Microscopy for biomedical research

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Keywords: endoplasmic reticulum, pre-Golgi intermediate, protein folding, ERAD, autophagy

In biomedical research, microscopical techniques are indispensable tools in combination with biochemical techniques. Although each of them may yield important results, it is the combination of the two tools that provides new and unmatched insight into complex cellular processes in health and disease. In this lecture, the focus will be on quality control of protein folding and how microscopic analysis has been instrumental in dissecting its subcellular topography under physiological and diseased conditions, in elucidating organelle changes due to the presence of misfolded proteins, and to evaluate the therapeutic potential of chemical chaperones in adjusting the effects of protein misfolding on protein traffic.

The quality of newly synthesized proteins is monitored in regard to proper folding and correct assembly in the early secretory pathway, the cytosol and the nucleoplasm. This type of quality control is not only of utmost importance as a regulatory house keeping function under physiological conditions but also under pathological conditions due to various forms of cellular stress (1,2). The importance of the protein quality control stems from the fact that folding and assembly of proteins and their function depend on each other, and this becomes much visible under pathological conditions associated with protein misfolding (3,4). The quality control for proteins in the early secretory pathway can be divided into three major stages: (i) recognition and retention, (ii) dislocation (or retrotranslocation), and (iii) degradation (5). Specific machinery proteins have been identified for each of the different stages, that, in terms of microscopy, represent useful marker proteins for analyzing their organization at organelle and sub-organelle level.

In addition to various cellular chaperones aiding proteins to achieve their proper folding, various machinery proteins are involved in the recognition and retention of misfolded proteins. Among them, glucosidase II (Gls II) and UDP-glucose:glycoprotein glucosyltransferase (GT) in connection with the calnexin-calreticulin cycle are of eminent importance (6-8). The involvement of mono-glucosylated oligosaccharides generated by Gls II in the recognition of misfolded glycoproteins is well documented. By confocal immunofluorescence, Gls II not unexpectedly exhibited a pattern typically observed for ER proteins, which by immunoelectron microscopy could be definitely established. In addition to the rough ER including the nuclear envelope and the transitional ER, the smooth ER was also positive for Gls II. Moreover, Gls II was additionally found in tubulo-vesicular clusters between transitional ER and the *cis* Golgi apparatus. They represent pre-Golgi intermediates involved in antero- and retrograde transport of cargo. Glycoproteins, if not correctly folded, will be targeted by GT, which has a two-fold function as a folding sensor and as a glycosyltransferase. After recognition and re-glucosylation by GT, misfolded glycoproteins will be retained in the ER by entering a new round of the calnexin-calreticulin cycle. When the subcellular distribution of GT was studied by confocal immunofluorescence and

M.A. Pabst, G. Zellnig (Eds.): MC2009, Vol. 2: Life Sciences,
DOI: 10.3217/978-3-85125-062-6-152, © Verlag der TU Graz 2009

immunoelectron microscopy, its labeling pattern was alike that observed for Gls II (9). Notably, the pre-Golgi intermediate immunogold labeling for GT was approximately twice that of rough ER. Together, these results provided new insight into the *in situ* subcellular organization of some quality control machinery proteins involved in the recognition and retention of misfolded glycoproteins. Significantly, they were not only present in the rough ER, as expected from biochemical analysis, but in addition in the smooth ER and unequivocally detectable in pre-Golgi intermediates. This pattern of immunolabeling was found in different mammalian cell lines and tissues as well as Drosophila tissue and cell lines and, therefore, seems highly conserved between insects and mammalian cells. Looked at from a broader perspective, these findings provided strong evidence for the involvement of pre-Golgi intermediates in protein quality control.

