Characterization of brefeldin A induced vesicular structures containing cycling proteins of the intermediate compartment/cis-Golgi network

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Abstract Residence of luminal ER proteins is mediated by a cyclic process which involves binding of escaped proteins to a KDEL receptor in a post-ER compartment and redistribution of the ligand-receptor complex back to the ER. We examined the relocation of the KDEL receptor after treatment with the fungal metabolite brefeldin A and compared this with the retrograde transport of the KDEL receptor observed after ligand or receptor overexpression. Incubation with brefeldin A led to the formation of vesicular structures containing the KDEL receptor and ERGIC-53, a marker for the ER-Golgi intermediate compartment. Immunoelectron microscopy revealed that these structures are composed of tubulo-vesicular clusters. The brefeldin A induced vesicular structures were morphologically and biochemically distinct from the ER-Golgi hybrid compartment as demonstrated by double immunofluorescence microscopy and subcellular fractionation. Overexpression of the receptor itself or a lysozyme-KDEL construct led to a shift of the KDEL receptor together with ERGIC-53, an intermediate compartment marker to the ER but not to structures resembling BFA induced vesicular structures. Moreover, overexpression of the receptor resulted in the partial redistribution of marker proteins of the medial Golgi and the trans-Golgi network to ER-like structures. We conclude that the effects of brefeldin A on the redistribution of the KDEL receptor do not reflect physiological events occurring during increased occupancy of the receptor with ligands.

Key words: Brefeldin A; KDEL receptor; Golgi apparatus; Endoplasmic reticulum; Intermediate compartment; Cis-Golgi network

1. Introduction

The Golgi complex is proposed to consist of at least three different compartments: the cis-most side of the Golgi/cis-Golgi network (CGN) associated with an array of tubules, the medial Golgi and the trans Golgi/trans-Golgi network (TGN) (for review see [1]). The membranes and proteins transported out of the ER reach the CGN via the intermediate compartment (IC). The IC, also termed ER-Golgi intermediate compartment (ERGIC) [2], appears to consist of an extensive tubular network spread over large areas of the cytoplasm. The precise characterization of the CGN and IC is limited by the paucity of accepted marker proteins and the fact that these proteins are able to move between different compartments. Examples are ERGIC-53 [2] or its rat homologue p58 [3] for the IC and the KDEL receptor [4,5] or gp74 [6] for the CGN.

Insight into the characteristics and dynamics of anterograde and retrograde transport has come from studies using the drug brefeldin A (BFA), which causes Golgi membranes to extend long uncoated tubules that fuse with the ER [7] resulting in an ER-Golgi hybrid compartment. Whether all parts of the Golgi complex fuse under BFA treatment with the ER is a matter of debate [8]. BFA reversibly blocks the first step in the formation of non-clathrin coat protein (COPI) coated transport vesicles [9,10]. However, formation of COPII coated buds and vesicles which are involved in anterograde ER-Golgi transport in yeast are not inhibited by BFA [11]. A BFA-like fusion of Golgi cisternae with the ER was observed by overexpression of bovine KDEL receptor [5] or a c-*myc* epitope tagged isoform of the KDEL receptor (ELP-1) [12]. These results raised the possibility that the effect of BFA and the phenotype of receptor overexpression are mechanistically related. In the course of our studies concerning the dynamics of the KDEL receptor and the nature of the retrograde traffic we directly compared all three mechanisms known to induce the redistribution of the KDEL receptor from the Golgi to the ER: treatment with BFA, the ligand induced redistribution of the KDEL receptor, and the overexpression of the receptor itself.

2. Materials and methods

2.1. Materials

Brefeldin A was obtained from Epicenter Technologies (Madison, WI, USA) and uridine-5′-bisphospho-D-[^{3}H]galactose from Amersham-Buchler (Braunschweig, Germany). For transient expression of lysozyme-KDEL or the KDEL receptor (hERD2) the pCMV2 vector of Anderson et al. [13] was used, which was kindly provided by Dr. G. Thiel (Institute for Genetics, University of Cologne, Germany). The expression vector pBEH was a gift from Dr. C. Peters (Department of Biochemistry II, Göttingen, Germany).

