microfuge at top speed. Transport and processing of VSV-G protein to the endoglycosidase H (endo H)-resistant forms was determined as described previously (Schwaninger et al., 1991).

## Results

### Digitonin Permeabilized Cells Transport VSV-G Protein from the ER to the Golgi

To establish conditions which allow access to the cytoplasm but retain the functional and morphological integrity of the ER and Golgi compartments, we examined the feasibility of using the glycoside digitonin to selectively permeabilize the cholesterol-rich plasma membrane, but not compartments of the early secretory pathway.

NRK cells infected with vesicular stomatitis virus (VSV) were incubated in the presence of increasing concentrations of digitonin for 5 min on ice, the detergent removed and cells incubated at 32°C in serum-free culture medium (Fig. 1 A) or in an in vitro cocktail which supports transport of VSV-G protein in semi-intact cells prepared by physical shear (Beckers and Balch, 1989; Beckers et al., 1987, 1990) (Fig. 1 B). Transport of VSV-glycoprotein (VSV-G) from the ER to Golgi compartments in vivo or in vitro can be detected by following the conversion of the two N-linked oligosaccharides found on VSV-G to the various endo H-resistant forms using SDS-PAGE (Schwaninger et al., 1991). Pretreatment of cells with up to 10  $\mu$ g/ml digitonin had no effect on the processing of VSV-G protein to the endo H resistant, terminally glycosylated (endo H<sub>T</sub>) form containing sialic acid when incubated in serum-free medium (Fig. 1 A, lanes b and c) or in the presence of an in vitro cocktail containing cytosol and ATP (Fig. 1 B, lanes b and d). In contrast, pretreatment of cells with 20  $\mu$ g/ml digitonin caused complete inhibition of transport and processing in cells incubated in serum-free medium (Fig. 1 A, lane d) or in vitro in the absence of ATP



**Figure 1.** Digitonin permeabilized cells transport VSV-G protein to the endo H resistant form in vitro. (A) NRK cells in suspension were permeabilized with digitonin at the indicated concentrations as described in Materials and Methods and incubated in serum-free culture medium for the indicated time. After incubation, the amount of VSV-G processed to endo H-resistant form containing terminal sialic acid (*endo H<sub>T</sub>*) was determined as described in Materials and Methods. (B) NRK cells in suspension were permeabilized with digitonin and incubated for the indicated time in an in vitro cocktail in the presence (lanes a, b, d, f, h, and j) or absence (lanes c, e, g, i, and k) of ATP and cytosol. After incubation, the amount of VSV-G protein processed to the endo H-resistant intermediate (*endo H<sub>R</sub>*) form containing terminal GlcNAc was determined as described in Materials and Methods.

and cytosol (Fig. 1 B, lane g). In both cases, VSV-G protein remained in the endo H sensitive (S) (*endo H<sub>S</sub>*) form. However, incubation of digitonin permeabilized cells in a transport cocktail containing ATP, cytosol, and UDP N-acetylglucosamine (UDP-GlcNAc), a sugar nucleotide essential for processing of VSV-G oligosaccharides to the endo H-resistant forms resulted in the efficient (>80%) conversion of VSV-G protein to the endo H-resistant (*endo-H<sub>R</sub>*) form (Fig. 1 B, lane f). Addition of higher concentrations of digitonin resulted in the progressive inhibition of processing to the endo H<sub>R</sub> form in vitro even in the presence of ATP and cytosol. In the presence of 80 μg/ml digitonin VSV-G protein transport was inhibited by ~70–80% (Fig. 1 B, compare lanes f and j).

These results indicate that pretreatment of cells for 5 min on ice using digitonin in the concentration range of 20–40 μg/ml renders the plasma membrane permeable to macromolecules while maintaining the functional integrity of the ER and the *cis/medial*-Golgi compartments.

#### Digitonin Permeabilization Preserves the Morphological Integrity of Both the ER and Golgi Compartments

To examine the effects of digitonin on the morphological transport of protein we used the VSV mutant strain tsO45 which expresses a form of VSV-G protein which has a thermoreversible defect in export from the ER (Balch et al., 1986; Lafay, 1974). Infected cells incubated at the restrictive

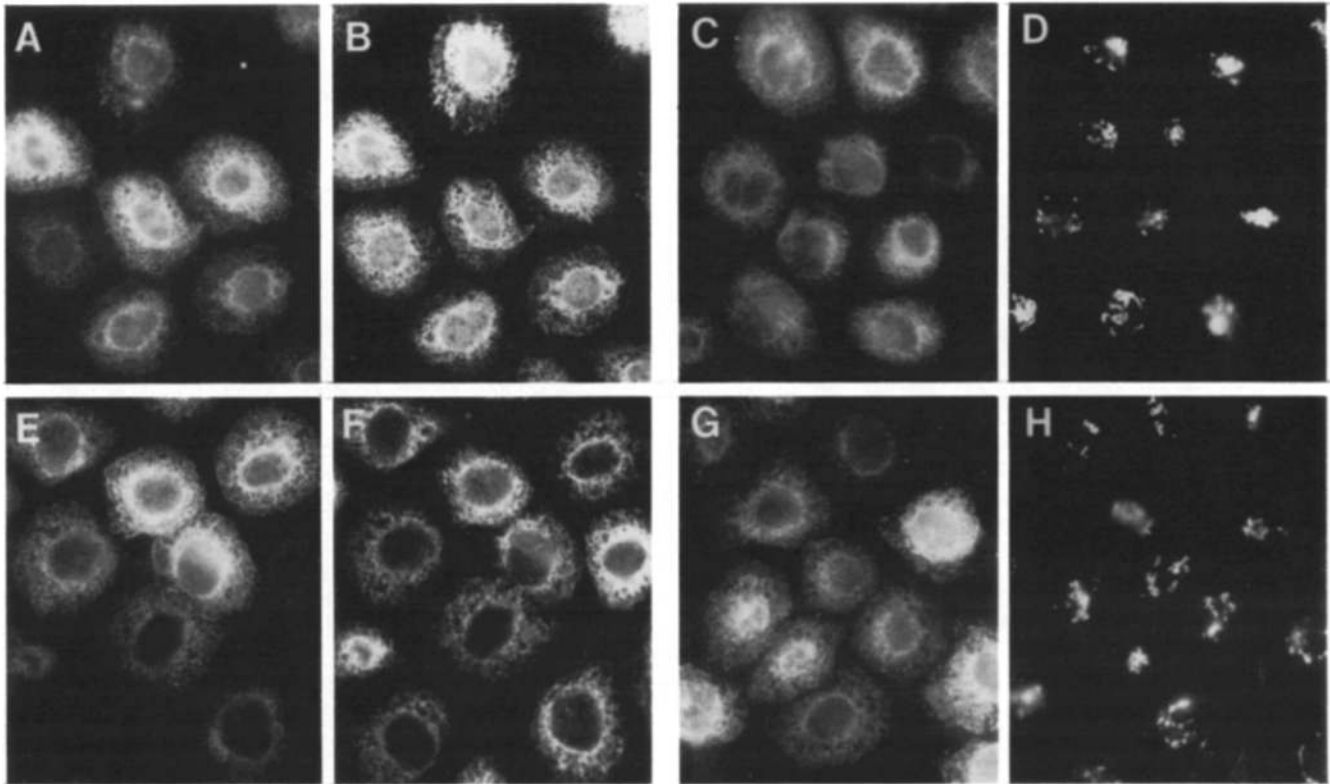
temperature (39.5°C) retain tsO45 VSV-G in the ER; transfer of cells to the permissive temperature (32°C) results in transport of VSV-G to the Golgi stack both in vivo (Balch et al., 1986) and in vitro (Beckers et al., 1987).

NRK cells grown on coverslips were infected with tsO45 VSV-G and maintained for 3 h at the restrictive temperature. Cells were then rapidly (<5 s) transferred to ice cold buffer to prevent export from the ER. As shown in Fig. 2, before permeabilization with digitonin, VSV-G showed a characteristic reticular distribution which colocalized with proteins recognized by a polyclonal sera raised against total ER membranes (anti-ER) (Fig. 2 A, VSV-G, and B, ER). At this time, VSV-G protein was not found in the perinuclear Golgi elements detected by an antibody which is specific for the *cis/medial* marker enzyme α-1,2-mannosidase II (Man II) (Fig. 2 C, VSV-G and D, Man II). After permeabilization with 25 μg/ml digitonin, but before incubation in vitro, the morphological structure of both the ER (Fig. 2, compare B, before, and F, after) and Golgi compartments (Fig. 2, compare D, before, and H, after) were retained, although the ER had a more swollen appearance than that observed in intact cells (Fig. 2, compare B and F). In addition, the distribution of VSV-G protein in digitonin permeabilized cells was identical to that prior to permeabilization (Fig. 2, compare A, before and E, after, or C, before, and G, after).

VSV-G is a transmembrane protein with a large NH<sub>2</sub>-terminal luminal domain and a 30 amino acids carboxyl-terminal cytoplasmic tail. To verify that the plasma membrane, but not the ER, was permeabilized by digitonin, we made use of mAbs which recognize epitopes in either the cytoplasmic tail or the amino-terminal luminal domain. As shown in Fig. 3, immediately after digitonin permeabilization VSV-G could be readily detected in the ER when fixed cells were pretreated with 0.1% saponin before addition of either of the antibody reagents (Fig. 3 A, anti-tail, and B, anti-lumen). In contrast, in the absence of saponin only the cytoplasmic tail specific antibody could detect VSV-G protein (Fig. 3 C, anti-tail, and D, anti-lumen). Cells which had not been pretreated with digitonin to permeabilize the plasma membrane also failed to react with either antibody (not shown). Thus, VSV-G remains in membrane-limited ER compartments in cells in which the plasma membrane has been largely perforated.

#### VSV-G Protein Is Transported from the ER to the Golgi Compartment in Digitonin Permeabilized Cells

The efficient processing of VSV-G protein to the endo H<sub>R</sub> form after digitonin permeabilization and incubation in the presence of ATP and cytosol (Fig. 1 B) suggested that VSV-G protein was being transported to the *cis/medial*-Golgi compartment(s). To verify this morphologically and to exclude the possibility that digitonin was triggering a retrograde flow of Golgi enzymes to the ER, NRK cells expressing tsO45 VSV at the restrictive temperature were transferred to ice, permeabilized with 25 μg/ml digitonin and subsequently incubated for 80 min in the presence of cytosol and ATP. As shown in Fig. 4, VSV-G undergoes a striking redistribution upon incubation in vitro. VSV-G was transported from the diffuse, reticular distribution observed before incubation to intensely labeled, punctate structures which varied markedly in size and were scattered throughout the cytoplasm of the cell (Fig. 4, A, C, E-G, and I-K, VSV-



**Figure 2.** VSV-G protein is restricted to the ER prior to incubation of digitonin permeabilized cells *in vitro*. The distribution of VSV-G protein, ER and Golgi compartments in intact NRK cells (A–D) or NRK cells permeabilized by digitonin (25  $\mu\text{g/ml}$ ) (E–H) were determined by indirect immunofluorescence as described. (A, E, C, and G) Distribution of VSV-G protein; (B and F) distribution of total ER; (D and H) distribution of Golgi compartments containing  $\alpha$ -1,2-mannosidase II (*Man II*).