The question then arose whether or not pre-Golgi intermediates are sites of accumulation of misfolded proteins. By combining morphometry and immunogold labeling, this issue could be positively answered. Misfolded proteins such as the cystic fibrosis causing delta F508 variant of the chloride channel (10), misfolded major histocompatibility complex class I protein (11,12) and misfolded proinsulin (13) have been shown to accumulate in expanded pre-Golgi intermediates. As a note of caution, however, it must be taken into consideration that depending on the protein studied, no organelle changes such as distension of ER cisternae and expansion of pre-Golgi intermediates may be observed at all, as was reported for Fabry disease-causing misfolded lysosomal alpha-galactosidase A (14,15) or renal diabetes insipidus-causing misfolded aquaporin 2 (16). At the other end of the spectrum positioned are misfolded glycoproteins, which form detergent-insoluble aggregates in the lumen of the rough ER, so-called Russel bodies (17), which by definition cannot be dislocated for degradation and provoke a strong ER stress response (18-20). Returning to the expanded pre-Golgi intermediates in which misfolded proteins can accumulate, the obvious question to answer is whether they represent also sites where protein dislocation occurs? This brings up the topic of proteins that function as a kind of gate opener by linking the calnexin-calreticulin cycle to ER-associated degradation (ERAD) (2,5). Such gate opener are represented by two lectin-like proteins at present: EDEM1 (yeast ortholog Htm1p/Mnl1p) and OS-9 (yeast orthologue Yos9p) (2,5). EDEM 1 has been shown to link the calnexin-calreticulin cycle to ERAD since its overexpression resulted in accelerated degradation of ERAD substrates (21,22). The subcellular distribution of endogenous EDEM1 in various mammalian cell types was established with a specific anti-peptide antibody (23). Unexpectedly, its immunofluorescence pattern did not correlate with that of calnexin and other ER marker proteins. Rather, an unusual pattern of well-distributed punctate structures along with some localized finger-like structures was revealed (Figure 1). Immunogold labeling and serial section analysis (Figure 2) revealed the presence of EDEM1-reactive buds along rough ER cisternae which apparently gave raise to ~150 nm vesicles. These buds and vesicles were devoid of a COPII coat (Figure 1), formed outside the canonical ER exit sites of the transitional ER (Figure 2) and were not found in the tubulo-vesicular clusters of pre-Golgi intermediates (Figure 2). Occasionally, EDEM1 luminal immunolabeling in limited parts of distended ER cisternae was observed, which accounted for approximately 11% of the immunogold labeling for EDEM1. Together, these data revealed the existence of a novel vesicular transport pathway out of the rough ER through which EDEM1 and the ERAD substrate Hong Kong Null alpha-1-antitrypsin became sequestered from the early secretory pathway. Through this ER vesicular exit pathway potentially harmful aberrant luminal proteins can be removed. These findings also indicate that the Gls II and GT containing pre-Golgi intermediates appear to be not involved in the dislocation of ERAD substrates. Subsequent analysis of EDEM1 was focused on its fate, namely whether it recycled or whether it became degraded (24). Immunoelectron microscopy unequivocally demonstrated

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DOI: 10.3217/978-3-85125-062-6-152, © Verlag der TU Graz 2009

that clusters of EDEM1 were engulfed by phagophores to yield autophagosomes (Fig. 3). Serial thin section analysis also revealed that the source of the phagophores was the rough ER. Ribosome-free cisternal parts continuous and contiguous with rough ER cisternae represented the EDEM1 engulfing phagophores. Biochemical analysis confirmed the microscopical findings by showing that EDEM1 was stabilized by inhibition of autophagosome formation through *ATG* siRNA knockdown. Thus, a protein quality control machinery protein is degraded by autophagy and not by proteasomes.

As mentioned, protein misfolding due to point mutations is the basis of many human congenital diseases such is cystic fibrosis, aquaporin2-caused renal diabetes insipidus, alpha-1-antitrypsin deficiency to name a few. Although being misfolded, many of the mutant proteins have biological activity and rescuing them from quality control-inflicted ERAD would be a means for therapy. To achieve this goal, chemical chaperones have been applied. Microscopical techniques provided proof of principal for the therapeutic potential of chemical chaperones in protein folding diseases as will be exemplified by Fabry disease.. Fabry disease, a clinically well-defined lysosomal storage disease, is due to lysosomal alpha-galactosidase A deficiency. Based on morphological and biochemical evidence that it actually represents a protein folding disease (14,15), we set out to test the efficiency of a chemical chaperone to rescue the trafficking defect of mutant alpha-galactosidase A (14,15). For this, 1-deoxygalactonojirimycin (DGJ), a competitive inhibitor of alpha-galactosidase A, was applied at subinhibitory dose. DGJ is known to from complexes with the enzyme at neutral pH, which is the pH in the ER lumen, and to dissociate form the enzyme at acidic pH, which is the pH in lysosomes. Following treatment with low concentrations of DGJ, which were not toxic to cells overexpressing the mutant enzyme, the mutant enzyme became detectable in most lysosomes as demonstrated by double confocal immunofluorescence for LAMP1 (Figure 3). By semiquantitative analysis of its co-distribution with LAMP1, the distribution pattern of mutant alpha-galactosidase A was close to that of normal cells. Immunolabeling of Fabry patient fibroblasts for glycosphingolipid globotriosylceramide (Gb3), the major glycosphingolipid species accumulating in lysosomes of Fabry patients, showed normalization of its lysosomal storage (Figure 3). By electron microscopy, both the lysosomal morphology and their size distribution were found to correspond to normal cells. Together, with the use of microscopical techniques it could be directly demonstrated that chemical chaperones have therapeutic potential for lysosomal storage disorders as well as for other genetic metabolic disorders caused by mutant but nonetheless catalytically active enzymes.