2.2. Antibodies

Antibodies against different proteins or epitopes were produced according to standard procedures in the following species: NH_2-CLYITKVLKGKKLSLPA-COOH (ERD2–4) in rabbit; NH_2-CDFFYLYITKVLKGKKLSLPA-COOH (ERD2–6) in sheep; chicken lysozyme in guinea pig. Antibodies directed against the following antigens were generous gifts: ERGIC-53 (G1/93) from H.P. Hauri (Department of Pharmacology, Basel, Switzerland) [14]; δ-COP from C. Harter (Department of Biochemistry, Heidelberg, Germany);

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Abbreviations: BFA, brefeldin A; BIVS, BFA induced vesicular structures; ER, endoplasmic reticulum; TGN, trans-Golgi network; CGN, cis-Golgi network; IC, intermediate compartment; GalTf, galactosyltransferase; Man II, α-mannosidase II; COP, coat protein

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rabbit antibodies against α-mannosidase II from K. Moremen (University of Georgia, Athens, GA, USA) and M.G. Farquhar (University of California, La Jolla, CA, USA) [15]; monoclonal antibodies against α-mannosidase II from G. Warren (Imperial Cancer Research Fund, London, UK) (hybridoma clone 53FC3) [16] and TGN38 (2F7.1) from G. Banting (University of Bristol, Bristol, UK) [17]. Monoclonal anti c-*myc* epitope antibodies (clone 9E10) as well as secondary antibodies coupled to fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC) or indigocarbocyanine (Cy3) were purchased from Dianova (Hamburg, Germany).

2.3. cDNA cloning

HepG2 RNA was purified according to [18] and used in a reverse PCR reaction with rTth polymerase (Perkin Elmer Cetus, Weiterstadt, Germany) with two 27-mer primers corresponding to the C- and N-terminal sequences of the human KDEL receptor (hERD2) [19]. The product was cloned via the TA vector (Invitrogen, Leek, The Netherlands) into the mammalian expression vector pCMV2 [13]. The cDNA of $ERD2_{myc}$ was constructed [20] and cloned into the pBEH vector which allowed a moderate expression of the protein in COS cells.

2.4. Subcellular fractionation

Vero cells were grown to confluence on four 145 cm^2 Petri dishes (Greiner, Nürtingen, Germany), treated with or without brefeldin A (2 μg/ml) for 60 min and washed with ice-cold PBS. The cells were collected, washed twice in homogenization buffer (130 mM NaCl, 50 mM Tris-HCl pH 7.5, 30 mM KCl, 3 mM EDTA) and homogenized in the same buffer (0.5 ml/100 mg wet weight) by passing through syringe needles (5×22 G/0.7 mm, 5×24 G/0.55 mm). The homogenate was centrifuged twice for 10 min at 1000×*g* and the resulting post-nuclear supernatant (2 ml) layered on top of a step density gradient (2 ml 30%, 1.33 ml 20%, 1.33 ml 15%, 1.33 ml 10%, 1.33 ml 7.5%, 1.33 ml 5%, 1.33 ml 2.5% (w/v) Nycodenz) (Nycomed, Oslo, Norway). After centrifugation in a SW40 Rotor (Beckmann, Munich, Germany) for 15 min at 33000×g_{av}, nine fractions were collected from top to bottom. The marker enzymes rotenone-insensitive NADH cytochrome *c* reductase for the ER and UDP-galactosyltransferase (GalTf) for the Golgi were measured according to [21] and [22] respectively. Western and dot blot analyses were performed as given in [23] using a chemiluminescence kit (Boehringer Mannheim, Mannheim, Germany) for the detection of the peroxidase coupled secondary antibodies. The monoclonal antibody against ERGIC-53 does not recognize the protein in Western blots.

2.5. Immunoelectron microscopy

Vero cells were removed from the Petri dish (25 cm^2) by treatment with 1 ml 20 μg/ml proteinase K. After addition of 1 ml fixing solution (4% paraformaldehyde, 250 mM HEPES pH 7.4), cells were pelleted for 2 min at 1000×*g*. The supernatant was removed and 2 ml fresh fixing solution added. Subsequent processing was as described elsewhere [24].

2.6. Immunofluorescence

Cells grown on cover slips to about 50% confluence were transferred to 24 well plates (Greiner, Nürtingen, Germany). Treatment with brefeldin A was at 5 μg/ml for different periods of time. Methanol (−20°C, 2 min) was used for rapid fixation and permeabilization. Unspecific binding sites were blocked for 15 min with 0.2% (w/v) fish gelatine in PBS. Primary antibody incubations were performed for 1 h at room temperature in 4 mg bovine serum albumin/ml PBS and secondary antibodies in 0.2% (w/v) fish gelatine in PBS. Cover slips were mounted in Mowiol and viewed using a Zeiss Axioskop microscope equipped with a Pan-Neofluar 100×/1.30 objective.

2.7. Cell culture and transfection

Vero cells (African green monkey kidney cells, ATCC CCL-81) and COS cells (SV40 transformed African green monkey kidney cells, ATCC CRL-1650) were grown as described in [23]. Transfections were carried out with the calcium phosphate coprecipitation method according to [25]. Unless otherwise stated cells were processed for immunofluorescence 24 h after transfection.