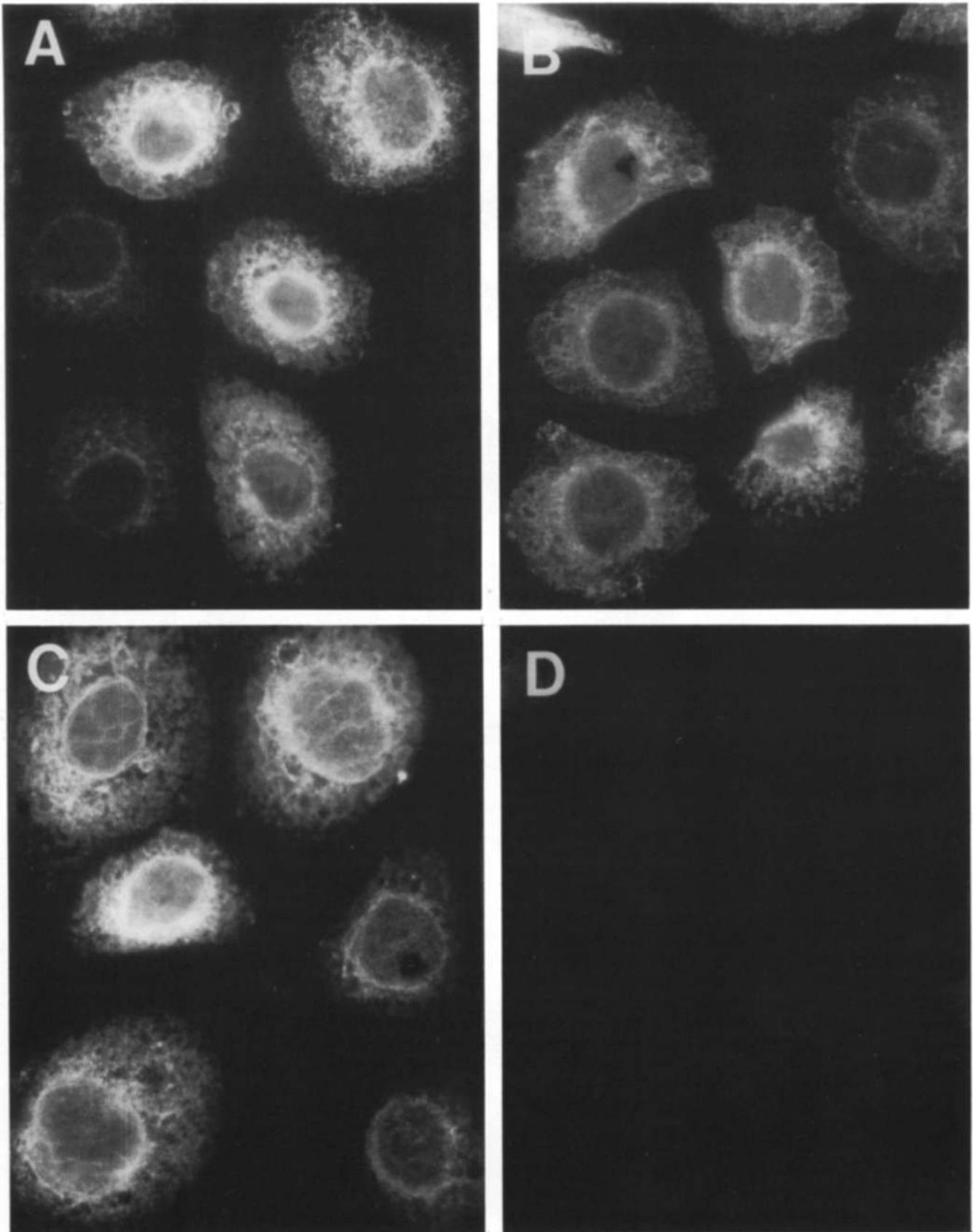
G). Furthermore, after 80 min of incubation *in vitro*, VSV-G protein remained in sealed compartments. VSV-G protein could not be detected using the luminal epitope specific antibody in the absence of saponin (Fig. 4 H, VSV-G). In contrast, the distribution of ER resident proteins remained largely unchanged albeit assuming a more swollen appearance (Fig. 4, B and D, anti-ER). The latter results exclude the possibility that concentration of VSV-G into punctate structures was a consequence of fragmentation of the ER.

To determine if VSV-G protein was transported to Golgi compartments, the distribution of VSV-G after 80 min of incubation was compared to the distribution of Man II. As shown in Fig. 4, colocalization was observed between a subset of the VSV-G containing punctate structures and Man II containing structures (Fig. 4, compare I and K, VSV-G, with J and L, Man II, boxed regions and arrows) although a significant fraction of punctate structures lacked Man II based on indirect immunofluorescence. Typically, greater than 95% of the cells on any coverslip incubated under these conditions transported VSV-G protein to structures which colocalized with the Golgi marker Man II within 80 min.

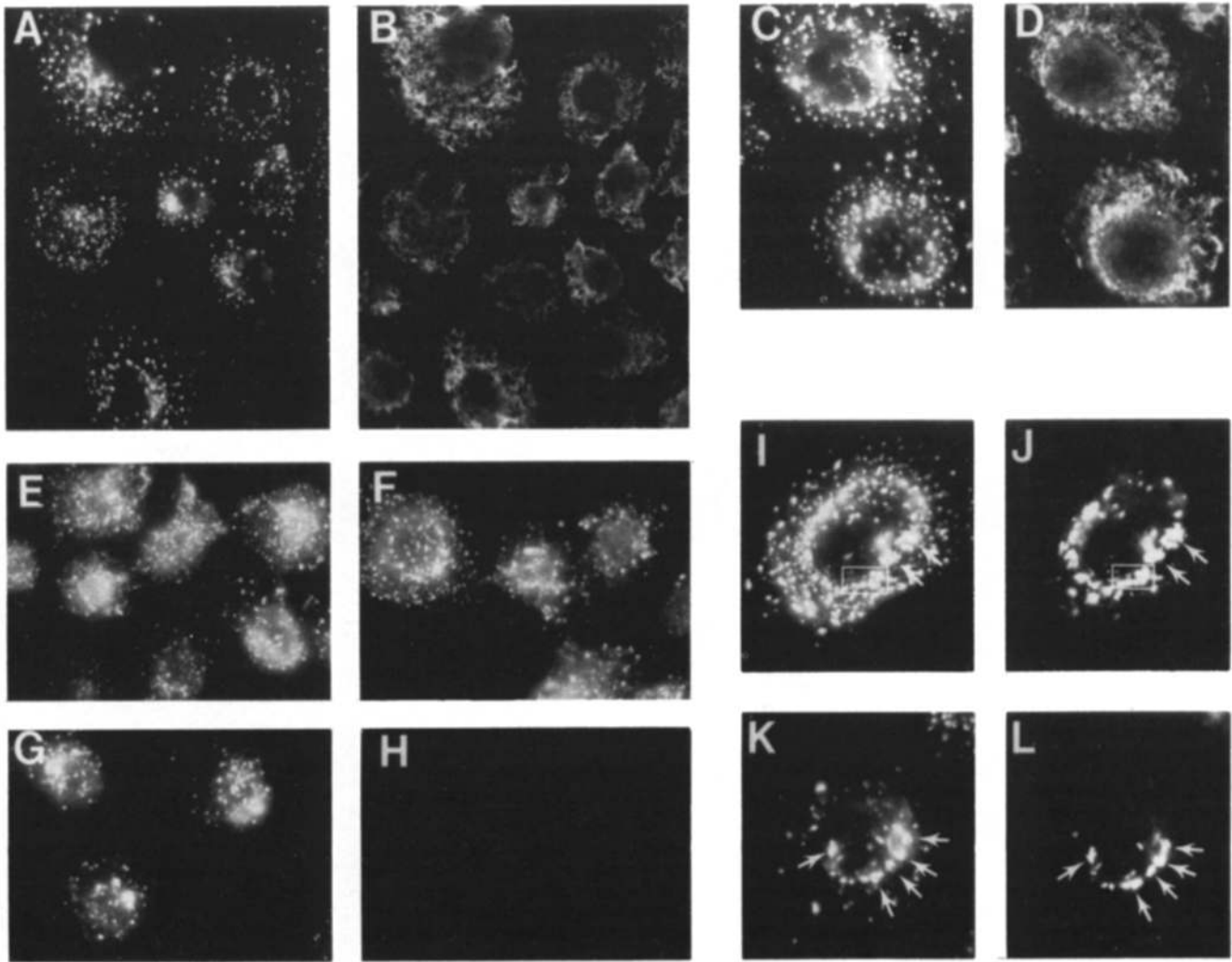
In contrast to results observed by incubating permeabilized cells in the presence of cytosol and ATP, processing of VSV-G to the endo H-resistant form was not observed when permeabilized cells were incubated in the absence of either cytosol or ATP (Fig. 1 B). Consistent with this result, when permeabilized cells were incubated for 80 min in the absence of either ATP (Fig. 5 A, VSV-G, and B, Man II) or cytosol (Fig. 5 C, VSV-G, or D, Man II), VSV-G protein did

not exit the ER, although the Golgi compartments in both cases remained largely intact (Fig. 5, B and D). The latter results rule out the trivial possibility that lack of processing of VSV-G to the endo H<sub>R</sub> form was due to loss of Golgi compartments from digitonin permeabilized cells. Importantly, Man II was not transported to the ER (Fig. 4, compare J and L, Golgi, to B and D, ER) making it unlikely that processing of VSV-G protein oligosaccharides was a consequence of a retrograde flow of Golgi enzymes to the ER. However, we have consistently noted that Golgi compartments appear more fragmented and dispersed, losing their perinuclear localization after 80 min of incubation. The latter result suggests that cytoskeletal components such as microtubules, which are essential for maintenance of the perinuclear localization of the Golgi complex *in vivo* (Thyberg and Moslkalewski, 1985) may be depolymerizing or altered upon incubation *in vitro*.

To assess the contribution of the cytoskeleton to the morphology of digitonin permeabilized cells, we examined the distribution of microtubules (MT) (using an anti-tubulin antibody) intermediate filaments (IF) (using an anti-vimentin antibody), and microfilaments (MF) (using the actin specific reagent phalloidin) before and after incubation *in vitro*. As shown in Fig. 6, IF, MF, and MT networks could be readily detected immediately after permeabilization by digitonin (Fig. 6 A, MT, C, MF, and E, IF) and were for the most part identical in structure to those observed prior to permeabilization (not shown). However, after 45-min incubation in the complete cocktail containing cytosol and ATP, both the MT



**Figure 3.** The plasma membrane but not the ER reticulum is perforated in digitonin permeabilized cells. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin, fixed, and incubated in the presence (*A* and *C*) or absence (*B* and *D*) of 0.1% saponin and with mAbs directed to either the cytoplasmic tail of VSV-G protein (*A* and *B*) or the amino-terminal luminal domain (*C* and *D*) as described in Materials and Methods.