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25. The research summarized here was supported by the Swiss National Science Foundation, the Wolfermann-Nägeli Foundation, the Velux Foundation and the Bonizzi-Theler Foundation.

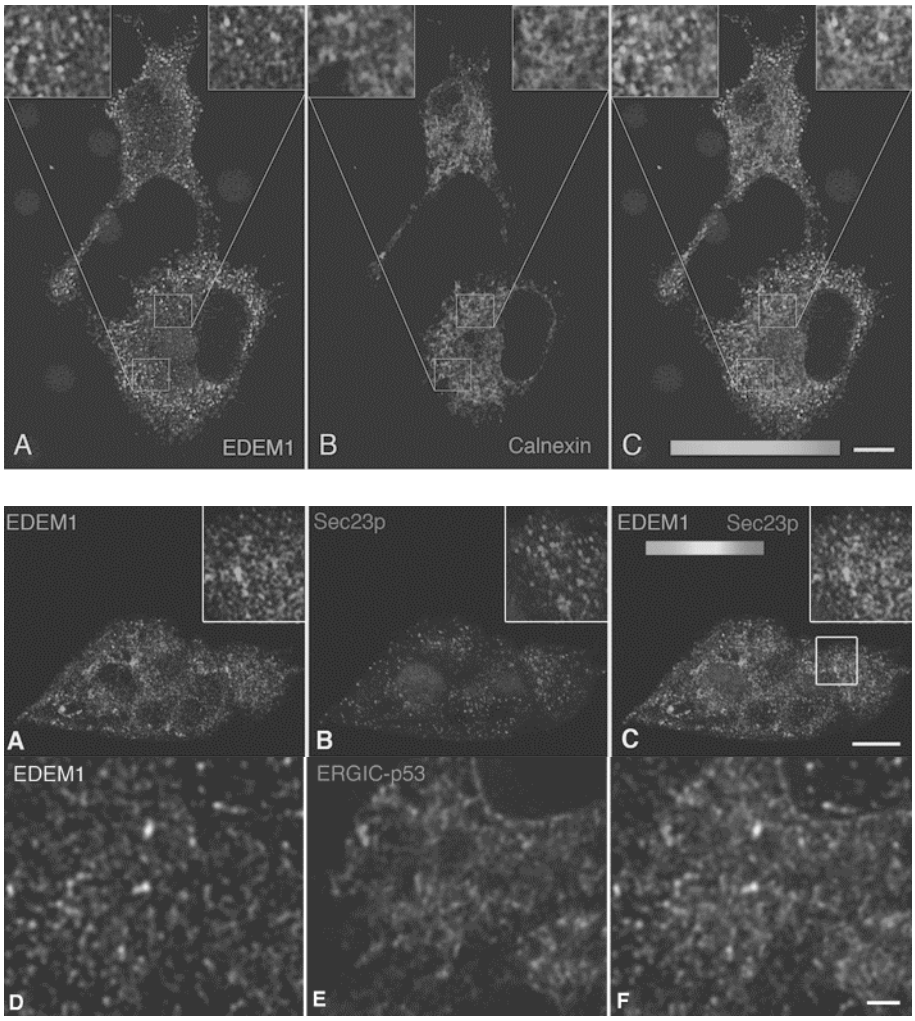

Figure 1. EDEM1 exhibits a non-ER localization (upper panel) and does not co-distribute with the COPII protein sec23 (middle panel) or with the pre-Golgi intermediate protein ERGIC-53 (lower panel) (from ref. 23).