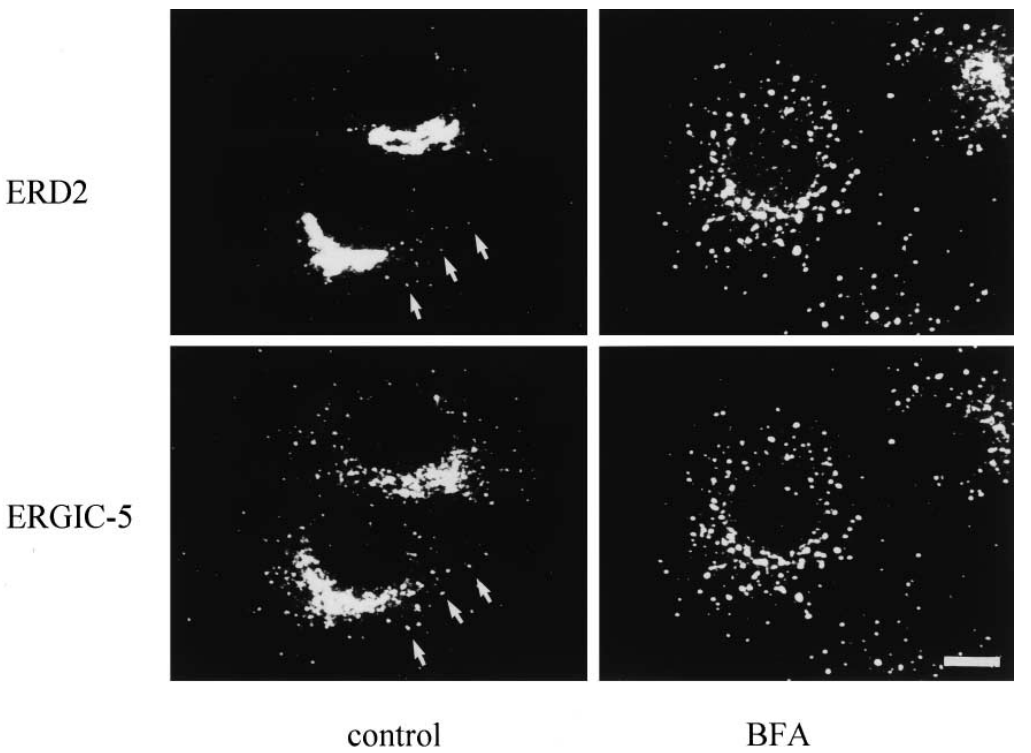

Fig. 1. Double immunofluorescence of ERD2 (2–4) and ERGIC-53 (G1/93) in Vero cells before (control) and after BFA treatment for 30 min. The images were obtained with a laser scan microscope. In untreated cells ERGIC-53 (intermediate compartment) overlaps partially with ERD2 (arrows). Application of 5 μg/ml BFA for 30 min leads to a striking colocalization in BFA induced vesicular structures (BIVS). Bar, 10 μm.

3. Results and discussion

3.1. Different subcellular distribution of Man II and GalTf as compared to ERD2 and ERGIC-53 following BFA treatment

In untreated cells ERD2 showed the known predominant localization in the perinuclear region, which was partially overlapping with ERGIC-53 (Fig. 1). In addition, a significant colocalization between the peripheral punctuated elements of ERD2 and ERGIC-53 could be observed (Fig. 1, arrows). Treatment with BFA resulted in a nearly perfect colocalization of both proteins in dotted structures throughout the cytoplasm (Fig. 1) which are composed of clusters of tubulovesicular structures with a lumen of 50–80 nm as shown by immunoelectron microscopy (Fig. 2). These BFA induced vesicular structures (BIVS) were different from the 'BFA bodies' described by [26] as they did not stain with δ-COP antibodies (data not shown). These BFA bodies have been described as a subcompartment of the ER and the site of accumulation of coatomer including δ-COP when the assembly of COP coated vesicles is prevented by BFA. However, BIVS are probably identical to the 'Golgi related' remnants after BFA that contained preferentially ERGIC-53 [27] and to the 'smooth ER clusters' described by Saraste and Svensson [3] by immunoelectron microscopy. However, it is not clear whether BIVS comprise distinct structures or are somehow linked to the ER-Golgi hybrid compartment as suggested by results obtained in the presence of nocodazol and BFA [7].

Subcellular fractionation of Vero cells using Nycodenz gradients revealed that ERD2 and ERGIC-53 are mostly present in the light fractions containing the trans Golgi marker GalTf. Treatment with BFA resulted in a significant shift of GalTf to ER fractions (Fig. 3, left panel). Surprisingly, the distribution of ERD2 and ERGIC-53 in the gradient was only marginally affected.

