Distribution of the intermediate elements operating in ER to Golgi transport

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Summary

We have used a 58 kDa membrane protein (p58) as a marker to study the transport pathway between the rough endoplasmic reticulum (ER) and the Golgi apparatus. Immunolocalization of p58 in fibroblasts showed its presence in a single cisterna and in small tubular and vesicular elements at the *cis* side of the Golgi apparatus. In addition, the protein was detected in large (200–500 nm in diameter) tubulovesicular structures, clustered in the Golgi region but also found in peripheral locations. These represent intermediates in ER to Golgi transport since they contained newly synthesized viral glycoproteins, arrested in cells at 15 °C. The peripheral structures accumulated at low temperature but reclustered rapidly to the Golgi region upon shift of cells back to 37 °C. This movement involved long intracellular distances and was efficiently inhibited by nocodazole, indicating that it requires the integrity of microtubules. In contrast, reclustering was unaffected by brefeldin A (BFA), suggesting that this compound affects ER to Golgi transport prior to the temperature-sensitive step. In BFA-treated cells p58 was localized to scattered, tubular, smooth ER clusters, found in close association with rough ER cisternae. The cellular distribution of the intermediate elements indicates that the sites of protein exit are widely distributed within the rough ER network. We suggest that the smooth ER locations where p58 accumulates in BFA-treated cells could represent such peripheral exit sites.

Key words: endoplasmic reticulum, Golgi apparatus, microtubules, organelle translocation, protein transport.

Introduction

Studies of specialized cells active in protein secretion resulted in the original description of the compartments that participate in the secretory pathway (Palade, 1975). Related to the early events of this process, electron microscopy revealed special domains of the rough endoplasmic reticulum (ER), the transitional elements, located close to the *cis* face of the Golgi complex (Jamieson and Palade, 1967; Palade, 1975). Transport of proteins between the rough ER and the Golgi apparatus is thought to be mediated by vesicles that shuttle between these transitional elements and the *cis* Golgi cisternae (Tartakoff, 1980; Farquhar, 1985; Pfeffer and Rothman, 1987). The morphology of the transitional elements suggests vesicle budding or fusion and the cytoplasm at the ER–Golgi interface is occupied by numerous small vesicles (Jamieson and Palade, 1967). Recent work with different cell types has suggested that such vesicles are closely associated with a tubular membrane system at the *cis* face of the Golgi apparatus (Lindsey and Ellisman, 1985; Merisko *et al.* 1986; Saraste *et al.* 1987; Schweizer *et al.* 1988; Rambourg and Clermont, 1990). The special characteristics of the *cis* Golgi compartment already became apparent from experiments showing its differential staining during heavy-metal impregnation or prolonged osmification (Friend and Murray, 1965; Rambourg *et al.* 1974).

The rate of transport from ER to Golgi varies for different proteins (Lodish, 1988; Rose and Doms, 1988) but is in many cases very rapid, making the morphological and biochemical characterization of this transport step more difficult. However, the development of systems that reconstitute protein transport *in vitro* in both yeast and mammalian cells have provided new insights into the biochemical mechanisms of membrane traffic in the early steps of the biosynthetic pathway (Balch, 1990; Hicke and Schekman, 1990; Rothman and Orci, 1990). Also, recent morphological studies have produced new data on this transport step. Immunoelectron microscopic studies of the synchronized movement of newly synthesized viral membrane proteins at reduced temperature (15 °C) showed the accumulation of the proteins in intermediate, tubulovesicular elements between the ER and the Golgi stacks (Saraste and Kuismanen, 1984). Studies on the intracellular maturation of a coronavirus suggested that early virus budding occurs at smooth membranes between the rough ER and Golgi, which may also represent the sites of addition of *O*-linked glycans to the viral proteins (Tooze *et al.* 1984; Tooze *et al.* 1988). It has been proposed that such an intermediate compartment functions in the receptor-mediated retention of a major class of soluble proteins within the ER lumen (Munro and Pelham, 1987; Warren, 1987; Pelham, 1988; see Pelham, 1989, for a review) and experiments using the drug brefeldin A (BFA) have provided the first evidence for the existence of such recycling pathways (Lippincott-Schwarz *et al.* 1990). BFA blocks transport from ER to Golgi (Takatsuki and Tamura, 1985; Misumi *et al.* 1986; Fujiwara *et al.* 1988) but allows

the redistribution of Golgi proteins to the ER system (Lippincott-Schwarz *et al.* 1989; Doms *et al.* 1989; Ulmer and Palade, 1989). Both the function of the intermediate elements and the apparent rapid and constitutive cycling of components (Wieland *et al.* 1987) emphasize the complexity of the pathways of membrane traffic between the rough ER and the Golgi apparatus.

A better understanding of the transport events between the ER and Golgi requires the identification and study of resident proteins of the intermediate membranes. Recently, Schweizer and colleagues (1988) described an unglycosylated 53 kDa membrane protein and obtained evidence of its enrichment in tubules and vesicles near the *cis* side of the Golgi apparatus, which also appear to contain the small GTP-binding protein rab2 (Chavrier *et al.* 1990). Through the production of antibodies against membrane proteins of a heavy Golgi subfraction from rat pancreas, we identified a 58 kDa protein (p58), which was shown to be concentrated in the *cis* Golgi cisterna and in tubulovesicular membranes apparently corresponding to the osmiophilic *cis* Golgi elements (Saraste *et al.* 1987). The present studies indicate that p58 is also a marker protein for the intermediate elements that operate in protein transport between the rough ER and the Golgi apparatus and suggest that cycling between ER and *cis* Golgi may determine its cellular localization. The pre-Golgi elements appear to arise at widely distributed ER sites and their movement to the central Golgi region requires the integrity of microtubules. Our results also indicate that, in cells treated with microtubule inhibitors, the dispersal of the Golgi apparatus is directed towards peripheral sites where the p58-containing intermediate elements accumulate. This would explain the contradiction between the present results and previous studies showing that protein transport from ER to Golgi is largely unaffected by microtubule-active drugs.

Materials and methods

Materials

Materials were obtained from the following sources: all cell culture media were from Gibco (Grand Island, NY); alkaline phosphatase-coupled anti-rabbit IgG and the enzyme substrate reagents from Promega (Madison, WI); FITC- or TRITC-coupled or biotinylated secondary antibodies against rabbit and mouse IgG, as well as fluorochrome–avidin conjugates, from Biosys (Compiegne, France) or Zymed (San Fransisco, CA); peroxidase-coupled $F(ab)_2$ fragments of anti-rabbit IgG from Immunotech (Marseille, France), and Protein A–Sepharose CL-4B from Pharmacia (Uppsala, Sweden). All other reagents were purchased from Sigma. BFA was kindly provided by Akiro Takatsuki (Department of Agricultural Chemistry, University of Tokyo), Jeff Ulmer and George Palade (Yale University, New Haven), as well as Jennifer Lippincott-Schwarz and Richard Klausner (NIH, Bethesda, MD).

Preparation of cell fractions

The procedures for the isolation and fractionation of total microsomes from rat pancreas have been described previously (Saraste *et al.* 1986). Microsomal subfractions recovered from isopycnic sucrose gradients were diluted in unbuffered 0.3 M sucrose containing a cocktail of protease inhibitors (100 units ml^{-1} Trasylol, 1 mM PMSF (phenylmethylsulfonyl fluoride), 10 $\mu g\,ml^{-1}$ soybean trypsin inhibitor, and 10 $\mu g\,ml^{-1}$ each of chymostatin, leupeptin, antipain and pepstatin), pelleted by a 60 min centrifugation at 100 000 g_{av} and then resuspended in 0.25 M sucrose containing the above protease inhibitors. To remove content proteins, the microsomal vesicles were extracted by addition of an equal volume 0.2 M sodium carbonate (pH 11.8) followed by a 30 min incubation on ice (Howell and Palade, 1982). Membranes were then concentrated by a 90 min centrifugation at 100 000 g_{av}. SDS–PAGE followed by protein staining and immunostaining of the gels with affinity-purified anti-p58 antibodies showed that p58 was a well recognizable, major protein in these membrane preparations and was predominantly concentrated in the two heavy Golgi subfractions (B2 and B3).

Purification of p58

The proteins in the alkaline-extracted B2 and B3 membranes were separated by preparative SDS–PAGE, the p58-containing regions of the gels were localized with the help of prestained markers, and excised using a razor blade. Subsequent protein staining of the remaining gels was used to verify the accuracy of this procedure. The protein was eluted from the gel slices using an Isco model 1750 electrophoretic concentrator (Isco Inc., Lincoln, NE) as described by Bhown *et al.* (1980). After lyophilization, SDS was removed from the samples by extraction according to the method of Henderson *et al.* (1979). The precipitated protein was concentrated by centrifugation. Samples of the protein were dissolved in SDS–PAGE sample buffer (to test for purity) or in sterile PBS (for immunization).

Antibodies

For the preparation of monospecific anti-p58 antibodies, the purified protein was injected into the popliteal lymph nodes of two New Zealand white rabbits. The overall immunization procedure was essentially the one described by Louvard and coworkers (1982) with the exception that the final booster injections were administered i.m. rather than i.v. During the immunization each rabbit recieved a total of 50–100 μg of purified protein. Both rabbits produced antibodies that detected a 58 kDa protein band in immunoblots of membrane preparations derived from different cell types. IgG fractions from the sera were prepared using a Protein A–Sepharose CL-4B column. Affinity-purification of the antibodies was carried out using the blot-method described elsewhere (Olmsted, 1986; Saraste *et al.* 1987).