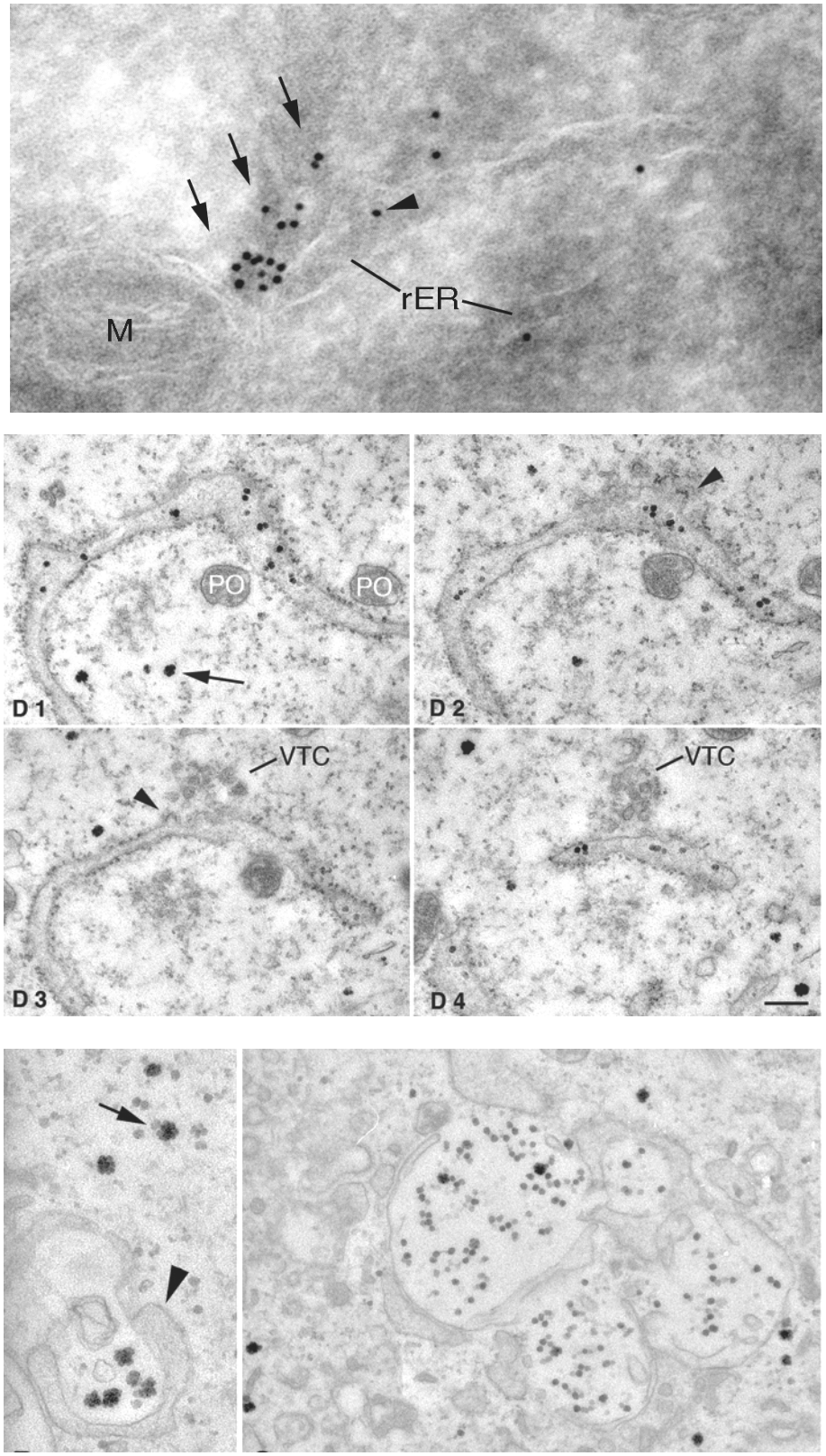

Figure 2. EDEM1 localization reveals a vesicular ER-exit pathway independent of COPII vesicles. Immunogold labeling for EDEM1, ultrathin frozen sections (upper panel; from ref. 23). Serial sections of an ER exit site showing no EDEM1 labeling in COPII buds (arrowheads) and vesicles (VTC) (middle panel; from ref. 23) EDEM1 degradation by autophagy, pre-embedding immunoperoxidase (lower panel; from ref. 24). PO: peroxisomes