It is well known that resident Golgi proteins like Man II redistribute to ER-like structures in the presence of BFA [7]. As the BFA induced redistribution of the KDEL receptor [5] and Man II [7] have been characterized only separately we reinvestigated the time course of BFA action and compared directly endogenous ERD2 and Man II using double immunofluorescence. For reasons of antibody specificity we used NRK cells. In control cells, the KDEL receptor shows a Golgi pattern very similar to Man II and some additional peripheral punctuated structures. The ERD2 containing BIVS observed after 30 min of BFA treatment (Fig. 4) are indeed different from the ER-Golgi hybrid compartment stained with Man II antibodies. Importantly, a colocalization of Man II with ERD2 could not be observed at any point of time during the BFA incubation. Even when the redistribution process was slowed down by incubating the cells at a reduced temperature (20°C) there was neither a temporal nor a morphological correlation between the distributions of ERD2 and Man II (results not shown). We cannot rule out the possibility that the portion of ERD2 that is present in Golgi structures beyond the CGN [28] becomes redistributed to the ER together with resident Golgi proteins but cannot be detected by immunofluorescence.

The effect of BFA on the redistribution of Golgi membranes is reversible [29]. In contrast to the situation observed during BFA treatment the reconstruction of the Golgi apparatus revealed a striking colocalization between the KDEL receptor and Man II during the first 5–10 min after removal of BFA (Fig. 4). The first intermediate transport structures (5 min) still resembled the BFA induced structures. This indicates that Golgi enzymes move in a first stage from the ER-Golgi hybrid compartment to the structures defined by pro-

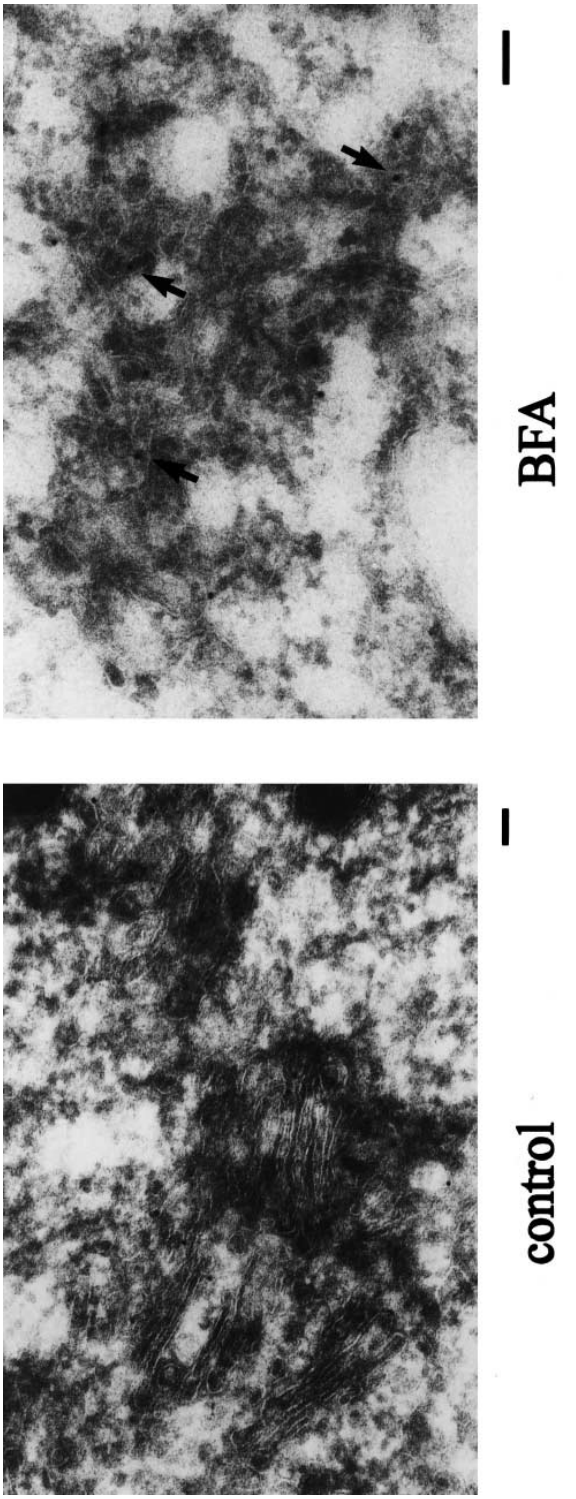

Fig. 2. Immunoelectron microscopy of Vero cells before and after BFA treatment for 30 min using antibodies against ERD2. In control cells the KDEL receptor is mainly localized to Golgi cisternae as described previously [28]. In BFA treated cells there is a cluster of tubulo-vesicular structures marked for ERD2. Bar, 100 nm.