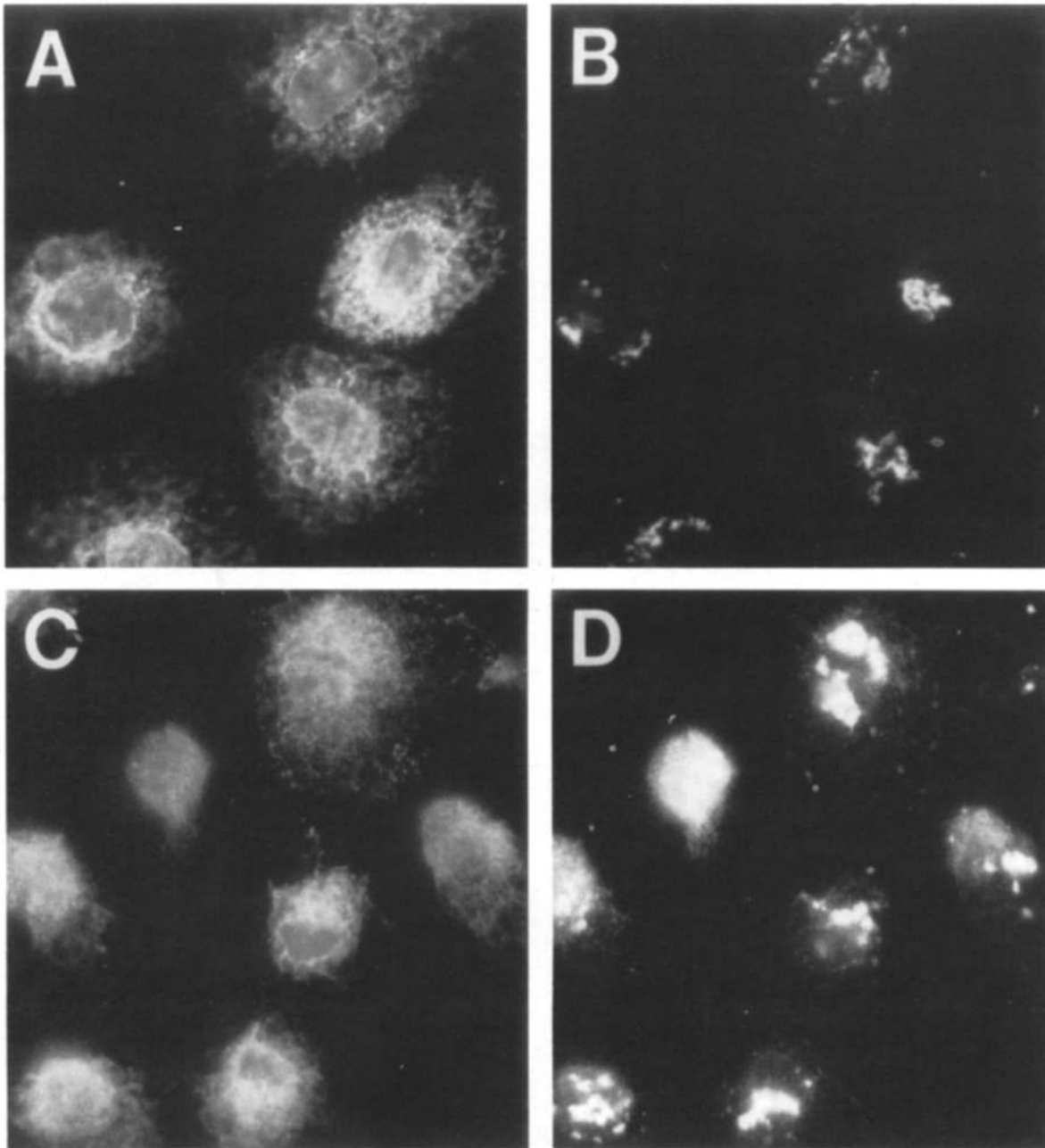
**Figure 4.** VSV-G protein is transported to the Man II containing Golgi compartments in permeabilized cells. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated in vitro for 80 min in the presence of ATP and cytosol as described in Materials and Methods. Cells were fixed and the distributions of VSV-G protein (A, C, I, and K), ER (B and D) and Golgi compartments (Man II) (J and L) determined as described in Materials and Methods. In E-H, fixed cells were incubated in the presence (E and G) or absence (F and H) of 0.1% saponin using antibodies directed to either the cytoplasmic tail of VSV-G protein (E and F) or the amino-terminal luminal domain (G and H).

and MF cytoskeletal networks were considerably altered, with the MT network being largely disassembled (Fig. 6 B, MT, and D, MF). In contrast, the distribution of the network recognized by the anti-vimentin antibody remained largely unchanged. While intermediate filaments may contribute to the stability of the overall architecture of the digitonin permeabilized cell, the altered structures of both the ER and Golgi compartments may reflect in part the reorganization of MT and/or MF networks during incubation in vitro.

#### ***VSV-G Is retained in the ER at the Restrictive Temperature but Accumulates in a Pre-Golgi Intermediate at 15°C***

To provide a direct test for the biochemical integrity of the folding environment of the ER in digitonin permeabilized cells, we examined whether such cells reconstitute the thermoreversible transport phenotype of VSV-G protein observed in vivo (Lafay, 1974). Thermosensitivity in vivo is

due to the misfolding of tsO45 VSV-G at the restrictive temperature (39.5°C) to form a BiP-associated aggregate; incubation at the permissive temperature results in a rapid ATP-dependent dissociation of the aggregate, trimerization, and export from the ER (Doms et al., 1987; de Silva et al., 1990). When permeabilized cells were incubated at the restrictive temperature (39.5°C) for 45 min in vivo (Fig. 7 A) or in vitro (Fig. 7 B), VSV-G protein was principally found in the ER, although a small amount of the VSV-G could also be detected in weakly staining punctate structures, presumably pre-Golgi intermediates (see below) (Fig. 7 A, in vivo, and B, in vitro, arrows). No transport to Golgi membranes was detected biochemically based on the appearance of endo  $H_x$  forms (not shown). Transfer to the permissive temperature after incubation in vitro for up to 20 min at the restrictive temperature resulted in efficient transport to the Golgi compartment, comparable to that observed in Fig. 4 (not shown). However, the efficiency of reversal was progressively reduced when cells were incubated for longer time



**Figure 5.** Export from the ER requires cytosol and ATP. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated in the absence of ATP (*A* and *B*) or cytosol (*C* and *D*). The distribution of VSV-G protein (*A* and *C*) or Man II (*B* and *D*) was determined as described in Materials and Methods.

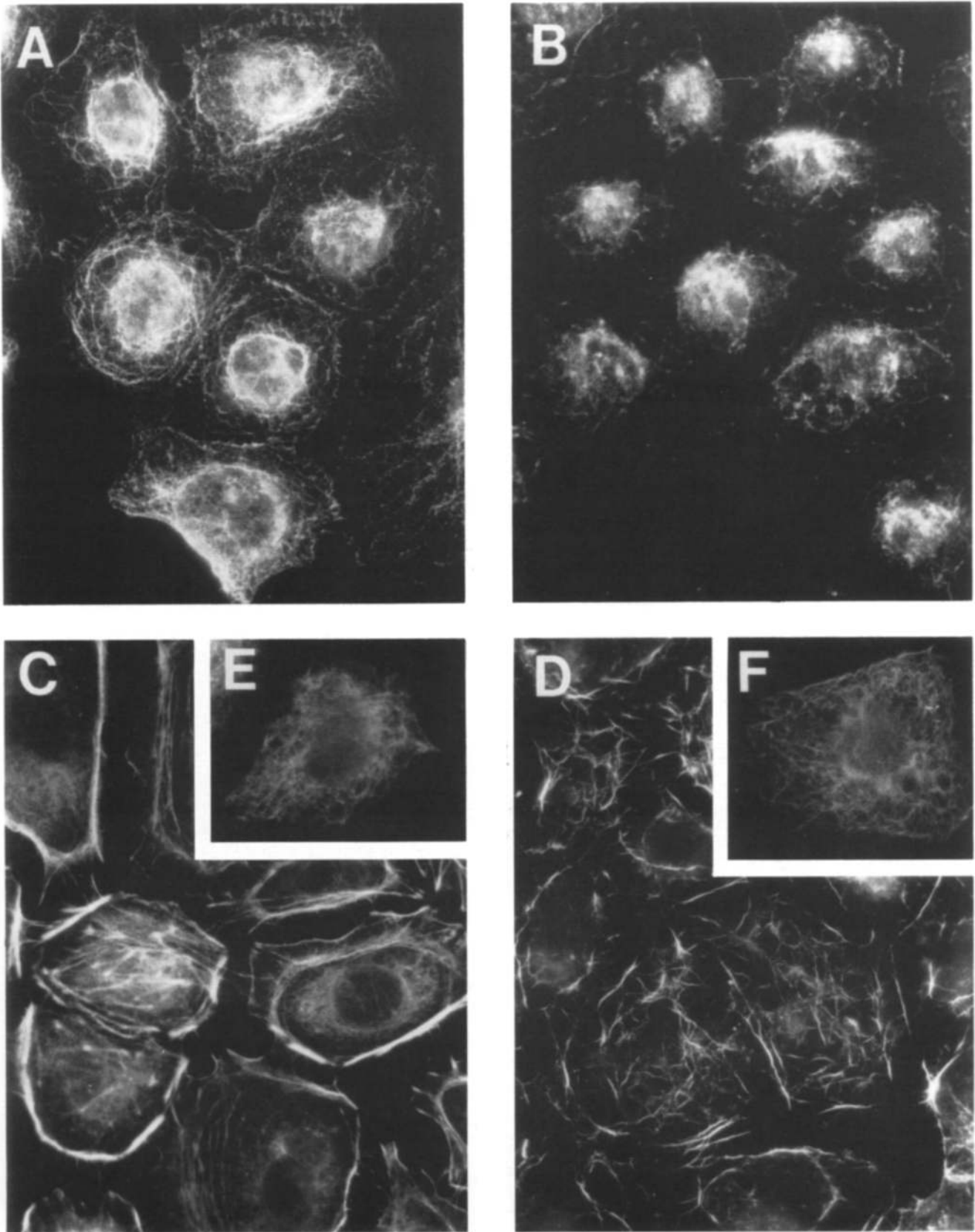
periods (>20 min) at the restrictive temperature in vitro (not shown), suggesting that the ER gradually loses export function under these conditions, even though targeting and fusion to acceptor Golgi compartments continues efficiently for at least 90 min.

Incubation of cells at reduced temperature (15–16°C) in vivo results in the accumulation of newly synthesized protein in punctate, pre-Golgi intermediates (Saraste and Kuismann, 1984; Schweizer et al., 1988; Saraste and Svensson, 1991). To determine whether VSV-G accumulates in similar structures in vitro, permeabilized cells were incubated for 80 min at 15°C. As shown in Fig. 7, while a small amount of VSV-G was detected in punctate structures in vivo (Fig. 7 *C*, *arrows*), a striking accumulation of VSV-G into intermedi-

ates was observed in permeabilized cells (Fig. 7 *D*, *arrows*). These punctate structures were frequently more peripheral and uniform (smaller) in size than VSV-G protein containing Golgi compartments observed after incubation for a similar time period at 32°C (Fig. 4, *I–L*). Consistent with their putative pre-Golgi disposition, these punctate intermediates did not colocalize with Man II by indirect immunofluorescence nor was VSV-G protein processed to endo  $H_R$  form after 80-min incubation at 15°C (not shown).