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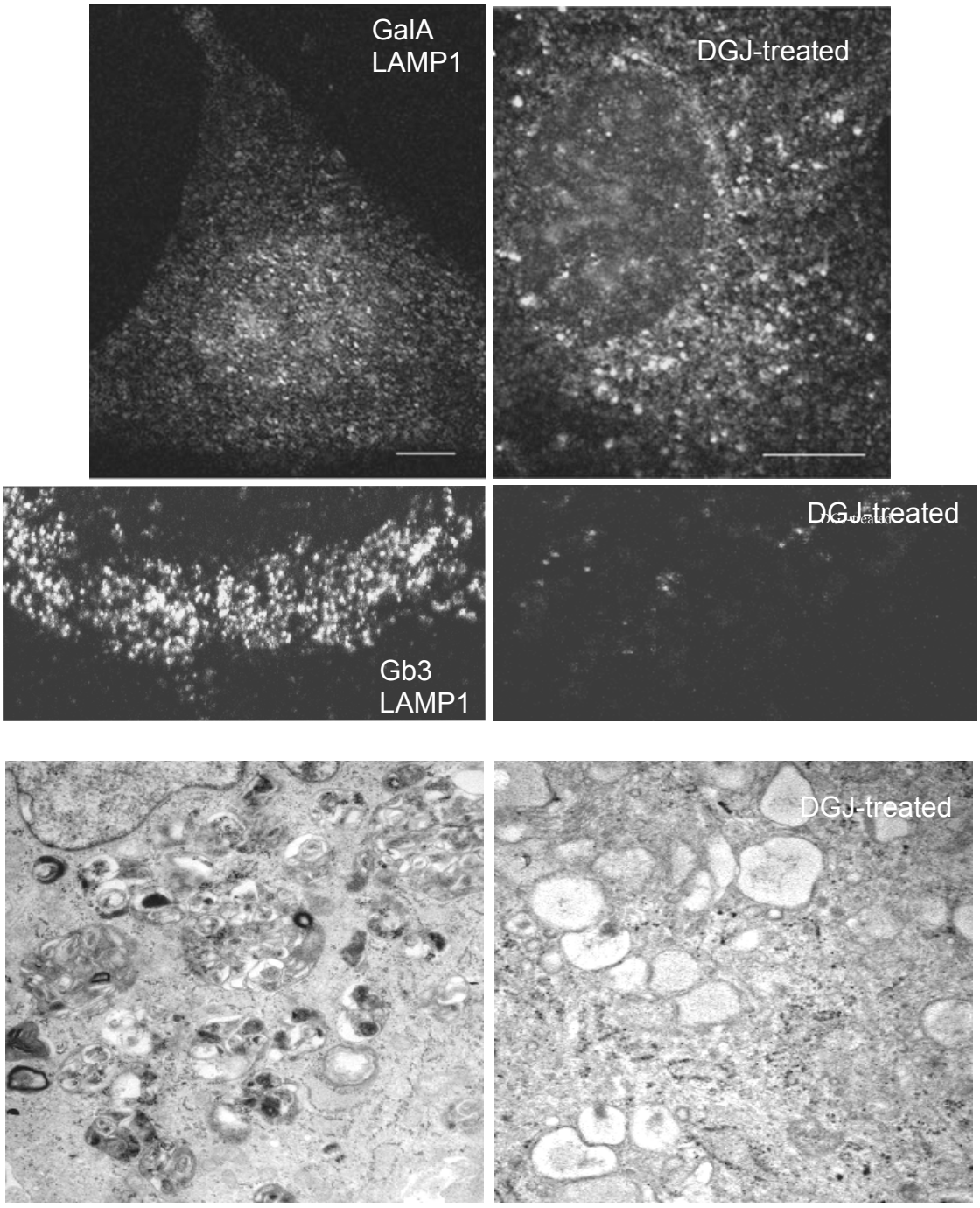

Figure 3. Fabry disease-causing mutant alpha-galactosidase A (GalA) is retained in the ER and can be rescued by chemical chaperone treatment. Mutant GalA (green) is in the ER (left in upper panel) and after treatment with DGJ in lysosomes (right in upper panel; from ref. 15). Gb3 accumulation in lysosomes (green) of Fabry patient fibroblasts (left, middle panel) is corrected after DGJ treatment (right, middle panel; from ref. 14). Effect of DGJ treatment on ultrastructural appearance of lysosomes in fibroblasts from Fabry patients (lower panel; from ref. 14).

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