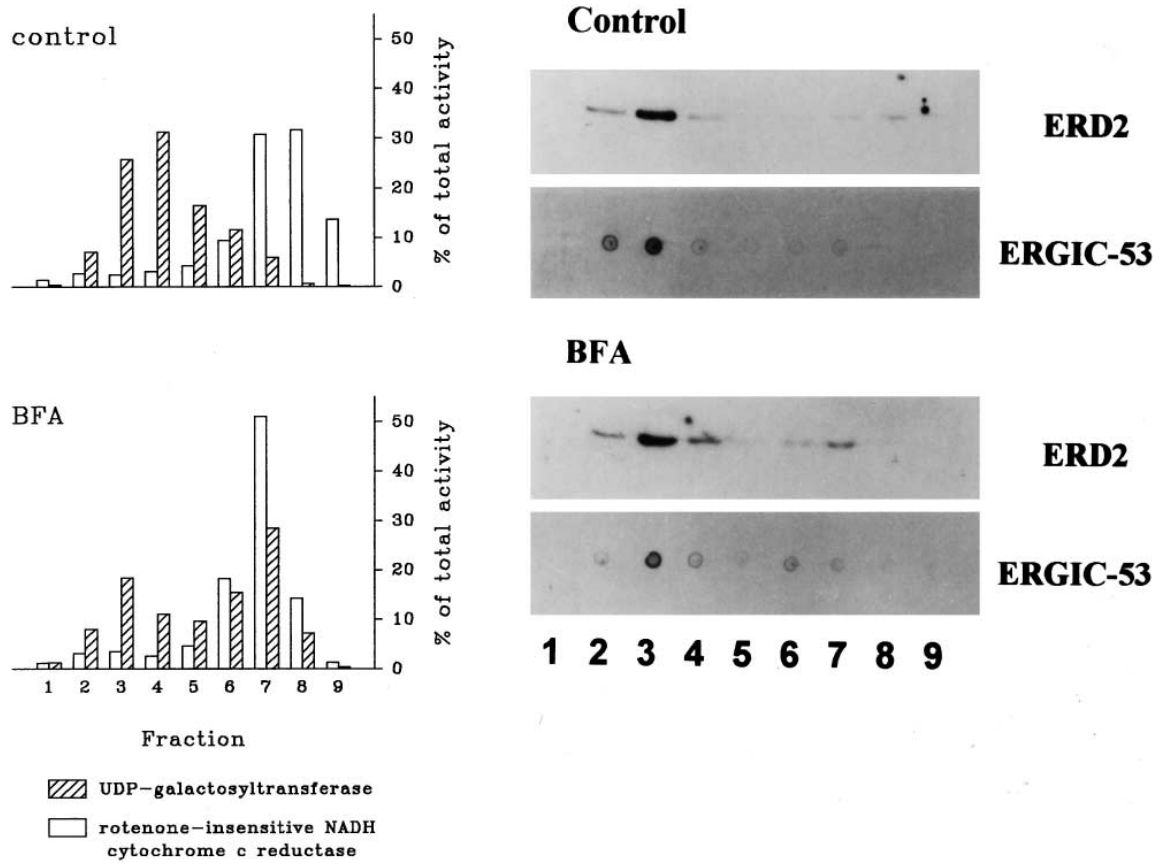

Fig. 3. Separation of BFA induced vesicular structures from the ER-Golgi hybrid compartment as revealed by subcellular fractionation of Vero cells. A postnuclear supernatant was subjected to a velocity controlled centrifugation on a Nycodenz gradient. Left panel: Rotenone-insensitive cytochrome *c* reductase (open boxes) denotes the position of ER membranes at the bottom of the gradient and GalTf (hatched boxes) the location of Golgi membranes in the top fractions. A significant part of GalTf activity cofractionates with the ER fractions after treatment with BFA demonstrating the fusion of Golgi membranes with the endoplasmic reticulum. Right panel: Distribution of ERD2 (Western blots) and the intermediate compartment marker ERGIC-53 (dot blots) was not markedly affected upon BFA treatment.

teins like ERD2 or ERGIC-53. The reconstruction of the Golgi was complete at about 30 min after removal of BFA as judged by the distribution of ERD2 and Man II. This suggests that under the conditions of BFA treatment there might be two different pathways for going back [30], but only one exit route after removal of the drug. There was no indication from our experiments that under these conditions the IC is somehow involved in the retrograde movement of resident Golgi proteins.