The monoclonal antibody (8.139) against the E1 spike glycoprotein of SFV (Semliki Forest virus) was provided by Wil Boere (State University of Utrecht, The Netherlands) and against the 135 kDa Golgi protein (53FC3; Burke *et al.* 1982) by Daniel Louvard (Pasteur Institute, Paris) as well as Brian Burke and Graham Warren (European Molecular Biology Laboratory, Heidelberg). The monoclonal antibodies against tubulin (1A2) and protein disulfide isomerase (ID3) were obtained from Thomas Kreis and Stephen Fuller (European Molecular Biology Laboratory, Heidelberg), respectively. The polyclonal antibody against mannosidase II was provided by Kelley Moremen (Massachusetts Institute of Technology, Boston, MA).

Cell culture and virus infection

NRK (normal rat kidney) cells were grown in DME supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 i.u. ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$). The growth medium of baby hamster kidney (BHK-21) cells was MEM containing 5% each of FCS, newborn calf serum, and tryptose phosphate broth, as well as L-glutamine and the antibiotics at the concentrations given above. For immunocytochemistry the cells were plated on glass coverslips on 35 mm plastic culture dishes and used after 2 days at about 50–70% confluency. The properties of the ts-1 mutant of SFV have been described (Kääriäinen *et al.* 1980; Saraste and Kuismanen, 1984). Infected BHK cells were grown for 4 h at 39.5 °C in MEM containing 0.2% BSA and 20 mM Hepes (pH 7.2) and then shifted for 60 min to 15 °C in the presence of 20 $\mu g\,ml^{-1}$ of cycloheximide.

Immunofluorescence microscopy

The basic procedures used for the processing of cells for immunofluorescence microscopy have been summarized elsewhere (Kuismanen and Saraste, 1989). Briefly, for the staining of membrane antigens (p58, 135 kDa Golgi protein) the cells were fixed for 30 min at room temperature with 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and thereafter permeabilized

for 15 min in PBS containing 0.05% Triton X-100. For the visualization of microtubules with anti-tubulin antibodies the cells, fixed as above, were permeabilized by immersion for 5 min in −20°C methanol. To detect the primary antibodies either fluorochrome-coupled or biotinylated secondary antibodies were used. The latter were detected *via* avidin–fluorochrome conjugates. In double-staining experiments no cross-reactivity between the secondary antibodies was detected. In double-staining with anti-mannosidase II antibodies, affinity-purified anti-p58 antibodies, coupled directly to FITC, were used. Finally, the cells were mounted in 80% glycerol in PBS supplemented with phenylenediamine and examined by epifluorescence in a Leitz Diaplan microscope equipped with the appropriate filters for FITC- and TRITC-derived fluorescence. Photographs were taken on Kodak Tri-X-Pan (400 ASA) film.

Vesicle translocation assay

During the temperature shift the 16°C medium was immediately replaced by a prewarmed 37°C medium containing 20 $\mu g\,ml^{-1}$ cycloheximide. The concentration of BFA was 5 $\mu g\,ml^{-1}$. In some experiments the cells were first incubated for 15 min on ice to depolymerize microtubules and then transferred to the 37°C medium containing also different concentrations of nocodazole (0.1–10 μM). After fixation at 37°C the cells were stained with anti-p58 antibodies and processed for immunofluorescence microscopy. The effect of the different treatments on the disassembly and reassembly of microtubules was controlled by staining with anti-tubulin antibodies. For the determination of vesicle numbers and intracellular distances, photographs were taken of random fields and printed to give a final magnification of ×1000. In both cases only the p58-positive, large vesicular structures located outside the Golgi region were measured. For each determination 15–20 cells were included. The distances of the peripheral vesicles were measured relative to the centers of the juxtanuclear Golgi areas, which could be easily determined in NRK cells after staining with anti-p58 antibodies. Control measurements indicated that the different treatments did not result in changes in cell shape that would have significantly affected the obtained values.

Immunoelectron microscopy

The details of the procedures used for the fixation, permeabilization, immunoperoxidase staining and processing of cells for electron microscopy have been described in detail elsewhere (Brown and Farquhar, 1989; Kuismanen and Saraste, 1989). After dehydration the cells were embedded in a low-viscosity epoxy resin (Spurr). Thin sections were stained with lead citrate and examined and photographed in a JEOL 100 CX electron microscope operated at 60 kV.

Results

Localization of p58 to both cis *Golgi and peripheral elements in fibroblasts*

To localize p58 in cultured fibroblasts we have used two types of polyclonal anti-p58 antibodies, one affinity purified from the original antiserum (Saraste *et al.* 1987), and the other prepared against p58 purified from rat pancreas microsomal membranes. In immunoblots of membrane fractions prepared from NRK and BHK cells (Fig. 1), and from rat pancreas (not shown), both antibodies detected a single protein of the same molecular weight. This protein was also specifically detected by immunoprecipitation in detergent lysates of metabolically labeled NRK or BHK cells (data not shown). In immunolocalization experiments with BHK and NRK cells these two antibodies gave identical results.

Immunofluorescence microscopy of BHK cells showed the presence of p58 in vesicular and tubular structures that, although largely concentrated in the perinuclear

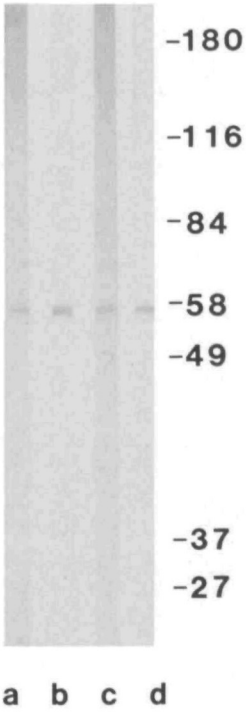


Fig. 1. Specificity of the antibodies. Immunoblotting of proteins of total membrane fractions (50 μg/lane) prepared from NRK (lanes a and b) and BHK cells (lanes c and d), with two anti-p58 antibodies, either affinity purified from the original anti-B2 serum (lanes a and c), or prepared against purified p58 (lanes b and d). Both antibodies reacted with a single protein band, which in these gels migrates slightly faster than the prestained 58 kDa marker (puruvate kinase).

Golgi area, were also seen outside the Golgi region and even close to the cell surface (Fig. 2A). The Golgi apparatus was visualized in the same cells by double-staining with monoclonal antibodies against a 135 kDa integral membrane protein (Fig. 2B), which was localized to Golgi cisternae in NRK cells (Louvard *et al.* 1982; Burke *et al.* 1983) and has recently been suggested to be identical with mannosidase II (Baron and Garoff, 1990). Although the two antibodies clearly stained different compartments, at many sites a close association of the p58-positive elements with the Golgi stacks was apparent.

In NRK cells, as in BHK cells, p58 was localized in vesicular elements that were concentrated in the Golgi region (Fig. 2C and D) but also found around the nucleus and in more peripheral locations. In NRK cells anti-p58 antibodies also gave a weak, reticular fluorescence that colocalized with the staining obtained with monoclonal antibodies against protein disulfide isomerase (PDI), a major luminal ER protein. Treatment of cells for up to 4 h with cycloheximide did not deplete this reticular staining, indicating that it was not due to a newly synthesized pool of p58 in the ER (data not shown).

To study the structure of the p58-containing elements and their relationships with other organelles in NRK cells we used immunoperoxidase electron microscopy (Figs 3 and 4). In the Golgi apparatus p58 was detected only in one cisterna (Fig. 3B), which, as in other cell types studied (Saraste *et al.* 1987; unpublished data), most likely corresponds to the *cis*-most Golgi cisterna. It also displayed the fenestrated morphology typical of the *cis* aspect the Golgi stack (Rambourg and Clermont, 1990). However, in the Golgi region of NRK cells p58 was most frequently localized in pleomorphic, tubulovesicular structures (Fig. 3 A and C), apparently giving rise to the vesicular Golgi pattern seen by immunofluorescence (Fig. 2). These elements were variable in both size (200–500 nm in diameter) and shape, and contained both vacuolar and tubular domains. By electron microscopy p58 was also detected in small Golgi vesicles, about 80 nm in diameter. These could represent either free vesicles or

