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The structure and function of a mannose 6-phosphate receptor-enriched, pre-lysosomal compartment in animal cells

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Summary

The characteristics of a distinct prelysosomal compartment, evident in many animal cells, are described. The main feature of this compartment is that it is an acidic, tubulo-vesicular late endosome structure that contains high concentrations of the cation-independent mannose 6-phosphate receptor. The pathway to the lysosomes from the early endosomes, as well as that of newly synthesized lysosomal proteins from the *trans*-Golgi network, appear to converge in this prelysosomal compartment.

The majority of membrane and soluble proteins that are synthesized by the rough endoplasmic reticulum of eukaryotes follow an identical pathway from this organelle through the compartments of the Golgi stack. Since the final destinations of these proteins can differ after leaving the Golgi complex, depending on the protein's function, it follows that the pathways must diverge at some point. The secretory machinery can somehow distinguish between at least three classes of proteins that are (1) targeted constitutively towards the plasma membrane (or specialized domains thereof), or (2) packaged into specialized secretory granules in exocrine and endocrine cells (the 'regulated' pathway), or (3) targeted to lysosomes. There is now compelling evidence that the sorting of these different classes of proteins into separate vesicles occurs in the last Golgi compartment, which we have recently termed the *trans*-Golgi network (TGN) (Griffiths & Simons, 1986; Dunphy & Rothman, 1985; Farquhar, 1985).

Our recent interest has focused on lysosome biogenesis. It is now becoming increasingly clear that the lysosome is the end-station for three distinct cellular pathways. First, newly synthesized lysosomal membrane and content proteins are targeted towards the lysosomes from the TGN (von Figura & Hasilik, 1986; Griffiths & Simons, 1986; Geuze *et al.* 1985). Second, there is the route from the extracellular medium *via* receptor-mediated and fluid-phase endocytosis (Mellman *et al.* 1986*a*,*b*) and finally, there is the more obscure autophagic pathway by which cells are able to digest their own organelles and cytoplasmic constituents (Gordon & Seglen, 1988; Seglen, 1987).

Lysosome biogenesis is especially interesting at present because it is the best characterized intracellular membrane pathway for which a sorting mechanism has been described at the molecular level; that is, a mechanism which enables the cell to target a molecule specifically from one membrane organelle to another. Early after

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synthesis in the endoplasmic reticulum, lysosomal enzymes, in contrast to all secretory proteins, are selectively phosphorylated on key mannose residues in their oligosaccharide side-chains (von Figura & Hasilik, 1986; Kornfeld & Kornfeld, 1985). This covalent modification, which most likely occurs before the *cis*-Golgi compartment, serves as an 'address signal' that enables these proteins to bind to the sorting receptor, the mannose 6-phosphate receptors (MPR), in the TGN (Kornfeld, 1987). The receptors, with bound lysosomal enzymes, are then delivered into some pre-lysosomal compartment of the endocytic pathway, thus effecting a meeting of the exocytic and endocytic pathways. There is agreement from both biochemical and immunocytochemical data that the lysosomes themselves are essentially devoid of MPR and that these receptors must recycle out of the pathway before the lysosomes are reached (see von Figura & Hasilik, 1986, for review).

There are two distinct MPRs, a large protein of about 300K ($K = 10^3 M_r$) which does not require divalent cations for binding to lysosomal enzymes (the cation-independent receptor CI-MPR), and a smaller 46K protein which does require divalent cations for binding (the cation-dependent receptor CD-MPR). The CI receptor has 15 homologous domains of about 150 amino acids although only two of these appear to be involved in binding oligosaccharides bearing two phosphorylated mannose residues on lysosomal enzymes (Lobel *et al.* 1987; Kornfeld, personal communication). This protein also has a high affinity binding site for the insulin-like growth factor II (Morgan *et al.* 1987; Roth, 1988) as well as a region that shows homology to collagen type II binding domain of fibronectin (Lobel *et al.* 1987). The significance of these observations is not yet clear. The CD receptor has only one domain that is homologous to each of the 15 domains of the large receptor (Dahms *et al.* 1987).