We conclude from our results that two compartments are generated in the presence of BFA, one by fusion of the Golgi with ER membranes to form an ER-Golgi hybrid and the other by fusion of IC with the CGN membranes to build an IC-CGN hybrid (BIVS). As a consequence of this interpretation all proteins of the CGN or the IC should be localized in BIVS after BFA treatment. This assumption is in line with reports on similar morphological changes after BFA treatment for proteins localized to the CGN: a peripherally associated Golgi protein termed p210 and a transmembrane glycoprotein named gp74 colocalize in the presence of BFA with p58 [6,31], the rat homologue to ERGIC-53 [32]. This argumentation predicts that all proteins which are concentrated in BIVS are localized either in the IC or in the CGN or in both compartments in the absence of BFA.

Interestingly, a C-terminal myc tag impaired the BFA induced localization of ERD2 to BIVS (not shown). The addition of BFA to COS cells which expressed $ERD2_{myc}$ at a moderate level resulted in a c-*myc* immunoreactivity in reticular ER structures but not in BIVS. Surprisingly, in the same cells the peptide specific KDEL receptor antibodies revealed additional dotted structures that resembled the ERD2 pattern of BIVS in non-transfected cells (Fig. 1). We assume that the c-*myc* tag interferes with an epitope necessary for correct localization in the CGN [28]. A shift in the distribution of the tagged receptor to later Golgi compartments might have the consequence that it becomes redistributed together with resident Golgi proteins to the ER after incubation with BFA. However, the function of the c-*myc* tagged yeast receptor to retain a HDEL tagged invertase fusion protein was not impaired [33]. The influence of the C-terminal c-*myc* epitope revealed in our study may therefore well be without consequence for the results obtained earlier using a c-*myc* tagged receptor [4,12].

3.2. Overexpression of ERD2 or lysozyme-KDEL is accompanied by redistribution of ERD2 and ERGIC-53 to the ER but not to BFA induced vesicular structures

Overexpression of an artificial KDEL receptor ligand was reported to cause the redistribution of the receptor from the Golgi to the ER [4,5]. We wanted to compare the ER structures of this approach with the BIVS. Transient expression of the secretory protein lysozyme tagged with a C-terminal