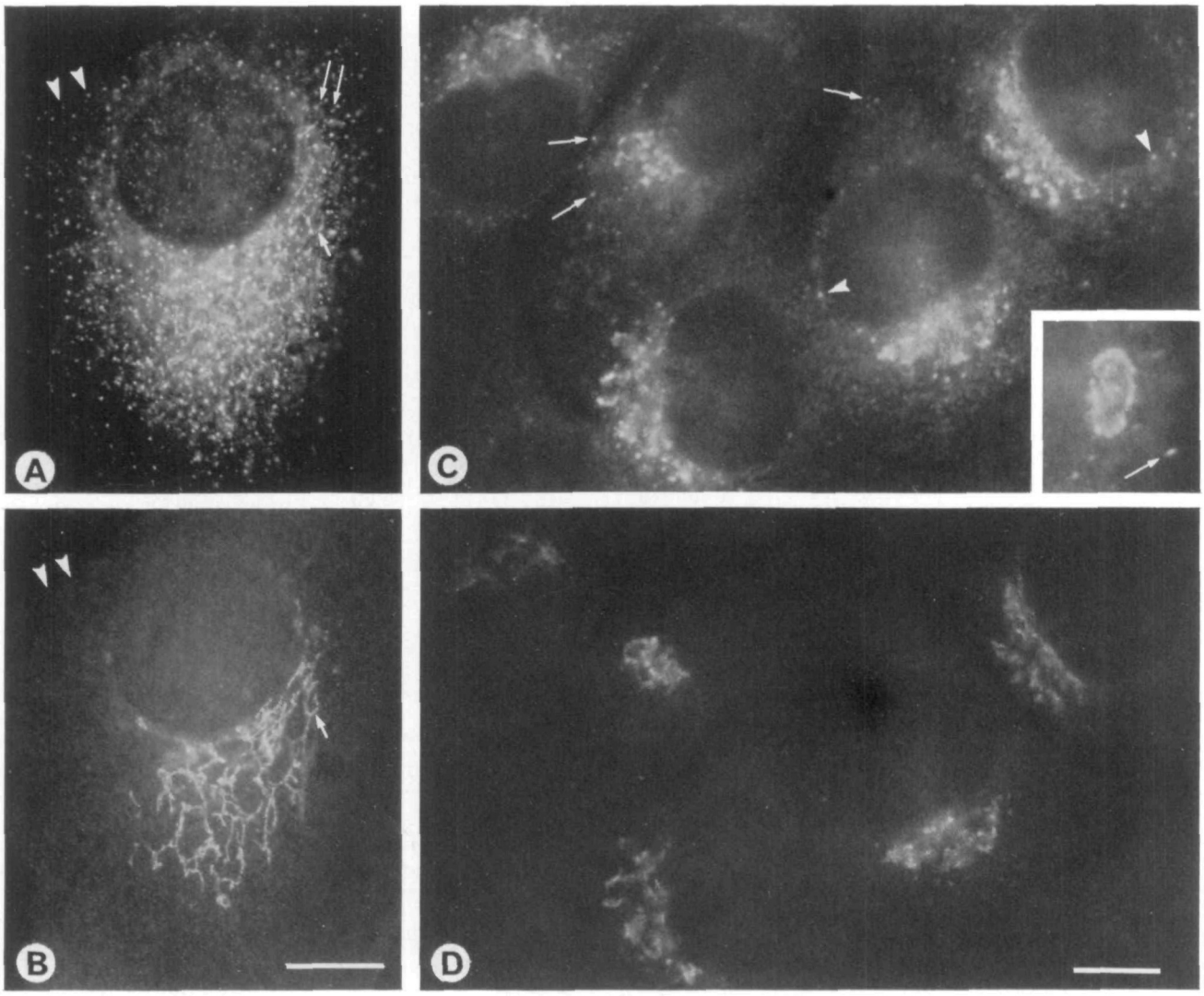


Fig. 2. Distribution of p58-containing elements. BHK (A and B) and NRK cells (C and D) were fixed at 37 °C, permeabilized and double-stained for immunofluorescence microscopy with affinity-purified rabbit anti-p58 antibodies (A and C) and monoclonal anti-Golgi antibodies (B and D). Note the concentration of the p58-positive vesicular or tubular (double arrows in A) elements close to the Golgi stacks labeled with the anti-Golgi antibodies (small arrows in A and B), next to the nuclear envelope (arrowheads in C), and in peripheral locations (long arrows in C), even to close the cell surface (double arrowheads in A and B). In NRK cells anti-p58 antibodies also give weak, reticular ER staining. The inset in C shows a cell with ring-like staining, indicative of the localization of the p58 at the *cis* aspect of the Golgi apparatus. Bars, 10 μm.

cross-sections of tubules extending from the pleomorphic elements or the *cis* Golgi cisternae (Saraste *et al.* 1987; Rambourg and Clermont, 1990).

The peripherally located p58-positive elements in NRK cells morphologically resembled the ones seen in the Golgi region (Fig. 4). As already indicated by light microscopy, these were often encountered close to the nuclear membrane (Fig. 4A) and the cell surface (Fig. 4B). In addition, electron microscopy revealed their frequent, close association with rough ER cisternae and unlabeled, smooth ER elements (Fig. 4E). Although they were often found next to morphologically identifiable components of the endocytic pathway, coated vesicles, multivesicular endosomes and lysosomes, no labeling of the latter was observed. In addition to the staining of the elements described above, anti-p58 antibodies also gave variable, but at most weak, labeling of rough ER cisternae and the nuclear membrane.

The peripheral p58-positive elements function in ER to Golgi transport

Previous work has shown that when Semliki Forest virus ts-1 mutant-infected cells were shifted from the restrictive temperature (39.5 °C) to 15 °C, newly synthesized virus membrane glycoproteins gained exit from the rough ER but accumulated in tubulovesicular structures between the rough ER and the Golgi apparatus (Saraste and Kuismanen, 1984). To determine if the above described p58-positive elements function in protein transport between the ER and Golgi, we used double-labeling of ts-1-infected BHK cells with antibodies against p58 and the E1 spike glycoprotein of SFV. In cells maintained at 39.5 °C, which causes the arrest the viral membrane proteins in the rough ER (Kääriäinen *et al.* 1980; Saraste and Hedman, 1983), negligible codistribution of the two proteins could be seen (Fig. 5A and E). In contrast, in cells shifted for 60 min to 15 °C, E1 could be detected in most of

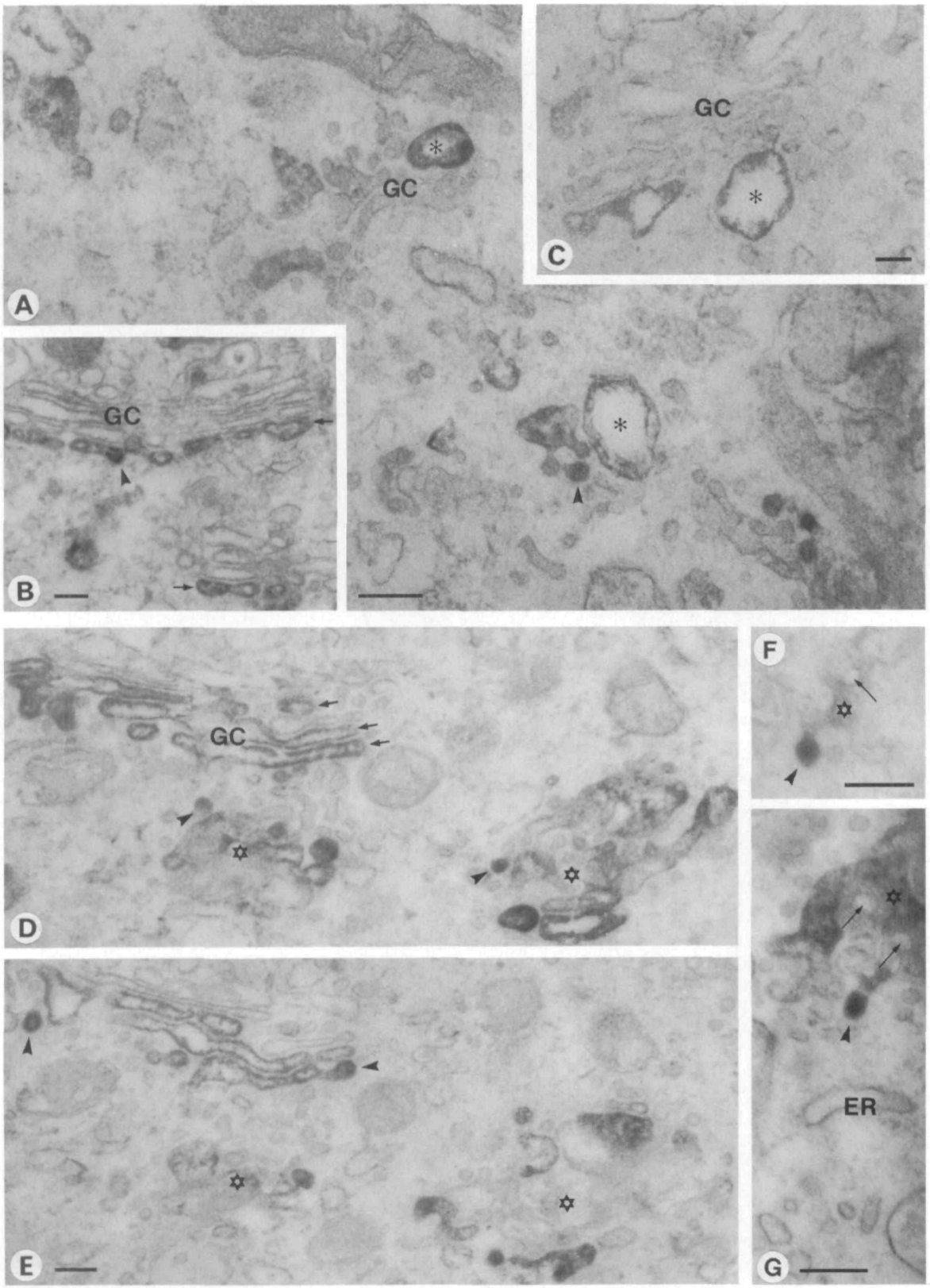


Fig. 3. Electron-microscopic localization of p58 in the Golgi region of NRK cells. The cells were fixed for immunoperoxidase staining after incubation at 37°C (A–C) or after 180 min incubation at 16°C (D–G). At steady state (37°C) p58 is detected in large, pleomorphic structures (asterisks in A and C) located close to the Golgi stacks (GC) and, less frequently, in a single cisternal element at one side of the Golgi stacks (B; small arrows). In contrast, at 16°C p58 is localized to several Golgi cisternae (small arrows in D) and in pleomorphic, fenestrated (long arrows in F and G) *cis* Golgi elements (stars in D–G). D and E are consecutive serial sections of the same Golgi area. At both temperatures, but especially at 16°C, p58 is also detected in small Golgi vesicles or buds (arrowheads). Bars, 0.2 μm.