Our recent immunocytochemical studies in normal rat kidney (NRK) cells have shown that the compartment to which lysosomal enzymes are delivered is most likely a specialized late endosome structure, which contains the bulk of the cell's cation-independent MPR. It is spatially very close to, but functionally distinct from, the TGN (Griffiths *et al.* 1988). Along the endocytic pathway it is the first compartment that stains significantly for acid phosphatase by the classical lead capture cytochemical reaction (Griffiths *et al.* unpublished). Further, our data using the acidotrophic reagent 3-(2,4 dinitro-anilino)-3′-amino-*N*-methyldipropylamine (DAMP) as a cytochemical probe for low pH compartments (Anderson *et al.* 1984; Anderson & Pathak, 1985) indicate that this pre-lysosomal compartment (PLC) has a pH that is significantly below that of the TGN (which is believed to have a pH between 6 and 6·5) and must be below that required to dissociate lysosomal enzymes from the MPR (below pH 5·5). It should be noted that detectable amounts of the CI-MPR are also observed on the plasma membrane in early endosomes and in the TGN. The amounts found by immunocytochemistry in the latter compartment appear to vary from one cell to another (Griffiths *et al.* 1988; Geuze *et al.* 1985; Brown & Farquhar, 1987).

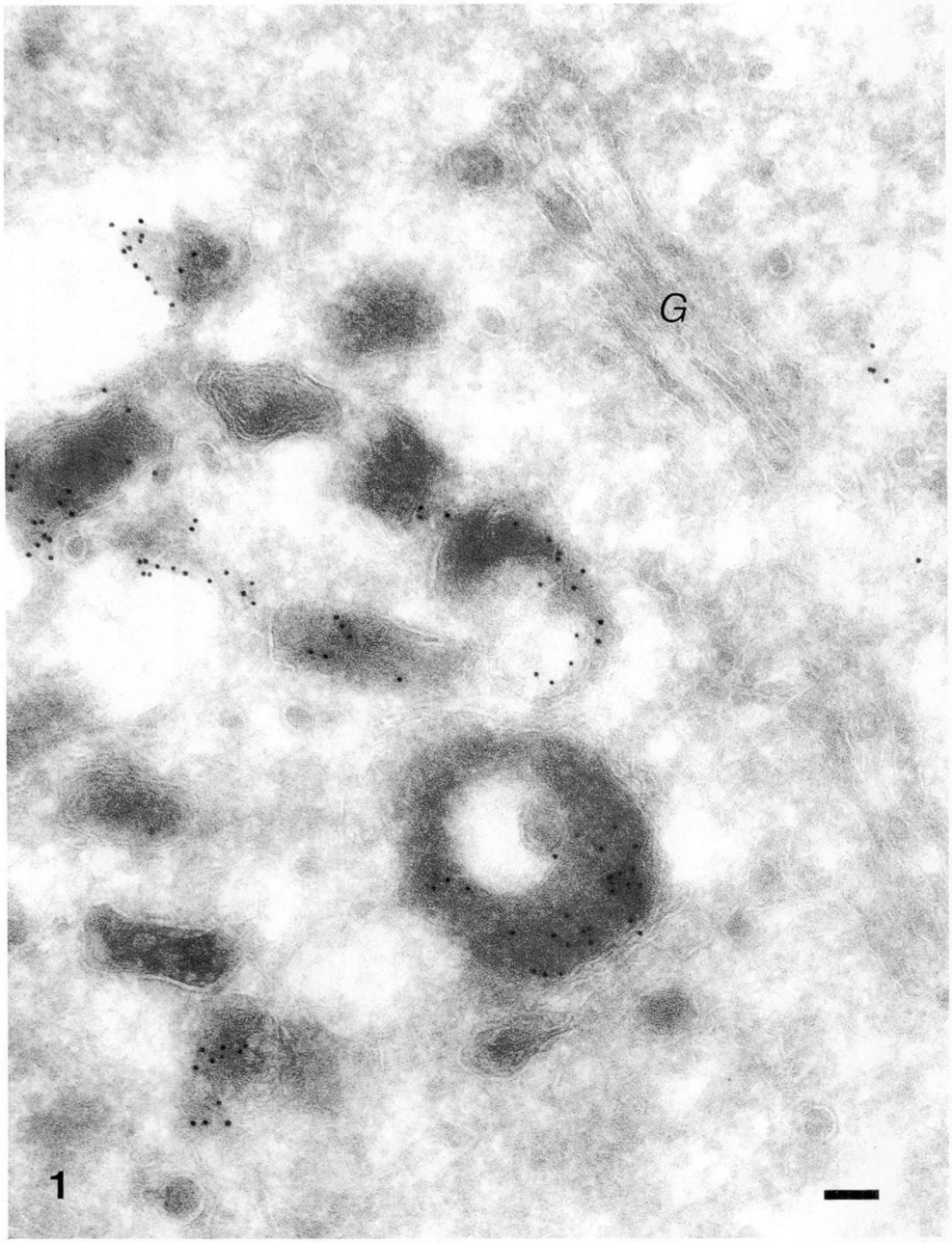
The PLC has unique structural features (Figs 1 and 2). It is a complex tubulo-reticular structure and the lumen of the vesicular parts are packed to high density