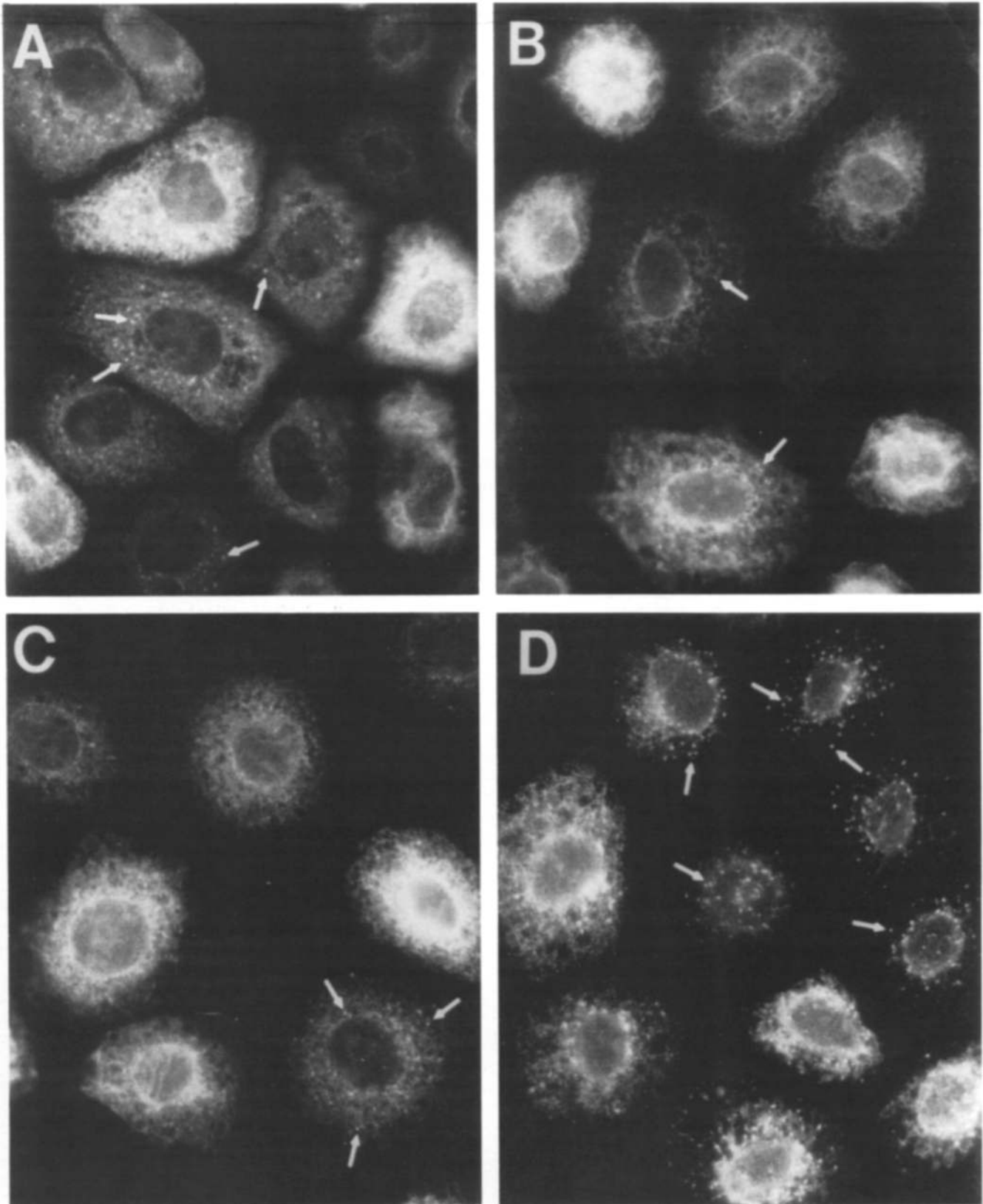
#### ***Transport In Vitro Occurs through the p58 Containing Compartment***

To begin to characterize the role of potential intermediate

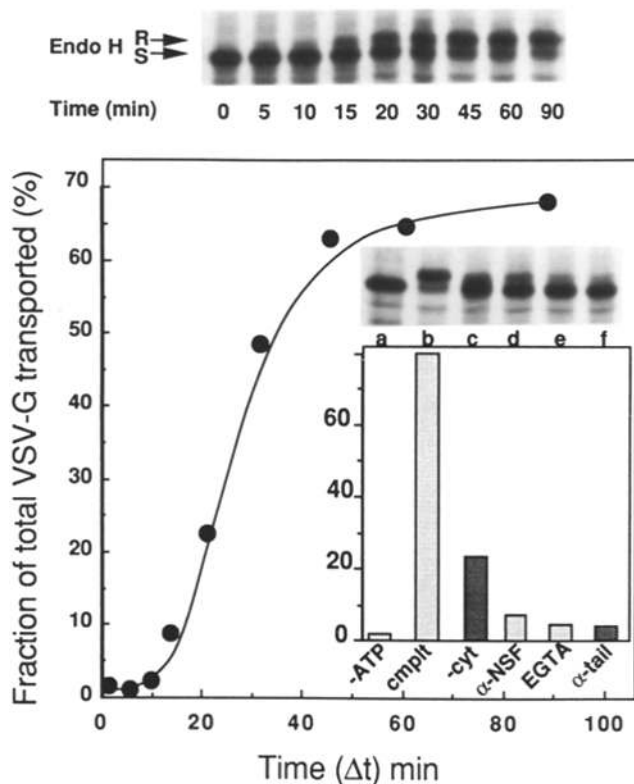


**Figure 6.** Organization of microtubules, microfilaments (actin) and intermediate filaments before and after incubation in vitro. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin. The distribution of microtubules (*A* and *B*), microfilaments (*C* and *D*) or intermediate filaments (*E* and *F*) were determined before (*A*, *C*, and *E*) or after (*B*, *D*, and *F*) incubation in vitro as described in Materials and Methods.





**Figure 7.** Effect of elevated (39.5°C) and reduced (15°C) temperature on transport of VSV-G protein. (A and C) NRK cells were incubated *in vivo* for 90 min at 39.5°C (A) or 15°C for 90 min (C) before fixation and analysis of the VSV-G protein distribution using indirect immunofluorescence as described in Materials and Methods. (B and D) NRK cells were permeabilized using 25  $\mu\text{g}/\text{ml}$  digitonin and incubated *in vitro* in the presence of cytosol and ATP at 39.5°C for 45 min (B) or 15°C for 90 min (D) before fixation and analysis of the distribution of VSV-G using indirect immunofluorescence.



**Figure 8.** Kinetics of transport of VSV-G in digitonin permeabilized cells. MRK cells in suspension were permeabilized using 25  $\mu$ g/ml digitonin and incubated for the indicated time prior to transfer to ice and analysis of the fraction of the total VSV-G protein processed to the endo H<sub>R</sub> form as described in Materials and Methods. (*Inset*) Permeabilized cells were incubated for 45 min in a cocktail containing ATP and cytosol (*cmplt*), in the absence of ATP (*-ATP*), in the absence of cytosol (*-cyt*), in the presence of 5  $\mu$ g of anti-NSF antibody ( $\alpha$ -NSF), in the presence of 10 mM EGTA (*EGTA*), or in the presence of 1  $\mu$ g of an antibody (P5D4) which recognizes the cytoplasmic tail of VSV-G protein ( $\alpha$ -tail). The fraction of the total VSV-G protein processed to the endo H<sub>R</sub> form is shown.

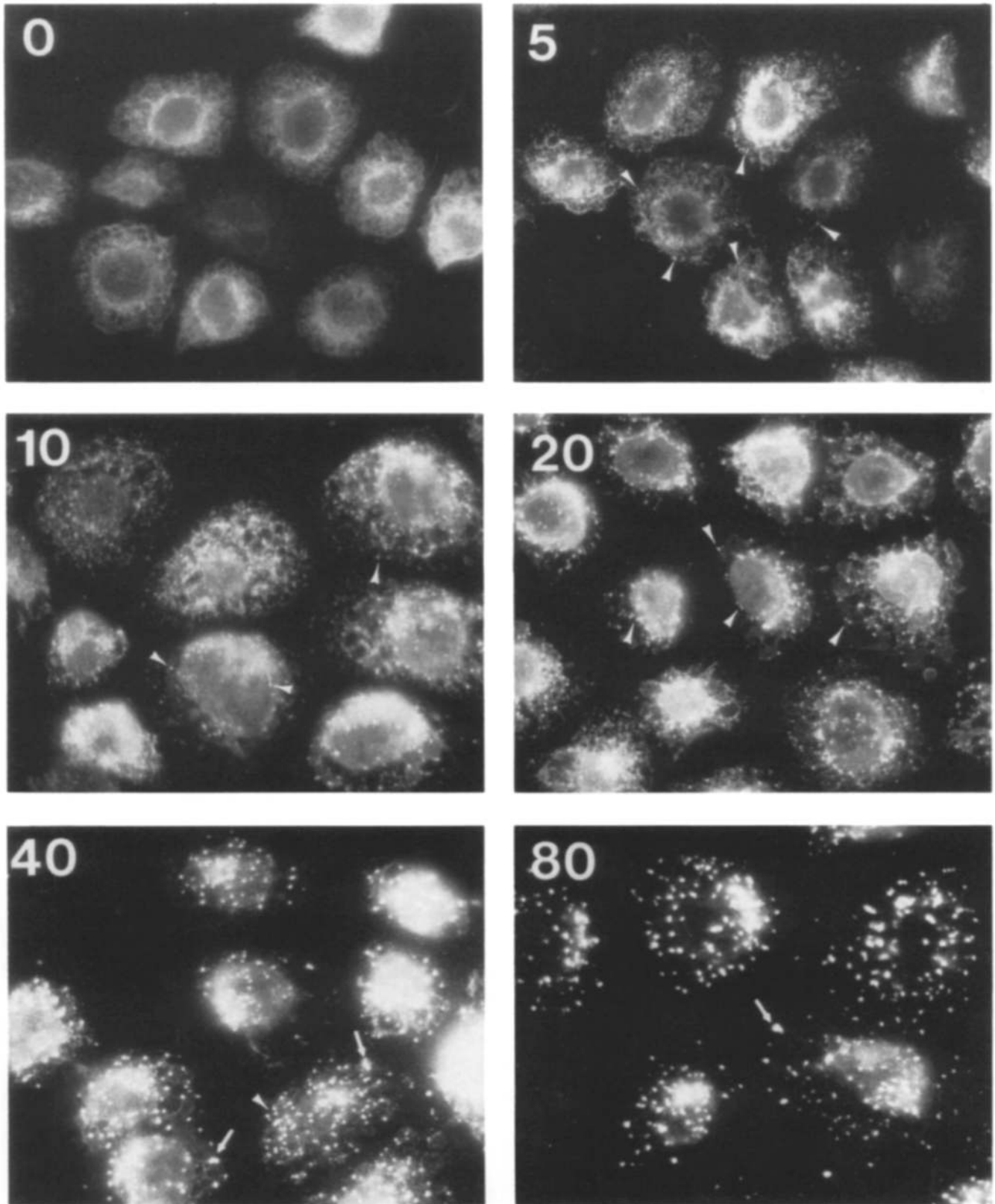
compartments in transport, we examined the processing and morphological distribution of VSV-G after short times of incubation. The kinetics of processing of VSV-G to the endo H<sub>R</sub> form are shown in Fig. 8. A ~10–15-min lag period was observed before the appearance of the first endo H-resistant form of VSV-G (Fig. 8). Processing of VSV-G to endo H-resistant forms reaches a maximum after 60–80 min of incubation with, in this instance, ~65% of the total VSV-G protein transported from the ER to the *cis/medial*-Golgi compartments. The efficiency of transport between different permeabilized cell preparations was found to vary from 50–80% (not shown). The  $t_{1/2}$  of processing from the endo H<sub>S</sub> to the endo H<sub>R</sub> form was ~30 min at 32°C. These kinetics are similar to those of the processing of VSV-G protein to the endo D sensitive (Man<sub>5</sub>) form when permeabilized cells were incubated in the absence of UDP-GlcNAc (not shown), indicating that the processing of VSV-G to an endo H<sub>R</sub> intermediate in NRK cells measures movement from the ER reticulum to early Golgi compartments (see Schwaninger et al., 1991; Davidson et al., 1992).