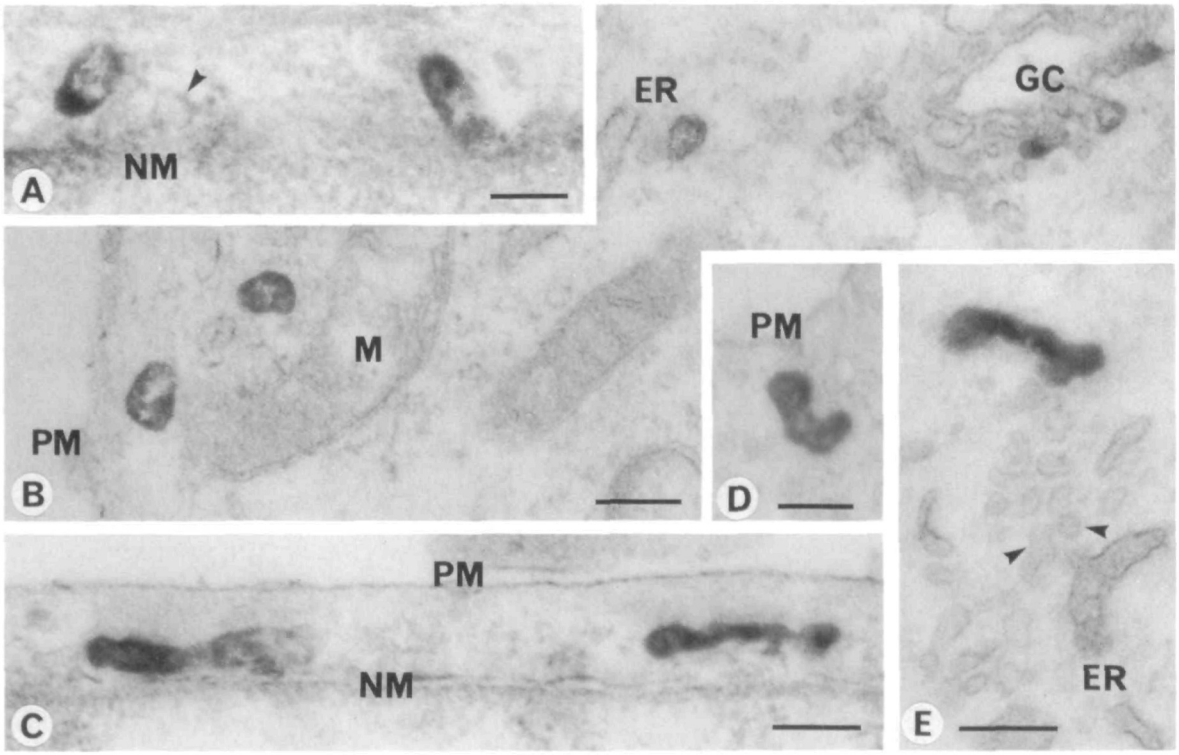


Fig. 4. Morphology of the peripheral pre-Golgi elements in NRK cells. For immunoperoxidase localization of p58 the cells were fixed at 37°C (A, B and E) or after a 180 min incubation at 16°C (C and D). At both temperatures p58 is localized to large, tubulo-vesicular elements outside the Golgi region (GC). These are located close to rough ER cisternae (ER; B and E), the nuclear membrane (NM; A and C), and the plasma membrane (PM; B and D). The arrowheads in A and E indicate unlabeled smooth ER elements located next to pleomorphic, p58-positive elements. M, mitochondria. Bars, 0.2 μm.

the p58-positive vesicular and tubular structures both in peripheral (Fig. 5B and F) and central (Fig. 5C and G) locations. The residual ER staining (Fig. 5B) and the detection methods used could explain why viral proteins were not detectable in some of the p58-positive vesicles. Also, some of the latter could be recycling vesicles and, therefore, would not be expected to contain the itinerary viral proteins.

As shown previously, return of cells from 15°C to 37°C for 5 min results in the transport of SFV membrane proteins from pre-Golgi elements into the Golgi stacks (Saraste and Kuismanen, 1984). We could visualize this transport event using immunofluorescence microscopy with anti-E1 antibodies. In contrast to E1, the temperature-shift did not appear to induce the movement of p58 into the Golgi apparatus. However, partial colocalization of E1 and p58 in vesicles in the Golgi region was still observed (Fig. 5D and H).

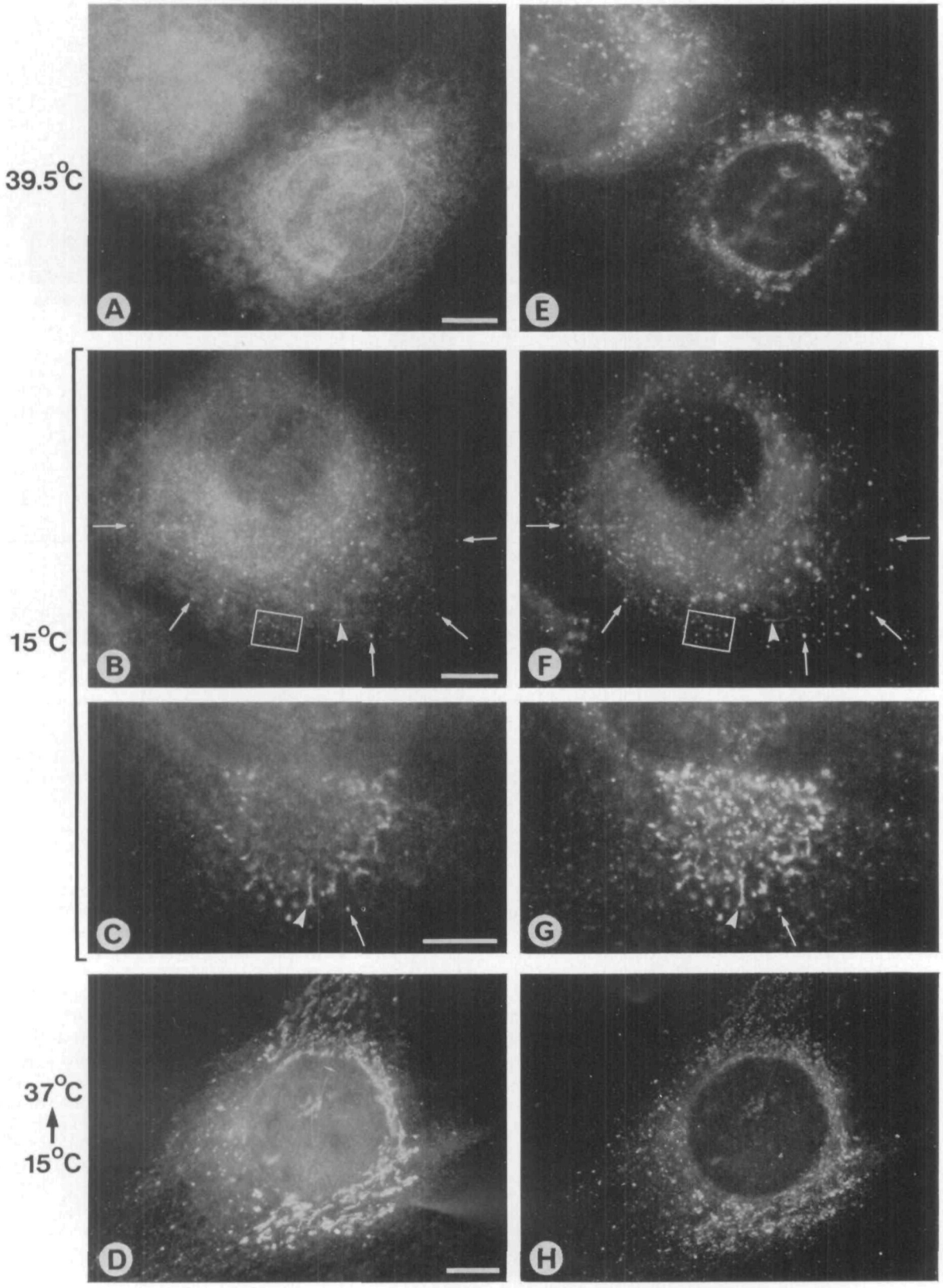
Peripheral pre-Golgi elements accumulate at low temperature

Since the peripheral p58-positive elements appeared to be involved in ER to Golgi transport and low temperature is known to affect this transport step (Kuismanen and Saraste, 1989), we examined how temperature reduction would affect the intracellular distribution of p58 in NRK cells. In contrast to the steady-state situation (Fig. 2C), in cells shifted to 16°C p58 was localized to a large number of vesicular elements in the perinuclear region as well as in more peripheral locations (Fig. 6B). The examination of cells shifted to 16°C for different times (1–3 h) showed that the number of these peripheral vesicles increased with time and, in parallel, the reticular ER staining was reduced (Fig. 6B, compare with Fig. 2C). Immunoelectron microscopy showed that this vesicular fluorescence was due to the accumulation of pleomorphic structures (Fig. 4C and D), similar in morphology to those seen in cells kept at 37°C. The 16°C incubation appeared to affect specifically the p58-positive elements, since immunofluorescence localization of the 135 kDa Golgi protein did not show changes in the distribution of the stacked Golgi elements in these cells (Fig. 6C and D).