with tubular membranes having a high concentration of the CI-MPR, and an outer limiting membrane that, in addition to the MPR, contains significant amounts of Lgp 120, a membrane protein purified from isolated lysosomal membranes (Lewis *et al.* 1985). In our model, the essential difference between the PLC and the lysosome compartment (both of which have Lgp 120) is that only the PLC has significant amounts of MPR. There are also small amounts of MPR on the plasma membrane and in peripheral endosomes as well as in the TGN. This agrees with biochemical evidence that the receptor is constitutively recycling between these compartments, although the reason for this extensive recycling is not yet clear (Duncan & Kornfeld, 1988; von Figura & Hasilik, 1986; Pfeffer, 1987).

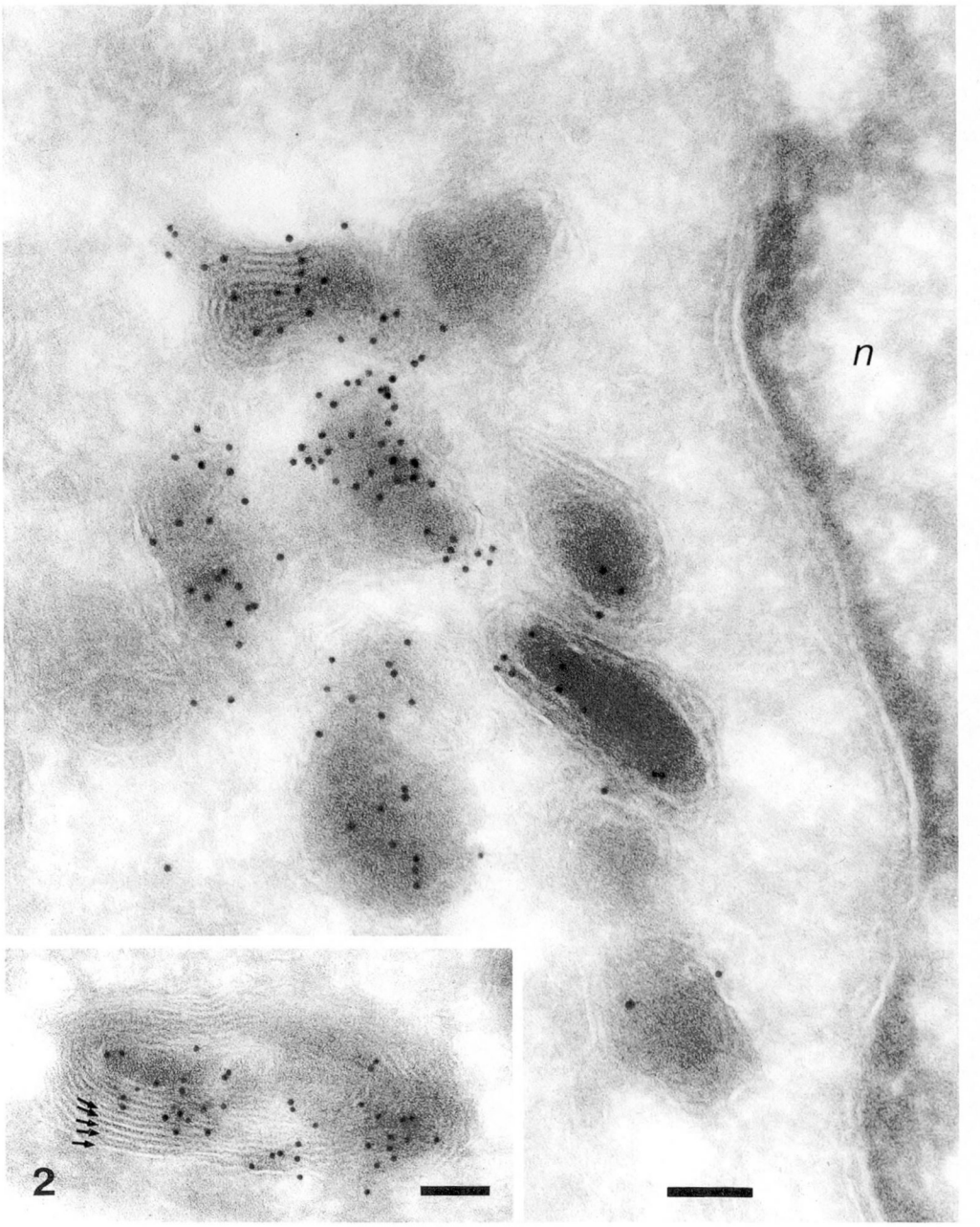
Our morphological studies have taken advantage of two well characterized low temperature blocks in transport. At 16–20°C, both the inward transport from the early endosomes (Marsh *et al.* 1986) as well as all outward traffic from the TGN (Griffiths *et al.* 1985) are effectively blocked. Using these blocks as tools to selectively mark the TGN (with newly synthesized G membrane protein of vesicular stomatitis virus) or early endosomes (with a variety of endocytic tracers) we have now developed the working model shown in Fig. 3 (Griffiths *et al.* 1988). Recent quantitative data using antibodies against horseradish peroxidase (HRP) on cryosections of cells that had internalized HRP at 37°C for varying amounts of time, indicate that the PLC is really an intermediate compartment between the early endosomes and lysosomes (Griffiths *et al.* manuscript in preparation).