The morphological distribution of VSV-G at early time points is shown in Fig. 9. After 5 min of incubation in vitro, VSV-G began to lose its diffuse reticular distribution and became concentrated in numerous small, punctate structures

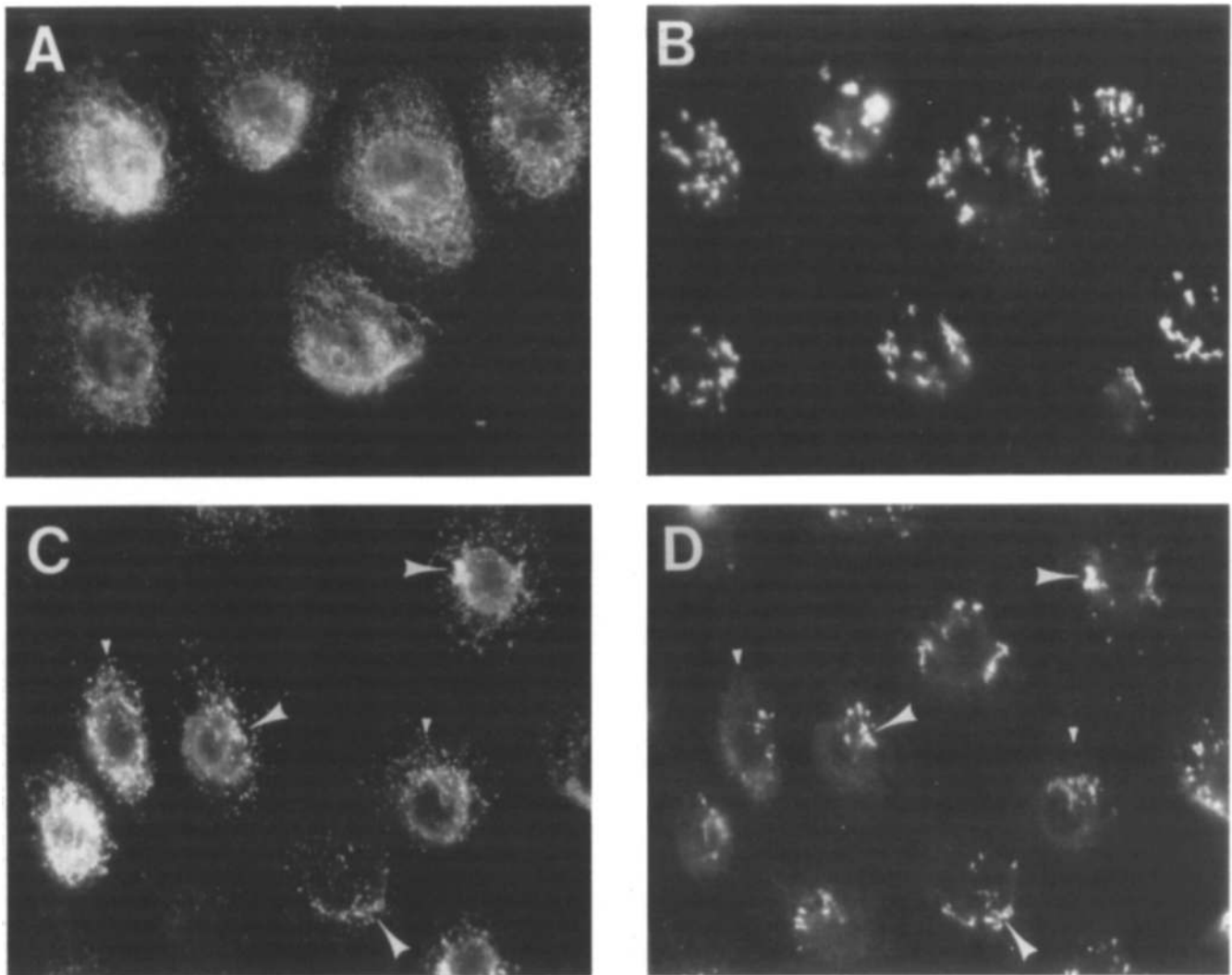
scattered throughout the cytoplasm of the cell (Fig. 9, 5 min, *arrowheads*). These punctate structures did not colocalize with Man II (Fig. 10, compare *A*, VSV-G, with *B*, Golgi). Concentration in these punctate structures increased during the subsequent 10–20 min of incubation (Fig. 9, 10 min and 20 min), followed by the accumulation in larger structures (40 min and 80 min, compare *arrowheads* to *arrows*) which colocalized with Man II (Fig. 4). Greater than 95% of cells on coverslips initiated export of VSV-G from the ER within the first 5–10 min of incubation in vitro. The mobilization of VSV-G into punctate structures at early times of incubation could reflect either: (*a*) concentration into export sites present in the transitional ER; (*b*) maturation of VSV-G protein into bona fide transport vesicles; and/or (*c*) transport of VSV-G protein via putative pre-Golgi intermediates to the Golgi stack.

To try to identify which compartments VSV-G was associated with at early time points, we compared its distribution with that of the p58 antigen (Hendricks et al., 1991; Saraste et al., 1987; Saraste and Svensson, 1991). The distribution of p58 after various times of incubation in vitro is shown in Fig. 11. Before incubation of permeabilized cells, p58 was located primarily in small punctate structures scattered throughout the cytoplasm (Fig. 11, *A*, p58, *small arrowheads*) and in more central reticular structures colocalizing with the Golgi marker Man II (Fig. 11, compare *A*, p58, and *B*, Man II, *large arrows*). These results are consistent with the distribution of p58 in intact cells (Hendricks et al., 1991; Saraste and Svensson, 1991). After a 10-min incubation in vitro, p58 was still present in the Golgi region (Fig. 11 compare *C*, p58 with *D*, Man II, *large arrows*). In addition, a more intense staining was observed in the punctate structures scattered throughout the cytoplasm (Fig. 11 *C*, *small arrowheads*). A similar distribution was observed after 45 min of incubation (Fig. 11 *E*, p58, and *F*, Man II, *small arrowheads*).

What is the fate of VSV-G protein relative to the distribution of p58 during incubation in vitro? As indicated previously, after 10 min VSV-G protein was concentrated in numerous small, punctate structures (Fig. 12 *B*, *small arrows*). However, only a small fraction (typically <5–10%) of these contained p58 protein (Fig. 12, compare *A*, p58, and *B*, VSV-G, *large arrows*). In contrast, a significantly different result was observed following an additional 10 min of incubation. After 20 min, a large percentage of the punctate structures containing VSV-G colocalized with p58 in both the central and peripheral regions of the cytoplasm (Fig. 12, compare *C*, p58, and *D*, VSV-G, *large arrows*). The extent of colocalization was more apparent using confocal microscopy. A merge of the data sets for the distribution of VSV-G (Fig. 13 *A*) and p58 (Fig. 13 *B*) after 20 min of incubation showed a striking colocalization with >50% of the peripheral compartments labeled with p58 also containing VSV-G (Fig. 13 *C*). By 60 min of incubation VSV-G protein was observed in both the p58 containing intermediates (Fig. 12 *E*, p58, and *F*, VSV-G, *arrowheads*) and the larger, morphologically distinct Golgi elements containing Man II (Fig. 12 *E*, p58, and *F*, VSV-G, *open arrows*) (Fig. 13). While colocalization with p58 was >75% at this time point, a number of punctate structures containing VSV-G protein did not colocalize with either p58 (Fig. 12, *E* and *F*, *boxed regions*) or Man II (Fig. 4). The latter compartments may represent the accumulation of VSV-G in ER export sites or nonfunc-



**Figure 9.** Time course of transport of VSV-G protein from the ER to the Golgi complex. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated for the indicated time, fixed and the distribution of VSV-G protein determined using indirect immunofluorescence as described in Materials and Methods. Small arrowheads indicate the distribution of small punctate structures during early times of incubation (5, 10, 20, and 40 min). Large arrows (40 and 80) indicate accumulation of VSV-G protein in structures which are larger and more variable in size, presumably Golgi compartments (see text).



**Figure 10.** Distribution of VSV-G protein and Man II containing Golgi compartments after 5 and 20 min of incubation in vitro. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated for 5 min (*A* and *B*) or 20 min (*C* and *D*), fixed and the distribution of VSV-G protein or Man II containing Golgi compartments determined using indirect immunofluorescence as described in Materials and Methods. Small arrows indicate small, peripheral punctate structures; large arrows indicate Man II containing Golgi compartments.

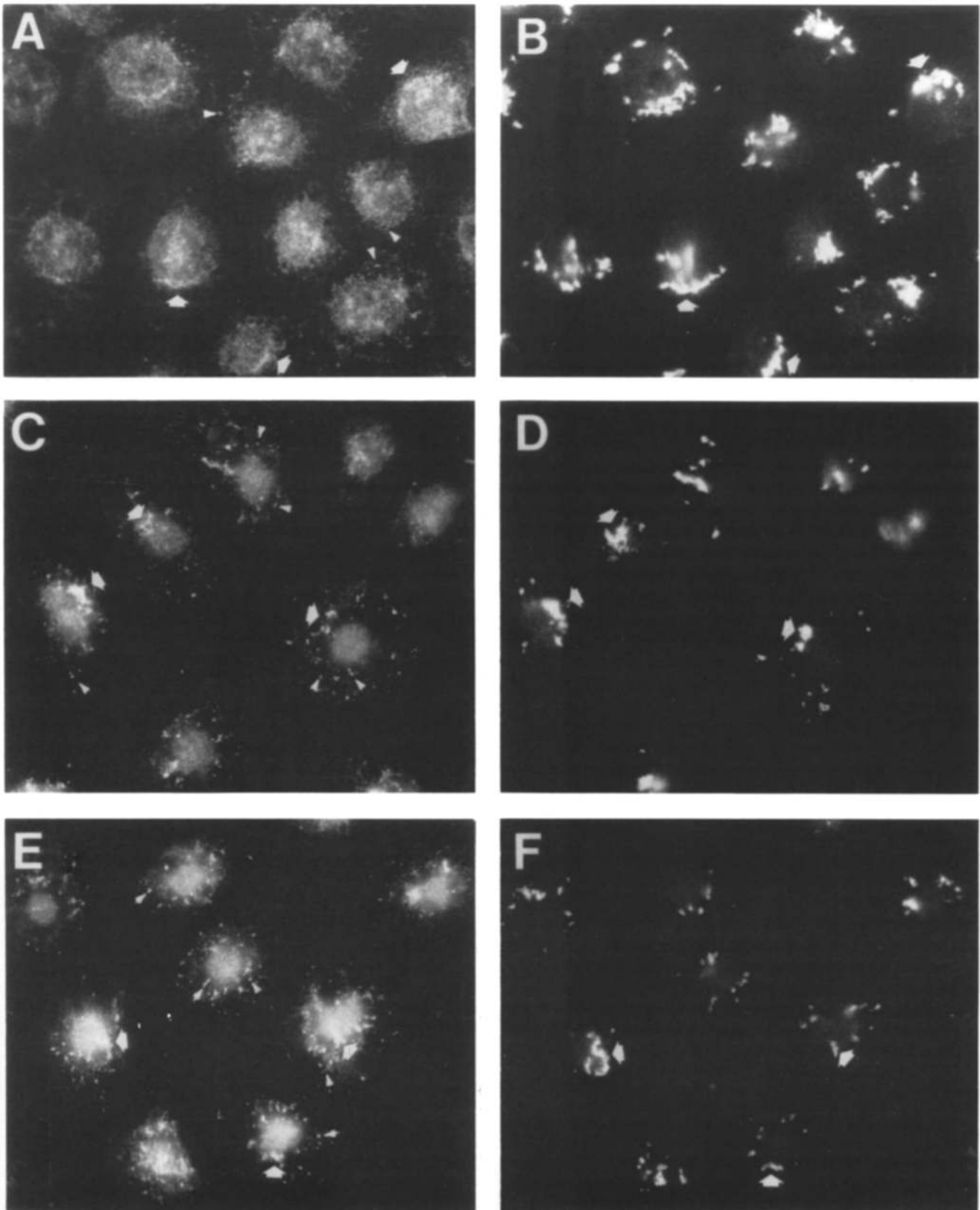
tional intermediates and are likely to account for the reduced efficiency of transport (50–80%) in vitro as measured biochemically (Fig. 8).