We also examined the effect of temperature reduction on the distribution of the pre-Golgi elements by counting the

Fig. 5. The peripheral p58-positive elements contain viral proteins arrested between the ER and Golgi at 15°C. For immunofluorescence microscopy the cells were fixed, permeabilized, and double-stained with monoclonal antibodies against the E1 glycoprotein of SFV (A–D) and polyclonal anti-p58 antibodies (E–H). BHK cells were infected with the ts-1 mutant of SFV and incubated for 4 h at the restrictive temperature (39.5°C), which causes the arrest of the viral glycoproteins in the rough ER. Negligible colocalization of E1 and p58 is seen (A and E). Some of the cultures were shifted for 60 min to 15°C in the presence of 20 μg ml^{-1} of cycloheximide. Note that at 15°C the SFV spike glycoproteins and p58 are colocalized in many vesicular (the boxed area and arrows) and tubular (arrowheads) structures seen in peripheral (B and F) and central locations (C and G). Some p58-positive vesicles appear to be devoid of or contain undetectable amounts of viral glycoproteins. (D and H) 5 min after the return of cells from 15°C to 37°C viral proteins have moved to the Golgi stacks (D) whereas p58 is mostly seen in vesicular structures in the Golgi region as well as in peripheral locations (H). Bars, 10 μm.

relative numbers of the p58-positive vesicles outside the Golgi region in cells shifted for 60 min to different temperatures below 37 °C (Fig. 6A). The accumulation of vesicles was already observed at temperatures between 20 °C and 30 °C, although the most prominent effect was seen at 15 °C. No increase in the number of the peripheral elements was seen at 10 °C, a temperature suggested to block early steps in vesicular transport (Marsh *et al.* 1980; Tartakoff, 1986). These experiments indicate that the accumulation of the peripheral, p58-positive structures is not an effect specific for the 16 °C incubation and suggest that temperature reduction slows down a dynamic process



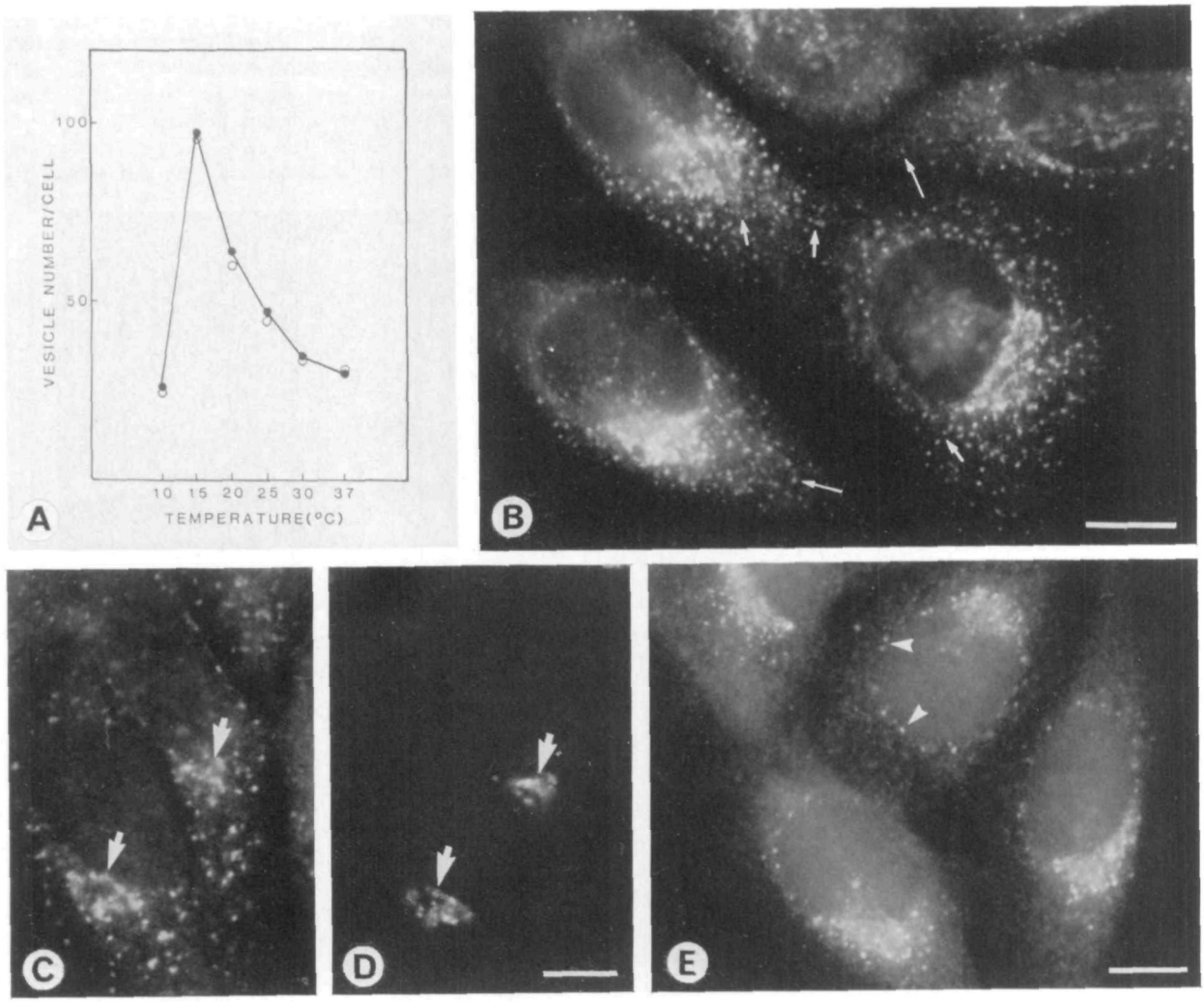


Fig. 6. Reversible accumulation of pre-Golgi elements at reduced temperature. (A) Relative numbers of p58-positive vesicles outside the Golgi region were counted from immunofluorescence photographs of NRK cells, shifted for 60 min to different temperatures below 37 °C before fixation, permeabilization and staining with anti-p58 antibodies. The open and closed circles indicate values obtained in two different experiments. (B–D) Accumulation of pre-Golgi elements in NRK cells incubated for 180 min at 16 °C. The cells were stained with anti-p58 antibodies only (B) or with both anti-p58 and anti-Golgi antibodies (C and D). Note the accumulation of p58-positive vesicles, sometimes in rows (small arrows in B), at peripheral locations (long arrows in B). Anti-p58 antibodies also give intensive Golgi labeling in these cells (arrows in C and D). (E) Reclustering of pre-Golgi vesicles to the Golgi region in cells shifted for 5 min from 16 °C back to 37 °C. The arrowheads indicate p58-positive structures next to the nuclear envelope that have failed to move to the Golgi region. Increased, reticular ER staining is also seen in these cells. Bars, 10 μm.

that maintains the steady-state distribution of these elements within cells.

Immunofluoresce microscopy showed intensive Golgi staining in cells incubated at 16 °C (Fig. 6C and D), suggesting the accumulation of p58 at this site. Surprisingly, immunoelectron microscopy showed that after a 180 min incubation at 16 °C p58 was redistributed within the Golgi and, in contrast to the situation at 37 °C, was now detectable in several Golgi cisternae (Fig. 3D and E). At 16 °C p58 was also localized in large pleomorphic structures and in small Golgi vesicles seen also at 37 °C. The former were fenestrated and thus seem to represent obliquely sectioned *cis* Golgi elements.

Microtubule-dependent clustering of pre-Golgi vesicles to the Golgi region

An advantage of the low-temperature-induced transport blocks is that they are readily reversible (Kuismanen and Saraste, 1989). Temperature-shift experiments could also be used to follow the movement of the peripheral p58-positive vesicles within cells. Already by 5 min after shift of NRK cells from 16 °C to 37 °C, p58 had resumed an intracellular distribution similar to that seen in control cells, suggesting that the peripheral elements, which had accumulated at low temperature, had moved to the Golgi region (Fig. 6E). By measuring the distances between individual vesicles and the center of the Golgi region we could reproducibly quantitate this process and, by studying cells shifted to 37 °C for short time periods, obtain evidence suggesting that the redistribution of p58 was due to the gradual movement of these pre-Golgi elements themselves rather than transport through another pathway (Table 1). The increased reticular staining in the cells shifted back to 37 °C indicated that some of the protein had been redistributed back to the ER (Fig. 6E; compare with Fig. 2C).

Table 1. *Reclustering of pre-Golgi elements to the Golgi region: effect of nocodazole and BFA*

Incubation	Average vesicle distance from Golgi (μm)	Inhibition of translocation (%)
37°C	7.6 (±0.2)	–
16°C, 180 min	14.3 (±1.2)	100
Shift 16°C to 37°C:		
2.5 min	11.1 (±0.6)	–
5.0 min	8.4 (±0.5)	–
10 min	7.8 (±0.2)	–
Shift 10 min+nocodazole:		
10 μM	15.3 (±1.7)	115
1.0 μM	13.9 (±1.0)	94
0.1 μM	9.9 (±0.4)	32
Shift 5 min+BFA	8.3 (±0.5)	0
Shift 10 min+BFA	11.2 (±1.3)	52

NRK cells were fixed at 37°C, after 180 min incubation at 16°C, or at different times (2.5–10 min) after shift from 16°C to 37°C in medium containing 20 μg ml^{-1} cycloheximide. The concentration of BFA in the shift medium was 5 μg ml^{-1}. To disrupt microtubules the cells, after incubation at 16°C, were placed for 15 min in ice-cold medium prior to shift back to 37°C in medium containing different concentrations of nocodazole. Vesicle distances were determined as described in Materials and methods. Values represent the mean (±S.D.) of three experiments.