The internal membrane structures of the PLC are highly reminiscent of bacterial mesosome structures, that is myelin-like invaginations of the plasma membrane that are evident in sections of many Gram-positive bacteria. Since it is now clear that mesosomes are somehow induced by aldehyde or osmium tetroxide fixatives (see Dubochet *et al.* 1983) it was important to show that the PLC in our cells is not a preparation-induced artefact. We therefore allowed cells to internalize gold particles conjugated with α-2 macroglobulin or transferrin. Conditions had already been worked out that enable us to selectively mark the PLC with these markers in living cells (Griffiths *et al.* 1988). Hydrated cryo-sections were then prepared. That is, the cells were vitrified (cooled rapidly such that all ice crystal formation was avoided), cryo-sectioned and the sections placed on grids and transferred to a cold stage of the electron microscope, without at any time raising the temperature above −140°C (see Dubochet *et al.* 1988 for a review of this approach). When these sections were examined under low electron dose conditions, structures labelled with the internalized gold particles showed clear indications of myelin-like arrays of membranes (McDowall *et al.* manuscript submitted). Thus, these internal membrane structures are evident in the absence of any chemical treatment.

The presence of a distinct tubulo-vesicular structure containing a high density of internal membranes that label extensively for the CI MPR (and all cases where the antibodies cross-react, also for Lgp) is evident in most cultured cells we have looked at as well as many, but not all cells in tissues. It is prominent, for example, in the epithelia of the proximal tubule of rat kidney, in rat spermatids and in one cell type only in the rat pituitary, the corticotrophs. It appears likely that the appearance of



Figs 1 and 2. Examples of cryo-sections in NRK cells immunolabelled with an antibody against the CI MPR and protein A–gold. Note the complex tubulo-vesicular appearance of the PLC structure which contains essentially all the gold particles of these images. Note also the close proximity of the Golgi complex (*G*), which is itself free of label (in Fig. 1) and the nucleus (*n* in Fig. 2). In Fig. 2 the internal membranes of the PLC are evident, especially in the example shown in the inset (arrows). Bars in all figures, 100 nm.



this structure is related to the amount of receptors that cells express. We consider it likely that the structures all represent a distinct late endosome compartment that may appear different from one cell type to another, both with respect to structural features as well as in the amount of MPR it contains. The latter may simply reflect the absolute amount of MPR that cells express. An interesting observation in this

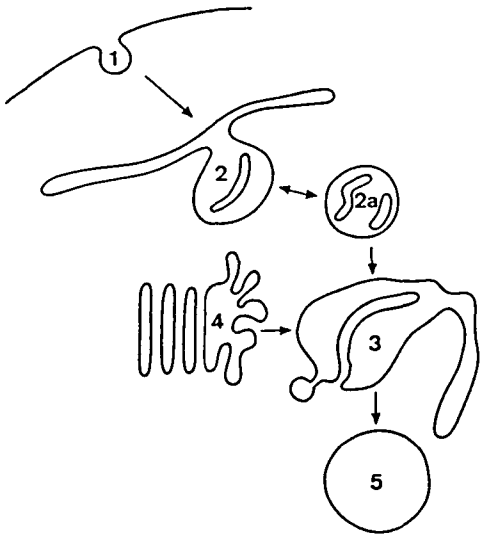


Fig. 3. Schematic diagram of our working model of the endocytic pathway and its relationship to the TGN (4). 1, coated pit; 2, early (peripheral) endosome; 2a, spherical vesicle, which we believe to be a transport vesicle between the early endosome (Gruenberg *et al.* 1989); 3, the MPR-lgp enriched prelysosomal compartment (late endosome); 5, MPR-negative/lgp positive lysosome.