***EGTA and an Inhibitory Antibody towards NSF Permit Export from the ER but Not Fusion with the Golgi***

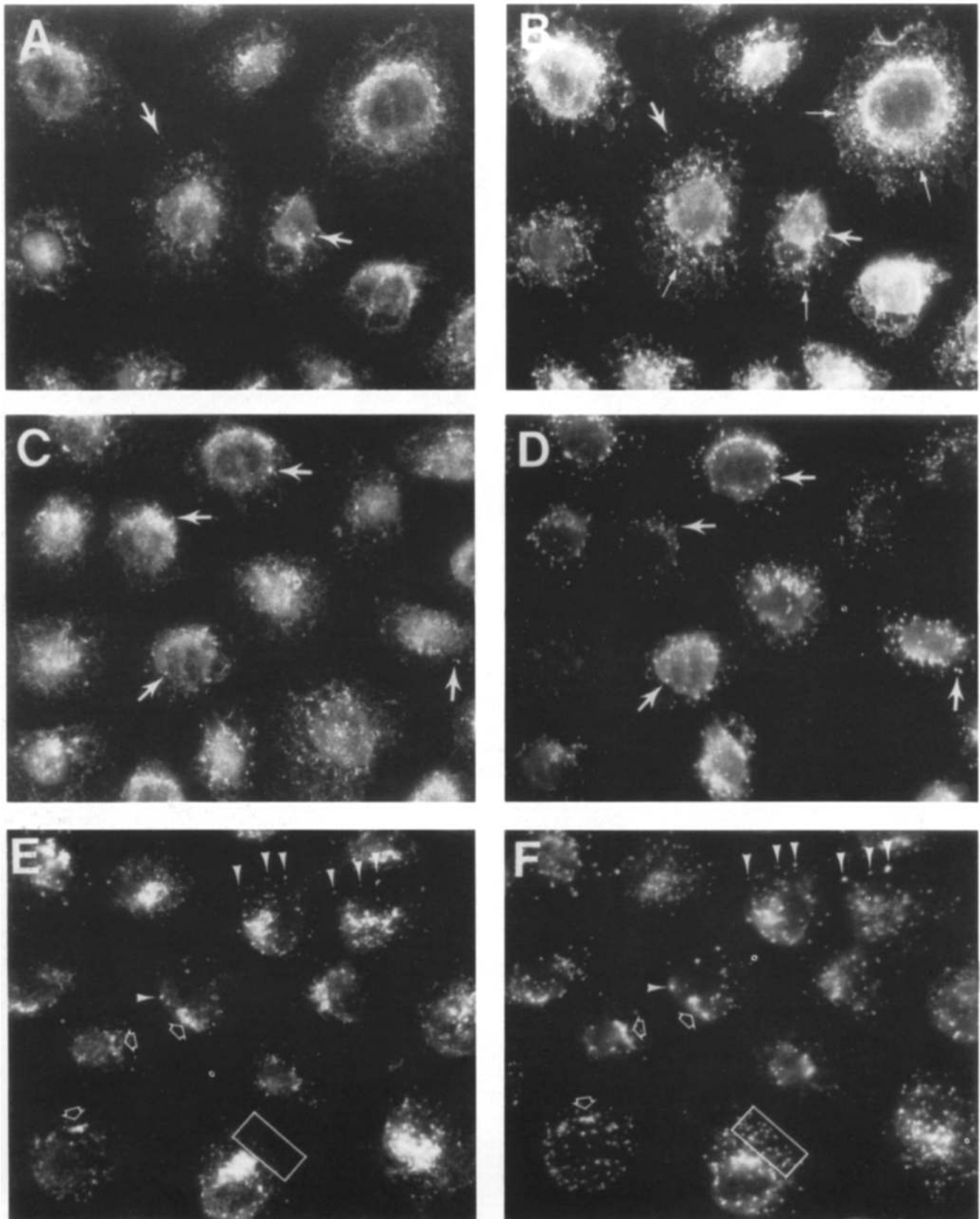
We have previously demonstrated that transport between the ER and the Golgi in semi-intact cells requires physiological concentrations of  $\text{Ca}^{2+}$  (0.1  $\mu\text{M}$ ). In the absence of  $\text{Ca}^{2+}$ , we suggested that delivery to Golgi compartments was inhibited, although we could not rule out the possibility that an earlier step related to export from the ER also required  $\text{Ca}^{2+}$  (Beckers and Balch, 1989). As shown in Fig. 8 (*inset*), incubation of permeabilized cells in the presence of EGTA inhibited processing of VSV-G to the endo  $\text{H}_R$  form. However, EGTA did not block export from the ER. Incubation for 45 min in the presence of cytosol and EGTA resulted in maturation of VSV-G into punctate structures in both the peripheral cytoplasm and the central Golgi region (Fig. 14).

These structures showed strong colocalization with p58 in the punctate peripheral elements and partial colocalization with p58 in the central Golgi region. However, strong colocalization of VSV-G with Man II observed in the control incubations was generally not observed, supporting our interpretation that  $\text{Ca}^{2+}$  is required for a late fusion-related step in transport to the Golgi stack (Beckers et al., 1990).

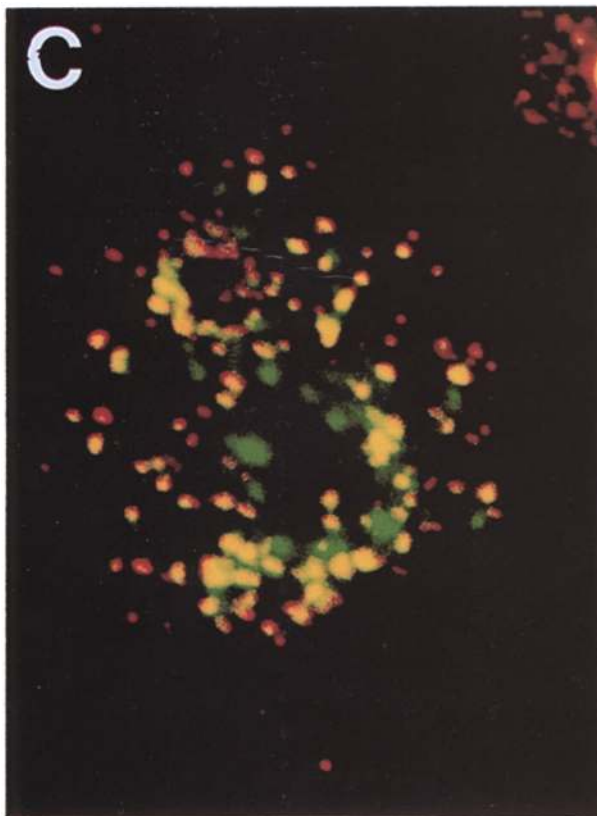
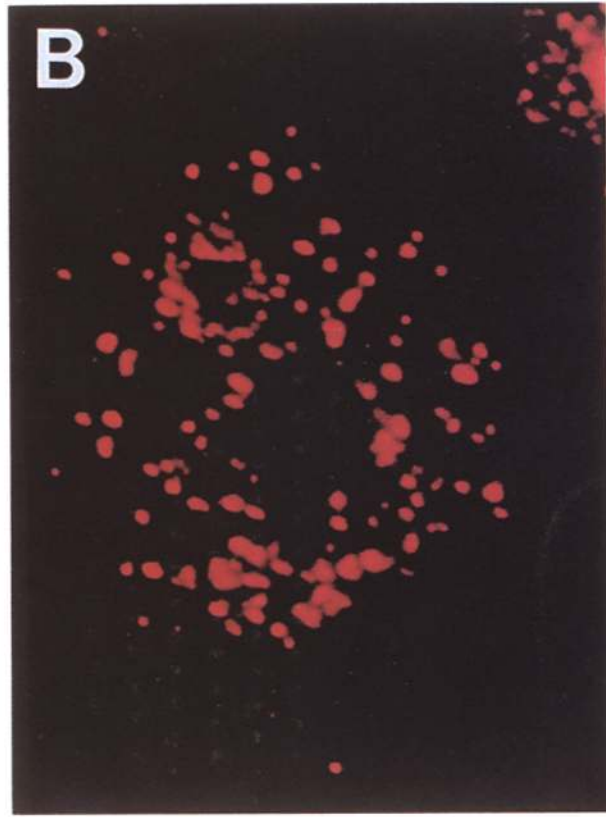
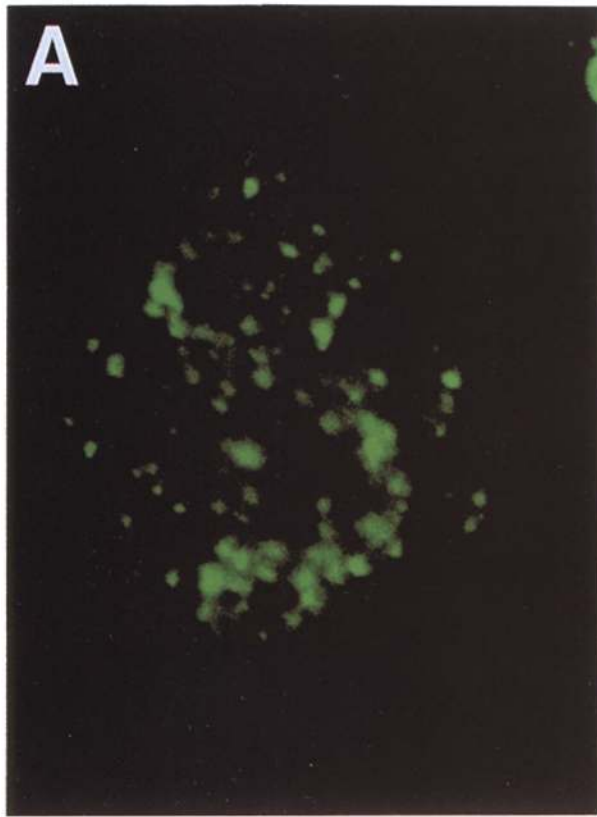
NSF and its yeast homologue SEC18 are required for ER to Golgi transport in mammalian cells (Beckers et al., 1989) and yeast (Kaiser and Schekman, 1990; Rexach and Schekman, 1991), and for transport through multiple compartments of the Golgi stack (Graham and Emr, 1991; Rothman and Orci, 1992; Waters et al., 1991; Wilson et al., 1989). NSF is a multi-subunit complex which is believed to be required for a late vesicle fusion step (Wilson et al., 1992). An antibody neutralizing NSF function (Wilson et al., 1989) inhibits ER to Golgi transport in semi-intact cells (Beckers et al., 1989). To assign a role for NSF in transport of VSV-G protein from the ER to the Golgi, we examined the distribution of VSV-G after incubation of digitonin permeabilized



**Figure 11.** Colocalization of the p58 containing intermediate compartment and Man II containing Golgi compartments. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated for 10 min (*C* and *D*) or 45 min (*E* and *F*) in the presence of cytosol and ATP, fixed and the distribution of p58 containing intermediate compartments (*A*, *C*, and *E*), or Man II containing Golgi compartments (*B*, *D*, and *F*) determined using indirect immunofluorescence as described in Materials and Methods. The distribution of p58 and VSV-G in permeabilized cells before incubation in vitro is shown in *A* and *B*. Small arrowheads indicate small, peripheral punctate structures; large arrows indicate Man II containing Golgi compartments.