The above measurements also showed that the translocation of the pre-Golgi elements involves long intracellular distances. We estimated that the distance that an average vesicle moves is at least 10 μm (Table 1). Therefore, we investigated whether the disassembly of microtubules could inhibit this process. Control immunofluorescence experiments using anti-tubulin antibodies, however, showed that even a 60 min treatment of cells at 16°C with nocodazole (De Brabander *et al.* 1976) failed to depolymerize the microtubules completely. For this reason the disassembly of microtubules was achieved by first incubating the cells briefly on ice prior to shift to 37°C. As shown in Table 1, nocodazole (1–10 μM), when added to the cells at the time of the shift 37°C, efficiently inhibited the reclustering of pre-Golgi elements to the central Golgi region, indicating that this process requires the integrity of microtubules. Even at very low concentrations (0.1 μM) the drug caused a partial inhibition in this assay. Preincubation of cells on ice as such did not affect the translocation process, since microtubules were completely repolymerized during the 10 min period following shift to 37°C in the absence of nocodazole.

When nocodazole was added to NRK cells during continuous incubation at 37°C, an accumulation of p58-positive vesicles in the periphery of the cells was observed already 10–15 min after addition of the drug, preceding the dispersal of Golgi elements. In a similar experiment we used double staining to localize p58 and the Golgi marker protein mannosidase II in BHK cells, treated for 15–120 min with 5 μM nocodazole. Accumulation of the peripheral, p58-positive structures was observed already by 15–30 min (Fig. 7B) after the addition of nocodazole whereas the dispersal of the mannosidase II-positive Golgi elements proceeded more slowly (Fig. 7F–H; see also Hiller and Weber, 1982; Rogalski and Singer, 1984; Sandoval *et al.* 1984). However, with time an increasing overlap in the distribution of the two markers was observed and after a 120 min nocodazole treatment p58 and mannosidase II showed almost complete colocalization in punctate structures scattered throughout the cells (Fig. 7D and H). Anti-p58 antibodies also gave increased diffuse, reticular staining in the drug-treated cells (Fig. 7B and C).

BFA affects ER to Golgi transport before the temperature-sensitive step

BFA is a fungal antibiotic that has been shown to inhibit protein transport from ER to Golgi in mammalian cells (Takatsuki and Tamura, 1985; Misumi *et al.* 1986) and cause the rapid, but reversible, disappearance of most morphologically identifiable Golgi elements as well as the redistribution of Golgi proteins into the ER (Fujiwara *et al.* 1988; Lippincott-Schwarz *et al.* 1989; Doms *et al.* 1989; Ulmer and Palade, 1989). We applied the vesicle translocation assay to map the inhibitory effect of BFA on ER to Golgi transport. As shown in Table 1, the presence of BFA during the first 5 min after the shift of cells to 37°C did not inhibit the reclustering of p58-positive elements to the Golgi region, suggesting that this compound affects transport before the temperature-sensitive step.

To examine further the effect of BFA we studied the distribution of p58 in NRK cells incubated for different times at 37°C in the presence of this drug. Immunofluorescence microscopy of cells treated for 5–15 min with BFA indicated rapid redistribution of p58 to reticular ER elements, including the nuclear membrane, as well as to punctate structures, scattered throughout the cells (Fig. 8B). During longer incubation with BFA (60 min; Fig. 8C) the reticular ER staining became somewhat weaker and the punctate elements in many cells assumed a more central position. By about 15 min after the removal of the drug the intracellular distribution of p58 returned to that seen in untreated control cells (Fig. 8A).

Immunoperoxidase localization of p58 in the BFA-treated cells (Fig. 9) verified the redistribution of the protein to the rough ER elements (Fig. 9A). The scattered, punctate staining seen by light microscopy appeared to be due to the presence of p58 in tubulovesicular smooth ER clusters located close to rough ER cisternae. A close association between the two types of structures indicated direct membrane continuities (Fig. 9B and E). In accordance with previous reports (Fujiwara *et al.* 1988; Lippincott-Schwarz *et al.* 1989; Ulmer and Palade, 1989), electron microscopy showed the complete absence of typical juxtanuclear Golgi stacks in NRK cells treated for only 10 min with BFA. At later time points dilation of the rough ER cisternae was also observed.

On the basis of these results, the partial inhibitory effect of BFA, observed at the 10 min time point in the translocation assay (Table 1), could be explained by assuming that some of the protein had now moved to the ER and subsequently accumulated at the peripheral smooth ER sites that, at light-microscopic resolution, are indistinguishable from the vesicular p58-positive structures accumulating at low temperature.

Discussion

The intermediate elements

In the present study we demonstrate that a 58 kDa membrane protein (p58), which was previously localized to *cis* Golgi elements in mouse myeloma cells (Saraste *et al.* 1987), is also a marker protein for large, tubulovesicular structures located outside the Golgi region. These peripheral elements contained newly synthesized viral spike glycoproteins, arrested in cells incubated at 15°C, and therefore correspond to the pre-Golgi structures that have

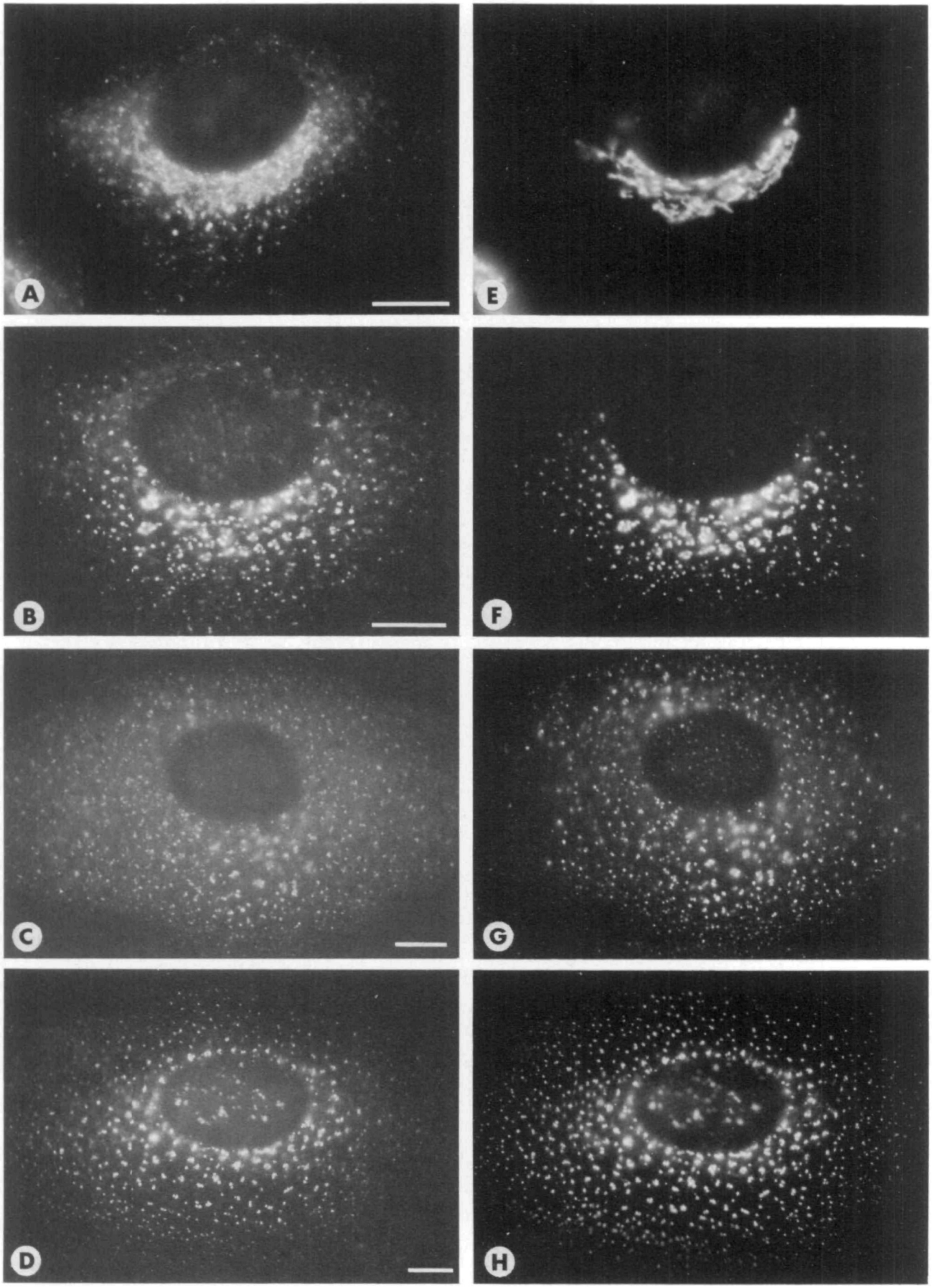

A
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Fig. 7. Double-immunofluorescence localization of p58 and the Golgi protein mannosidase II in nocodazole-treated cells. BHK cells were untreated (A, E) or treated with nocodazole (5 μM) at 37°C for 30 (B, F), 60 (C, G) or 120 min (D, H) prior to staining with antibodies against p58 (A–D) and mannosidase II (E–H). Note the rapid accumulation of p58-positive peripheral structures in the drug-treated cells. The fragmentation of Golgi elements, marked by anti-mannosidase II antibodies, appears to proceed more slowly. The resulting scattered Golgi staining is partly (30 and 60 min) or almost completely (120 min) coincident with the staining obtained with anti-p58 antibodies. Bars, 10 μm.

been shown to operate in protein transport between the rough ER and the Golgi apparatus (Saraste and Kuismanen, 1984). The present results obtained with fibroblasts are in agreement with the localization of p58 in other cell types. For example, in the acinar cells of rat pancreas p58 was seen to be concentrated both in *cis* Golgi elements and in tubulovesicular elements located close to the rough ER cisternae at peripheral locations (Saraste *et al.* unpublished results). In addition, the quantitation of p58 in microsomal subfractions derived from these cells (Saraste *et al.* 1986) showed that it is concentrated in two heavy Golgi fractions, banding at different densities (Lahtinen *et al.* unpublished data). In the present study we have used p58 as a marker protein in experiments involving transport inhibitors to map the pathway of protein transport between the rough ER and Golgi apparatus. A schematic summary of our results is presented in Fig. 10.