respect comes from recent unpublished studies in collaboration with Stuart Kornfeld's group. All macrophages examined until now appear to be essentially devoid of the CI receptor although they have structures in the vicinity of the Golgi complex that appear similar to the MPR-enriched PLC in other cells. When an MPR-negative L-cell line is transfected with a high copy number of a cDNA coding for the CI-MPR receptor, the distribution of this (functional) receptor in this cell type by immunocytochemistry is indistinguishable from that seen in NRK cells. That is, low amounts of the receptor are evident on the cell surface, Golgi complex and putative early endosome structures while the bulk is present in perinuclear structures indistinguishable from the PLC in NRK cells (Lobel *et al.* unpublished data).

Recent unpublished data obtained by Robert Parton, a postdoctoral fellow, in collaboration with Kai Simons's group, show that the CI-MPR-enriched structure is also present in the polarized epithelial cell line MDCK grown on filters. By observing the internalization of different markers from the apical and basolateral media it is clear that distinct apical and basolateral early endosome structures exist. However, fluid phase markers that have first entered these early compartments, co-localize in the MPR-enriched peri-nuclear compartment at later times after internalization (after 30 min at 37 °C). At still later times a significant amount of these markers appears to 'chase' into MPR-negative structures. This suggests that the endocytic pathways from the two plasma membrane domains meet in a pre-lysosomal compartment prior to transport into a common set of lysozomes (Parton *et al.* unpublished data).

The bovine kidney epithelial cell line MDBK also has an MPR-enriched pre-lysosomal compartment. In this cell type we have been able to localize the regulatory (R) subunit of the cAMP-dependent protein kinase type II to the cytoplasmic surface of the PLC (in collaboration with E. Nigg and H. Hilz, see Nigg *et al.* 1985). This R subunit, which itself binds the catalytic subunit of the kinase, is also present in the TGN as well as in early endosomes and the plasma membrane of these cells (as shown by double labelling using various markers for these compartments). Significantly, however, MPR-negative 'late' endocytic structures which we define as lysosomes do not label with this anti-R. Although the overall functional significance of this result is not clear, it suggests that all compartments involved in recycling of receptors contain the R subunit but that lysosomes do not. It is a further argument for a functional difference between the PLC and the lysosomes.

A puzzling feature of the PLC is the fact that it contains lysosomal enzymes in detectable amounts, and is acidic. An obvious question which arises is whether this organelle is itself active in degradation. A clue may come from recent work we have done in collaboration with Jean Gruenberg and Kathryn Howell. This project involves the localization of the vesicular stomatitis virus (VSV) G-protein that has been inserted by low pH fusion of the virions into the plasma membrane of cultured cells and then allowed to be internalized (see Gruenberg & Howell, 1987). Under appropriate conditions the bulk of the G-protein will be degraded in lysosomes. An intriguing finding is that at intermediate times after endocytosis, and coincident with the G-protein reaching, first, early endosomes and, second, the PLC (as shown by immunocytochemistry), there are two sequential proteolytic cleavages which occur resulting in the removal of about 40K from the 67K G-protein (Gruenberg *et al.* 1989). This opens the possibility that the PLC and/or the early endosomes may play a role in a controlled (partial) proteolysis of proteins (see also Diment & Stahl, 1985; Roederer *et al.* 1987). It is a reasonable guess that one or both of these compartments plays a role in antigen processing in cells such as macrophages.

In collaboration with Iris Killisch and Hartmut Beug, we have found that in chicken erythroblasts transformed with avian erythroblastosis virus, detectable amounts of transferrin and transferrin receptors can be immune-localized to the MPR-enriched PLC, although the bulk of these receptors are present in early endosomes and the plasma membrane. This suggests that the PLC may be involved in the 'late' recycling of receptors (in contrast to the 'early' recycling events that most likely occur in early endosomes) as well as possibly being on the recycling route back to the TGN, where re-sialylation of de-sialylated receptors is expected to occur (Snider & Rogers, 1985; Duncan & Kornfeld, 1988).

Our current view of the PLC is that it represents a distinct late endosome compartment that receives newly synthesized lysosomal enzymes from the TGN as well as material from the early endosomes taken in by endocytosis. We favour the idea that the early endosome as well as the PLC, like the TGN, are pre-existing sorting compartments rather than transient structures formed *de novo* which somehow 'mature' into lysosomes by the continual acquisition of lysosomal enzymes (see Helenius *et al.* 1983). It is fair to mention, however, that the latter hypothesis is still

favoured by many workers and that no definitive experiments have yet been carried out that could distinguish between the two models.

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