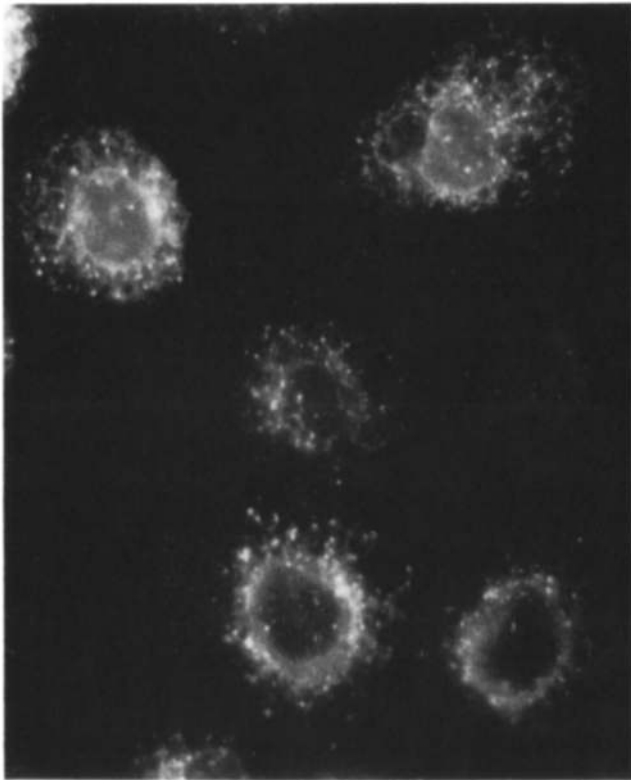
**Figure 12.** Colocalization of the p58 containing intermediate compartment and VSV-G protein after increasing time of incubation. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated for 10 min (*A* and *B*), 20 min (*C* and *D*) or 45 min (*E* and *F*) in the presence of cytosol and ATP, fixed, and the distribution of p58 containing intermediate compartments (*A*, *C*, and *E*) or VSV-G protein (*B*, *D*, and *F*) determined using indirect immunofluorescence as described in Materials and Methods. (*A-D*) Large arrows indicate punctate structures containing both p58 and VSV-G; small arrows indicate peripheral punctate structures containing VSV-G but not containing p58 (*B*). (*E* and *F*) Arrowheads indicate peripheral structures containing both p58 and VSV-G protein; open arrows illustrate distribution of VSV-G and p58 to the more central Golgi region; boxed region indicates punctate structures which contain VSV-G but do not contain p58.



**Figure 13.** Confocal scanning microscopy of VSV-G protein in the p58 containing intermediate compartment. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated for 20 min in the presence of ATP and cytosol, fixed and the distribution of p58 containing intermediate compartments (*A*) or VSV-G protein (*B*) determined using confocal scanning laser microscopy as described in Materials and Methods. A merge of the two confocal data sets is shown in *C*. Yellow indicates colocalization of the two proteins.

cells in the presence of anti-NSF antibody. As shown in Fig. 8 (*inset*), addition of anti-NSF antibody to the assay before initiation of transport inhibited the processing of VSV-G protein to the endo  $H_R$  form. However, the anti-NSF antibody

did not block export of VSV-G protein from the ER. As shown in Fig. 15, in the presence of the anti-NSF antibody VSV-G protein accumulated in uniform punctate structures principally found in the peripheral regions of the permea-



**Figure 14.** EGTA does not inhibit export from the ER. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated for 45 min in the presence of ATP and cytosol, fixed and the distribution of VSV-G protein determined using indirect immunofluorescence as described in Materials and Methods.

bilized cell (Fig. 15 C). These punctate structures strongly colocalized with p58 in the peripheral cytoplasm (Fig. 15 D, p58). In contrast, no colocalization with Man II was detected (Fig. 15 A, VSV-G, and B, Man II).

We next examined if an antibody to the cytoplasmic tail of VSV-G would also perturb transport between the ER and the Golgi. Two previous studies have demonstrated the inhibitory effects of various anti-tail antibodies on VSV-G protein export from the ER *in vivo* (Arnheiter et al., 1984; Kreis, 1986). When the anti-cytoplasmic tail antibody used to detect VSV-G protein by indirect immunofluorescence was added before the initiation of transport, processing to the endo  $H_R$  form was inhibited (Fig. 8, *inset*). When examined using indirect immunofluorescence, unlike the effects of either EGTA or  $\alpha$ -NSF, export of VSV-G protein from the ER was strongly inhibited. Even after 45-min incubation *in vitro*, VSV-G retained its diffuse reticular distribution observed prior to incubation (Fig. 16). In contrast, no inhibition of transport was observed in the presence of an antibody which recognized the luminal domain of VSV-G (not shown).

## Discussion

### Digitonin-permeabilized Cells

We have used the glycoside digitonin to selectively permeabilize the plasma membrane, but not compartments of the

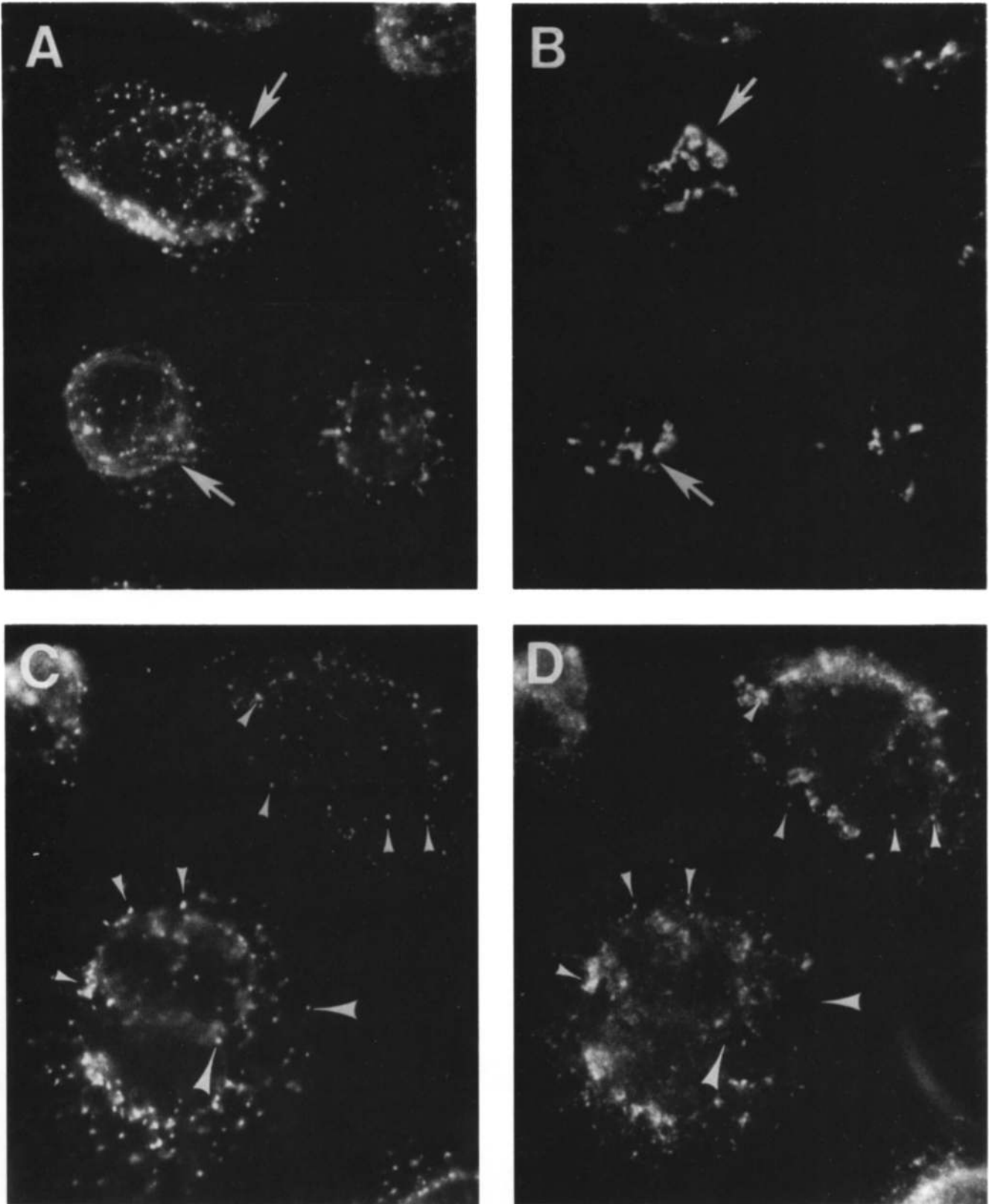
early secretory pathway. This allowed us to develop a model system which permitted both biochemical and morphological analysis of transport of protein from the ER to the Golgi complex. Our approach was motivated by the success of previous studies using cells permeabilized with digitonin and the related detergent saponin to analyze regulated exocytosis (Dunn and Holz, 1983; Wilson and Kirschner, 1983), lipid transport to mitochondria (Voelker, 1990), processing of oligosaccharides in primary hepatic cells (Wassler et al., 1990) and nuclear import (Adam et al., 1991; Greber and Gerace, 1992). Cells prepared by physical shear of the plasma membrane (Beckers et al., 1987) do not retain sufficient fine structure of the ER and Golgi compartments to facilitate a detailed morphological analysis of transport (W. E. Balch, unpublished results). In contrast, we have found that a broad range of cell lines (including CHO, BHK, HeLa, Vero, and L cells) can be permeabilized by 20–40  $\mu\text{g/ml}$  digitonin yet efficiently transport VSV-G protein from the ER to the Golgi. In all cases, permeabilization of the plasma membrane by digitonin was very efficient since transport was rendered completely dependent on exogenous cytosol and could be inhibited by selected mAbs. One such reagent (anti-NSF) was an IgM with a molecular weight of  $\sim 900$  kD indicating that large protein complexes can gain rapid access to the interior of the permeabilized cell.

Although the plasma membrane was permeabilized, VSV-G protein remained in sealed ER and Golgi compartments throughout the duration of the experiment. This observation was consistent with the efficient processing of VSV-G protein to the endo  $D$ -sensitive  $\text{Man}_5$  form and the endo  $H_R$  forms by enzymes concentrated in the lumen of Golgi compartments. Although we followed the appearance of the partially processed endo  $H_R$  intermediate to focus on ER to Golgi transport, VSV-G can be efficiently processed to the terminally glycosylated endo  $H_T$  form containing sialic acid *in vitro* (W. E. Balch and H. Davidson, manuscript in preparation), suggesting these cells reconstitute transport from the ER through multiple cisternae of the Golgi stack. Processing of VSV-G protein to the endo  $H_R$  intermediate was coordinate with its processing to the  $\text{Man}_5$  form in the absence of UDP-GlcNAc, suggesting that both measure transport from the ER to early Golgi compartments as discussed previously (Schwaninger et al., 1991; Davidson et al., 1992).

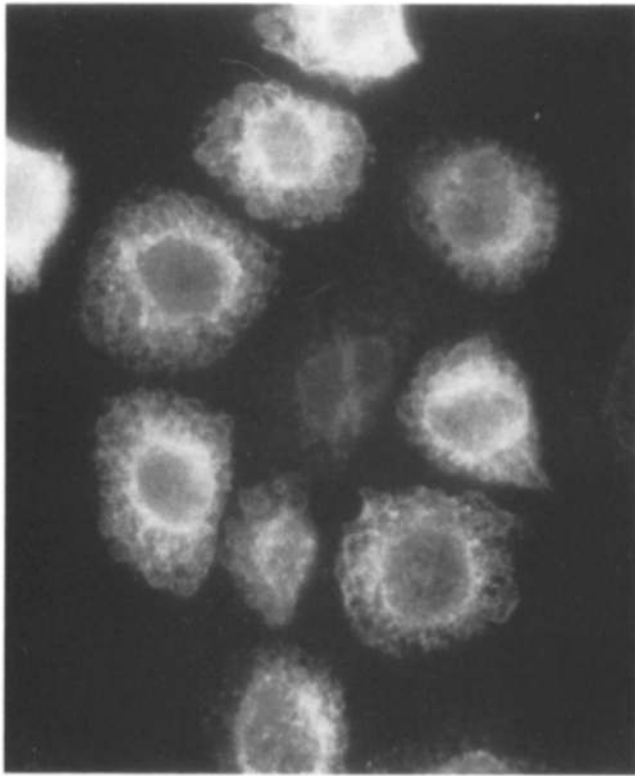
Digitonin permeabilization was found to be highly reproducible and provided us with an experimentally flexible system. Attesting to its reproducibility, we have found that over 95% of the cells on the coverslips responded identically to various incubation conditions. Since the cells remained bound to coverslips during incubation *in vitro*, morphological analysis of transport was amenable to the use of indirect immunofluorescence. Alternatively, radiolabeled cells could also be permeabilized in suspension for biochemical analysis of transport. A common pool of digitonin permeabilized suspension cells ( $\sim 10^7$  cells) from one 10-cm plate can be dispensed into at least 25 different incubation conditions. This allows for comparative biochemical and kinetic analyses of transport from related cell preparations, which is particularly important for the biochemical fractionation of transport components and for kinetic analyses.