The concentration of p58 both in *cis* Golgi and in the peripheral elements suggests that these two types of structures may have compositional and functional similarities. This raises the possibility that many of the events that create the organelle boundary between the rough ER and the Golgi apparatus already take place at the peripheral sites where the formation of the pre-Golgi elements appears to occur. Our results suggest that the intermediate elements are dynamic structures and, in the context of protein transport, could be considered as 'vesicular' carriers themselves. Another possibility is that they form an intermediate compartment that has distinct compositional and functional properties, and represents a way station that interacts with both the rough ER and *cis* Golgi *via* shuttling vesicular carriers. It should be noted that the discontinuity between the peripheral and central p58-positive elements, depicted in Fig. 10, is tentative. Further morphological studies and examination of additional markers are required to reveal whether the observed peripheral structures are separate entities or part of a complex *cis* Golgi membrane system.

Possible cycling of p58 between the ER and cis *Golgi*

We observed the redistribution of p58 into the rough ER in BFA-treated NRK cells, but also in untreated cells a cycloheximide-resistant pool of the protein was detected in this com-partment. In addition, the detection of p58 in subcellular fractions derived from rat pancreatic acinar cells has indicated that at steady state considerable amounts of the protein are also present in the rough ER (Lahtinen and Saraste, unpublished results). These observations suggest that the concentration of p58 in the early compartments of the secretory pathway could result from its continuous cycling between *cis* Golgi and the ER (Fig. 10). The accumulation of p58 in several Golgi cisternae at 16°C could be explained by assuming that low

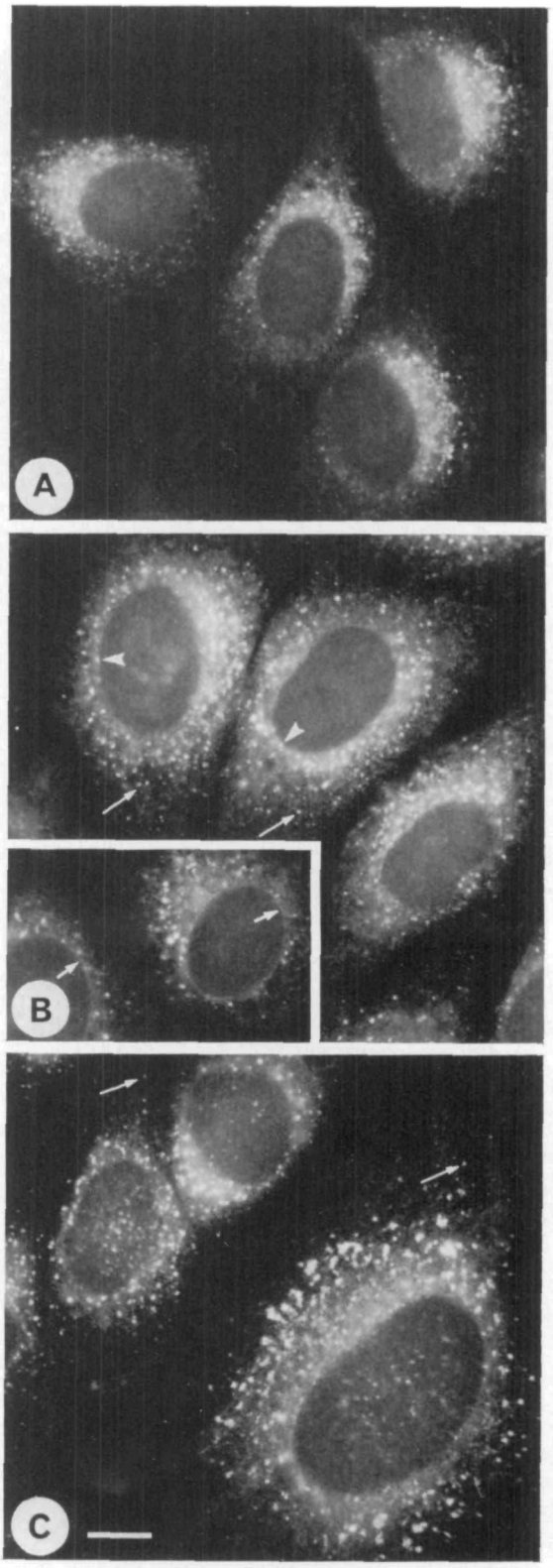

Fig. 8. Immunofluorescence localization of p58 in BFA-treated cells. NRK cells were untreated (A) or incubated for 10 min (inset in B), 15 min (B), or 60 min (C) at 37°C in medium containing BFA (5 μg ml^{-1}). BFA induces the redistribution of p58 to reticular ER (B), including the nuclear envelope (small arrows in the inset in B), and to punctate structures in the perinuclear region, near the nuclear membrane (arrowheads in B), and at the periphery of the cells (long arrows in B and C). Bar, 10 μm.

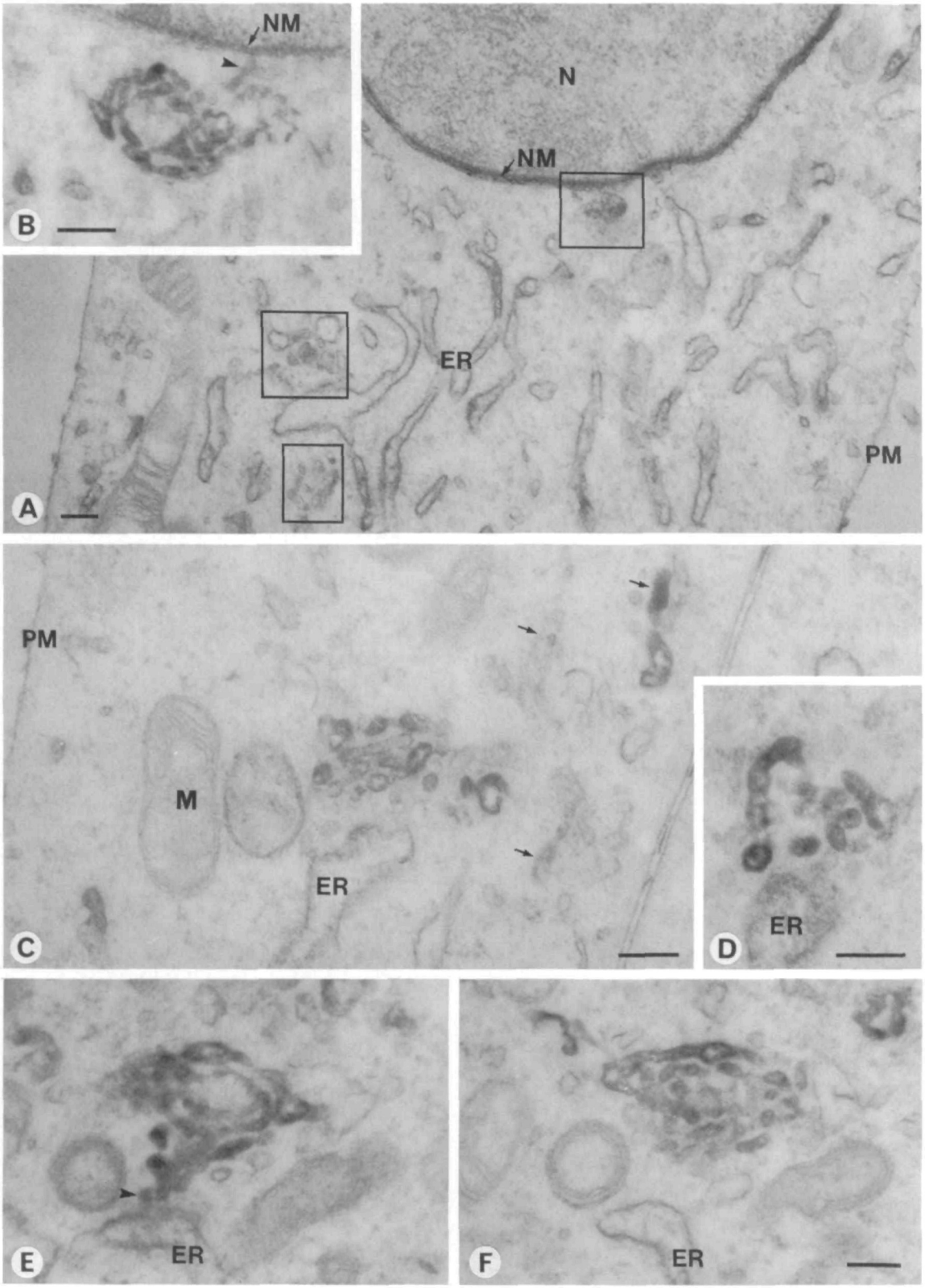

Fig. 9. Electron-microscopic localization of p58 in BFA-treated cells. NRK cells were fixed for immunoperoxidase staining at 15 min (A–C) or 60 min (D–F) after addition of BFA (5 μg ml^{-1}). Increased labeling of the rough ER (ER) and the nuclear membrane (NM) is seen (A). Both early and late after BFA addition p58 is detected in smooth ER clusters, located close to rough ER cisternae, and consisting of tubular (cisternal) and small vesicular elements. Three such, weakly positive, sites are boxed in A. In C long, tubular structures (small arrows) next to a rough ER-associated tubular cluster are also seen. The arrowheads in B and E indicate close connections between rough ER elements and the tubular, p58-positive membranes. E and F are consecutive serial sections. *N*, nucleus; PM, plasma membrane; M, mitochondria. Bars, 0.2 μm.