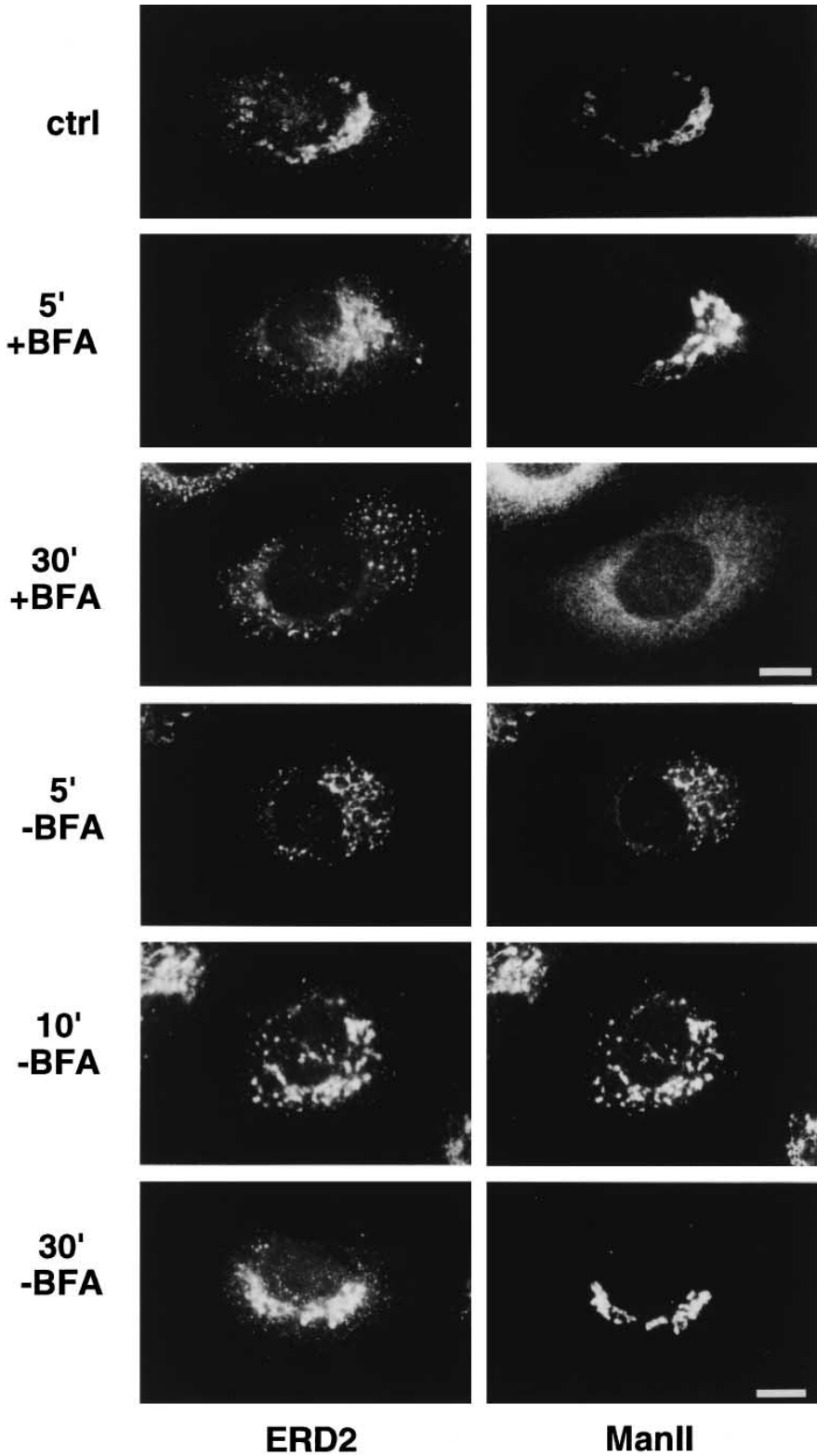

Fig. 4. Comparison of localization of endogenous ERD2 and Man II during treatment with brefeldin A. NRK cells were incubated with 5 μg/ml BFA for the indicated times (5, 30 min) and processed for double immunofluorescence. After 30 min ERD2 is concentrated in BFA induced vesicular structures (BIVS) that are clearly different from the ER pattern observed for Man II. After 30 min, BFA was washed out and cells examined after recovery for 5, 10 and 30 min. Despite their very different intracellular distribution before the BFA washout the KDEL receptor and Man II show a striking colocalization starting 5 min after BFA washout. After 30 min the distributions of both proteins were indistinguishable from control cells. Bar, 10 μm.