In our preliminary characterization of this model system we examined the potential role of the cytoskeleton in stabilizing the morphology of the permeabilized cell, and





**Figure 15.** An antibody which inhibits NSF function results in accumulation of VSV-G in pre-Golgi intermediates. NRK cells were permeabilized using 25  $\mu\text{g/ml}$  digitonin and incubated for 45 min in the presence of ATP and cytosol and a monoclonal antibody to NSF (4A6), fixed and the distribution of VSV-G (A and C), Man II (B) or p58 (D) determined using indirect immunofluorescence as described in Materials and Methods. (A and B) The large arrows indicate distribution of Golgi compartments; (C and D) the large arrowheads indicate punctate structures which contain VSV-G (C) but not p58 (p58); the small arrowheads indicate punctate structures which contain both markers.



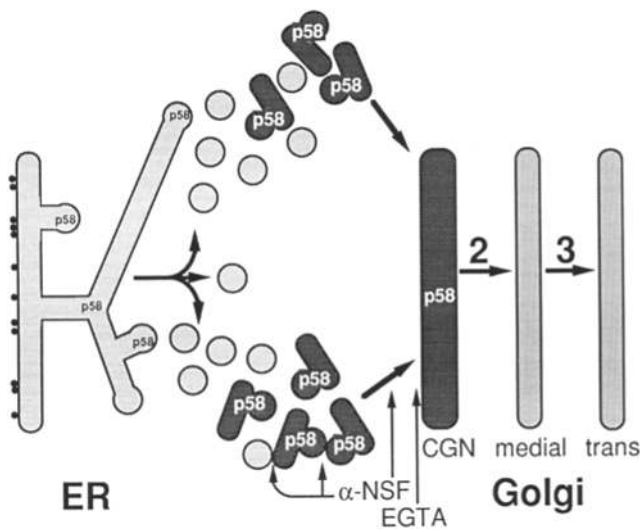
**Figure 16.** An antibody to the cytoplasmic tail of VSV-G protein inhibits export from the ER. NRK cells were permeabilized using 25  $\mu\text{g}/\text{ml}$  digitonin and incubated for 45 min in the presence of ATP, cytosol and 1  $\mu\text{g}$  of mAb P5D4 which recognizes the cytoplasmic tail of VSV-G protein. Cells were fixed and the distribution of VSV-G determined using indirect immunofluorescence as described in Materials and Methods.

structure of ER and Golgi compartments in vitro. Microtubules for the most part disassembled during the incubation. Given their importance in maintaining the ER network (Allan and Vale, 1991; Dabora and Sheetz, 1988; Vale and Hontani, 1988) and organization of the Golgi stack (Thyberg and Moskalewski, 1985), their disassembly is likely to account for both the more dispersed nature of the Golgi compartments and the swollen appearance of the ER after increasing time of incubation. In this regard, the delivery of VSV-G protein to peripheral punctate structures colocalizing with p58 at early time points may reflect in part a fragmentation of the *cis*-Golgi reticulum containing p58. Since microtubules are believed to play an important role in recycling components between the Golgi and the ER (Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991), the progressive loss of the capacity of the ER to export tsO45 VSV-G after increased time of incubation at the restrictive temperature could in part be due to disassembly of the microtubule network, a point we are currently pursuing. While actin filaments were also observed to undergo limited rearrangement, the distribution of intermediate filaments (vimentin) remained unchanged, consistent with their inability to undergo the rapid dynamic turnover characteristic of both actin filaments and microtubules. IF and/or actin networks may impart a degree of morphological integrity to the permeabilized cell during incubation in vitro.

### **Intermediate Steps in Transport of VSV-G Protein between the ER and the Golgi Compartment In Vitro**

Our focus in this study was to use digitonin permeabilized cells to define the morphological maturation of VSV-G protein from its reticular distribution in the ER to its concentration in the Golgi complex, and to relate this to biochemical and kinetic parameters we have previously documented using physically sheared cells (Beckers et al., 1990). We first established that transport reconstituted the vectorial steps observed in vivo. At the earliest time points (5–10 min) VSV-G protein became concentrated in small punctate structures which for the most part did not colocalize with p58. This step was inhibited by an antibody specific for the cytoplasmic tail of VSV-G and could represent redistribution of VSV-G in the ER reticulum prior to export, i.e., concentration in sites associated with recruitment of coat components involved in vesicle budding. After 15–20 min of incubation this distribution changed dramatically with >50% of the compartments containing VSV-G also containing p58. At this time point ~10–15% of the VSV-G protein has been processed by Golgi-associated forms. Subsequently, VSV-G protein appeared in Man II-containing compartments, correlating with its rapid processing to the endo  $H_R$  form. Importantly, Man II was retained in the morphologically distinct central Golgi compartments and was not transported in a retrograde fashion to the ER cisternae, a result which can be artificially generated in some cell lines by incubation of intact cells in the presence of the drug BFA (reviewed in Klausner et al., 1992).

The distribution of the p58 antigen used in our studies has been characterized extensively in vivo (Hendricks et al., 1991; Saraste and Svensson, 1991). The protein is not glycosylated with either galactose or sialic acid, but does contain terminal GlcNAc, suggesting that it fails to efficiently enter the later (*trans*-Golgi/TGN) elements of the Golgi stack (Hendricks et al., 1991). In support of this glycosylation pattern, the protein occupies both peripheral tubular-vesicular punctate elements and a central reticular compartment associated with the *cis* face of the Golgi at steady state (Hendricks et al., 1991; Saraste and Svensson, 1991) recently referred to as the CGN (Mellman and Simons, 1992; Rothman and Orci, 1992). The distribution of this protein is perturbed by prolonged incubation at 15°C, resulting in its accumulation in the peripheral punctate structures which also accumulate newly transported protein (Saraste and Svensson, 1991). Consistent with this observation, VSV-G accumulated in peripheral intermediates after a 90 min incubation at 15°C in vitro. A 53-kD antigen present in Vero cells behaves similarly to p58 (Schweizer et al., 1988). This protein has been used to establish a subcellular fractionation procedure to obtain highly enriched membrane fractions of the putative intermediate which contain a distinct protein composition from those of either the ER or Golgi membranes (Schweizer et al., 1990; Schweizer et al., 1991). Although we cannot presently distinguish whether the peripheral punctate intermediates containing p58 and VSV-G are specialized extensions of the ER involved in protein export or compartments distinct from the ER network, we strongly favor the latter interpretation based on three-dimensional reconstructions of these punctate intermediates using



**Figure 17.** Model for the role of p58 containing intermediates in ER to Golgi transport. VSV-G protein is exported from the ER via vesicle budding followed by fusion to peripheral and central punctate p58 containing compartments. Further transport of VSV-G to the Golgi occurs via coalescence of the p58 containing tubular-vesicular elements to form the CGN. In this interpretation, one vesicle fission step and multiple fusion events lead to the delivery of VSV-G to the central Golgi stack. The thick arrows show the vectorial flow of VSV-G protein to the cell surface. The thin arrows indicate the steps in transport sensitive to EGTA and an antibody ( $\alpha$ -NSF) which inhibits the function of NSF. Compartments enriched in p58 are labeled in bold. The potential recirculation (recycling) of p58 back to the ER is indicated by the small p58 label. The numbers 2 and 3 define the second and third vesicular transport steps required to transport VSV-G to the medial and trans Golgi compartments, respectively.

confocal microscopy and electron microscopy (W. E. Balch and M. G. Farquhar, unpublished results). Given the strong possibility that the p58 protein may recycle between the ER and Golgi (Klausner et al., 1992; Saraste and Svensson, 1991) this marker protein is likely to be a constituent of the same transport vesicles exporting VSV-G from the transitional ER. The lack of colocalization at the earliest time points may be due to the inability of the antibody to detect a low concentration of p58 at the export site. At later time points, the codistribution is more apparent after condensation of transport vesicles to form the larger tubular-vesicular elements characteristic of pre-Golgi punctate intermediates and the CGN.

### Compartments Involved in ER to Golgi Transport

Fig. 17 summarizes our current view of the steps in the early exocytic pathway. We suggest that transport from the ER to the Golgi involves one vesicle fission event responsible for export of protein from the ER followed by multiple fusion events leading to the delivery of vesicles to the CGN.

At the light microscopic level we cannot presently dissect the structure of pre-Golgi intermediates forming *in vitro*, however given the existing evidence (Saraste and Svensson, 1991; Schweizer et al., 1988, 1990) it is likely to consist of a collection of punctate tubular-vesicular elements. Since VSV-G protein accumulates at these sites when cells are incubated in the presence of an antibody which inhibits NSF function, a transport component which is likely to be in-

involved in vesicle fusion (Wilson et al., 1989, 1992; Waters et al., 1991; Rothman and Orci, 1992), we would propose that colocalization in the absence of NSF function is a consequence of concentration of transport vesicles emerging from the ER, but inhibition of their collective fusion to form the intermediate compartment. This interpretation is consistent with our inability to detect processing of VSV-G protein to either endo D-sensitive or endo H-resistant structures in the presence of anti-NSF antibody.

The mechanism involved in movement from the peripherally disposed punctate p58 containing compartments to the more centrally disposed CGN remains to be defined. One possibility is that a second round of vesicle formation is required. At the moment we are able to detect only one transport step between the ER and the more central p58 CGN, although this could simply reflect technical limitations due to the location of the enzymes processing VSV-G protein oligosaccharides used to assess transport which are unlikely to be concentrated in the intermediate (Schweizer et al., 1991). Alternatively, and an interpretation which we prefer, is that an additional round of vesicle fission and fusion is not required to move protein from the p58 containing punctate compartments to the CGN. Rather these compartments and the CGN define a biochemically related collection of tubular vesicular elements which ultimately condense to form the more central CGN found at the *cis* face of the Golgi stack. In particular, this interpretation is consistent with the striking accumulation of transported protein in the p58 compartment at reduced temperature (15–16°C), suggesting that a unique set of biochemical events distinct from vesicle fission is fundamental to delivery to the CGN. This coalescence could be similar to the recognized capacity of the ER to assemble from fragmented tubular-vesicular elements into extended reticular networks (Dabora and Sheetz, 1988), or the ability of the endocytic network to serve as a collection point for vesicles budding from the cell surface.

The structures observed morphologically reflect the dynamic nature of these tubular-vesicular elements in vectorial flow through the early exocytic pathway. Our interpretation is not to be confused with the cisternal progression model. At least two additional vesicular transport steps are required from the CGN to the *trans*-Golgi compartment (Fig. 17), and a third to the *trans*-Golgi network (Schwaninger et al., 1991). Given the evidence that recycling of proteins such as the KDEL receptor in mammalian cells occurs between the ER and the Golgi stack (reviewed in Pelham, 1989; Klausner et al., 1992), the CGN is likely to be the first “way-station” in vesicular trafficking from the ER and therefore a likely candidate to serve as the sorting compartment for this recycling step. It might be trivially referred to as an “exosome.”

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