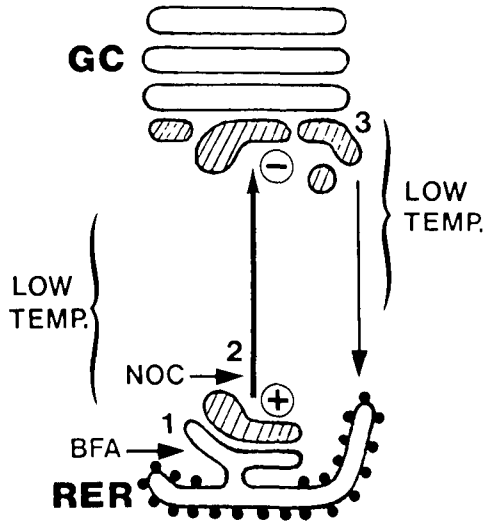

Fig. 10. A cyclic map of pre-Golgi events suggested by the effects of inhibitors on the distribution of p58. The peripheral and *cis* Golgi elements in which p58 is concentrated at steady state are shadowed. (1) Peripheral smooth ER exit sites in which p58 accumulates in the presence of BFA and where this drug propably blocks transport from the rough ER to the Golgi apparatus. (2) Nocodazole (NOC) inhibits the clustering of pre-Golgi elements to the central Golgi (GC) region, suggesting that this long-distance transport step involves microtubules. The putative plus to minus polarity of microtubules with respect to this (retrograde) transport step is indicated. This transport step is also slowed down at reduced temperature. (3) 16°C also causes the accumulation of p58 in the Golgi region and its redistribution into several Golgi cisternae, suggesting that low temperature efficiently blocks a recycling process that normally retrieves p58 from *cis* Golgi back to the rough ER.

temperature efficiently inhibits this recycling process, which normally rapidly turns the protein from *cis* Golgi back to the ER. It has been previously suggested that the 53 kDa marker protein (Schweizer *et al.* 1988) could normally utilize a recycling pathway that operates between the intermediate compartment and the ER (Lippincott-Schwarz *et al.* 1990; Schweizer *et al.* 1990).

Topography of ER to Golgi transport

Our results suggest that the steady-state distribution of the pre-Golgi elements is determined by their continuous movement towards the Golgi region. Temperature reduction, apparently simply by slowing down this process, resulted in the accumulation of pre-Golgi elements throughout the cells. Since morphologically similar elements could also be seen outside the Golgi region in cells maintained at 37°C, it is clear that the observed distribution does not result from an effect of low temperature on membrane organization. Topographically, the situation observed here (Fig. 10) could be compared to the events that occur along the endocytic pathway. Endosomes are present both at the cell periphery and in the central Golgi region and their cellular localization appears to be determined by a retrograde translocation process that is mediated by microtubules (see Kornfeld and Mellman, 1989, for a review).

The present results on the distribution of the pre-Golgi elements support the notion that the sites for protein exit from the rough ER are widely distributed within cells. These sites seem to follow the overall distribution of the rough ER network (Louvard *et al.* 1982; Terasaki *et al.* 1984) rather than being concentrated in the vicinity of the Golgi complex (Jamieson and Palade, 1967). Since the concentration of p58 normally takes place in the intermediate elements between ER and Golgi, their distribution reveals the long intracellular distances involved in this transport step (Table 1), but does not directly identify the exit sites of the rough ER. However, our results indicated that BFA affects an early step in ER to Golgi transport and causes the accumulation of p58 in tubular smooth ER membranes. The distribution of these BFA-sensitive sites, especially at short times after the addition of the drug, was similar to that of the intermediate elements that accumulate in cells at low temperature. However, morphologically these p58-containing smooth ER clusters, seen in BFA-treated cells, were completely different from the pleomorphic elements seen at 16°C and their close association with rough ER cisternae is in accordance with the idea that they represent the transitional regions of the ER. Interestingly, in both their distribution and morphology these BFA-sensitive membranes resemble the scattered Golgi clusters described in mitotic cells (Lucocq *et al.* 1987, 1989), where ER to Golgi transport is known to be arrested (Featherstone *et al.* 1985). Studies of other cell types have described rough ER-associated, smooth membrane elements that may correspond to the BFA-sensitive sites observed here (Tooze at al. 1988; Vertel *et al.* 1989).

The earliest known alteration in BFA-treated cells is the dissociation of a 110 kDa peripheral protein (Allan and Kreis, 1986) from Golgi membranes (Donaldson *et al.* 1990). It was recently reported that, in addition to central Golgi elements, this coat protein is also associated with peripheral vesicular structures in cells and colocalized with the vesicular stomatitis virus G-protein, arrested between the ER and Golgi at 15°C (Duden *et al.* 1991). These results support our proposal that the peripheral transitional regions of the ER could represent one of the cellular sites where BFA exerts its effect on transport.

Microtubules and ER to Golgi transport

A number of studies have documented the reversible effects of microtubule disruption on the organization of the Golgi apparatus (see Thyberg and Moskalewski, 1985; Kreis, 1989, for reviews). Upon microtubule breakdown, e.g. by nocodazole, the Golgi stacks are fragmented and dispersed throughout the cell. It has been suggested that these steps depend on energy and ongoing membrane traffic (Turner and Tartakoff, 1989). After the removal of nocodazole, the scattered Golgi elements are recentralized by retrograde movements along microtubules (Ho *et al.* 1989). More recently, studies carried out both *in vivo* and *in vitro* have suggested that microtubules are also involved in the dynamic movements of the ER (Terasaki *et al.* 1986; Dabora and Sheetz, 1988; Lee and Chen, 1988; Vale and Hotani, 1988; Lee *et al.* 1989). The question is how do the interactions between microtubules and these major organelles of the biosynthetic pathway relate to protein transport?

It has recently been suggested that microtubules facilitate the movement of membranes from the intermediate compartment and Golgi back towards the ER in cells treated with BFA (Lippincott-Schwarz *et al.* 1990), i.e. in the direction that is opposite to the one considered here. If a single microtubule-dependent pathway connects these compartments, the discrepancy between these and our results could be resolved by assuming that BFA acts

by simply reversing the direction of movement along microtubules. The flow of membranes back to the ER, seen in the drug-treated cells, could either represent an abnormal pathway or, alternatively, BFA could amplify an existing mechanism that normally retrieves membrane in a more regulated fashion. Our results, showing that BFA did not inhibit the reclustering of the intermediate elements back to the Golgi region (Table 1), could mean that the effect of BFA is manifested only at the two ends of the microtubule-dependent pathway and that the elements, which had already accumulated at 16°C, were insensitive to the action of BFA. The results of Donaldson *et al.* (1990) showing that, following a 1 min treatment with BFA, some of the Golgi membrane was committed to move to the ER even after the removal of the drug, would also support this possibility.

Experiments with live cells (Arnheiter *et al.* 1984; Kreis *et al.* 1989) and microtubule inhibitors (see Burgess and Kelly, 1987; Caplan and Matlin, 1989, for reviews) suggest that microtubules facilitate the transport of vesicles between the Golgi apparatus and the plasma membrane. However, these drugs at most only partially inhibit the transport of proteins along the biosynthetic pathway (Tartakoff and Vassalli, 1977; Kääriäinen *et al.* 1980; Rogalski *et al.* 1984; Burgess and Kelly, 1987; Kelly, 1990). Also, since the Golgi-specific glycosylation of transported proteins is largely unaffected by microtubule depolymerization (see e.g. Stults *et al.* 1989), it has been generally assumed, although there are exceptions (Busson-Mabillot *et al.* 1982; Pavelka and Ellinger, 1983), that protein transport between the rough ER and Golgi does not involve microtubules.

If microtubules play a role in ER to Golgi transport how can the ongoing transport and Golgi-specific processing of proteins in cells treated with microtubule-disruptive agents be explained? As suggested in Fig. 10, the observed redistribution of the Golgi apparatus could be due to a recycling pathway that operates between the Golgi and the peripheral exit sites of the rough ER. Then, in the absence of microtubule tracks the retrograde movement of membrane from ER to the Golgi would be blocked whereas the retrieval pathway, being less sensitive to the breakdown of microtubules, would direct Golgi components back to the ER. Thus, the apparent dispersal of the Golgi complex could involve the formation of small Golgi stacks (Thyberg and Moskalewski, 1985; Ho *et al.* 1989) at the peripheral sites. This, rather than random dispersal of existing elements, could re-establish a functional relationship between the ER and Golgi. Recent experiments showing the rapid and reversible effects of BFA on the organization of the Golgi apparatus support this idea. In fact, this model predicts that nocodazole and BFA, although by acting through different mechanisms, could exert a similar overall effect on the distribution of membranes at the ER–Golgi interface. Both would block net membrane movement from ER to Golgi but allow the backflow to continue. Finally, these considerations raise the possibility that protein transport from ER to the central Golgi region and the dynamic organization of the Golgi apparatus may involve the same microtubule-dependent mechanism.

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