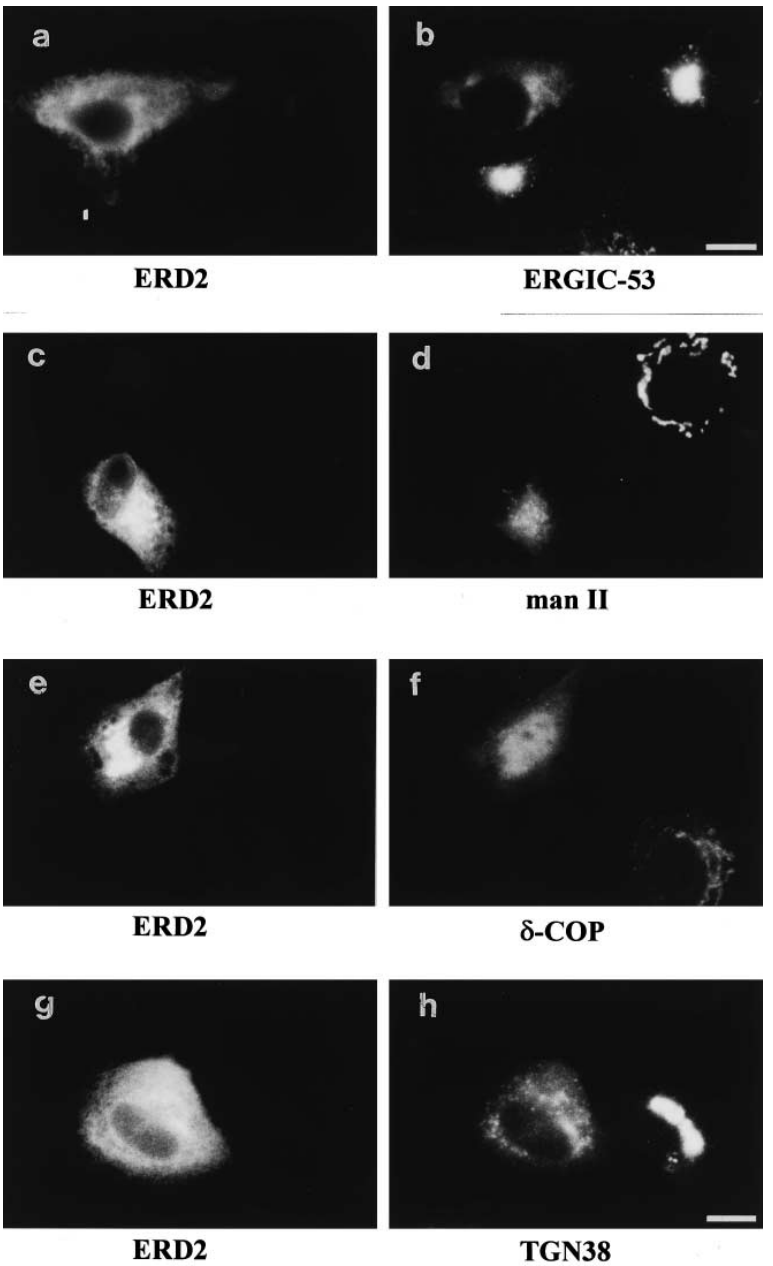

Fig. 5. Transient overexpression of hERD2 in COS and NRK cells causes location of ERGIC-53, Man II and TGN38 to the endoplasmic reticulum. Overexpression in COS cells under the control of the strong cytomegalovirus promoter of pCMV2 led to a redistribution of hERD2 together with ERGIC-53 to ER-like structures (a,b). The peptide specific antibody against ERD2 (ERD2–4) was used in a concentration that enabled the recognition of hERD2 overexpressing cells. Note that only the transfected cell shows a diffuse staining pattern for hERD2 and ERGIC-53 in the ER. Overexpression of hERD2 causes a partial redistribution of Man II (c,d), δ-COP (e,f) and TGN38 (g,h) in NRK cells. Double immunofluorescence was performed using ERD2–4 in combination with monoclonal antibodies against TGN38 (2F7.1) and Man II (53FC3) and ERD2-6 together with δ-COP antibodies. Bar, 10 μm.

KDEL sequence [20] led to the disappearance of the prominent Golgi staining of the KDEL receptor and the typical ERGIC-53 staining in transfected COS cells (not shown). A faint reticular staining colocalizing with lysozyme-KDEL indicates that the KDEL receptor and ERGIC-53 had been redistributed to the ER. However, no BIV-like structures could be observed.

An alternative way to induce retrograde transport is to overexpress the KDEL receptor itself. High level overexpression of the isoforms of the human ERD2, bovine p23 and human ELP-1 resulted in an ER-like co-staining of the Golgi marker *Lens culinaris* lectin in COS cells [5,12]. These results led to the suggestion that overexpression of ERD2 induces a phenotype identical to that observed during treatment with brefeldin A, and that both events might be mechanistically related [12]. As we observed that in contrast to the tagged $ERD2_{myc}$ the untagged KDEL receptor does not concentrate in the ER under BFA but colocalizes with ERGIC-53 in distinct vesicular structures (BIVS, Fig. 1), we reinvestigated this issue. We overexpressed an untagged version of human ERD2 under the control of the strong cytomegalovirus promoter and compared the localization of ERD2 with the following marker

proteins: ERGIC-53 for the IC, Man II for the medial Golgi, TGN38 for the trans Golgi network and δ-COP for coated structures. For reasons of species specificity of the antibodies we used COS (ERGIC-53) and NRK (δ-COP, Man II, TGN 38) cells. Most of the COS cells overexpressing hERD2 showed a general redistribution of the receptor to the ER (Fig. 5a,b). Double immunofluorescence with antibodies against ERGIC-53 demonstrated that in these cells the IC marker had shifted to a very similar diffuse ER pattern but not to dotted vesicular structures as was observed during BFA treatment [7] (see also Fig. 1). Transient overexpression of hERD2 in NRK cells led to a weaker phenotype regarding ERD2 since there always remained a Golgi-like staining in addition to the reticular ER pattern. The redistribution of hERD2 to the ER correlated with the redistribution of Man II (Fig. 5c,d). TGN38 was also redistributed to ER-like structures indicating that even the trans Golgi network was affected by the overexpression of hERD2 (Fig. 5g,h). The perinuclear staining of δ-COP shifted to a diffuse pattern representing either the ER or a cytosolic localization (Fig. 5e,f). Together these results suggest that the entire Golgi complex had been partially redistributed to the ER due to the increased amount of KDEL receptor. However there were no BIVS as observed for ERD2 and ERGIC-53 after BFA treatment.

In summary, we think it unlikely that the response to BFA and the overexpression of the receptor follow the same mechanism: First, in the presence of BFA proteins cycling between ER and Golgi like ERD2, ERGIC-53/p58 or gp74 are transferred not to the ER but to distinct structures (BIVS) and resident Golgi proteins do appear in the ER. Second, the ligand or receptor induced retrograde transport of ERD2 leads to the ER, and not to membrane structures resembling BIVS or IC. Third, recent evidence suggests that coatomer is an essential component for retrograde transport from the Golgi back to the ER [34], whereas BFA prevents the binding of coatomer to membranes.

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