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Heat shock and the sorting of luminal ER proteins

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When I arrived at the MRC Laboratory of Molecular Biology in 1981 I was keen to study the transcriptional regulation of a protein-coding gene; I had just spent two years working with Donald Brown on 5S rRNA synthesis and wanted to try something different. I began looking for a gene that might be regulated when transiently expressed in the newly developed monkey COS cell system. There were rather few to choose from, but a good candidate was the *hsp70* heat shock gene from *Drosophila*, which had just been shown to respond to heat shock when introduced into animal cells (Corces *et al.*, 1981).

Heat shock genes have a venerable history, having been discovered in fruit flies as early as 1962 (Ritossa, 1962), but only in 1978 did it become apparent that they existed in organisms other than flies (reviewed by Ashburner, 1982); it is now known that they are present in essentially all living cells. They encode a small family of evolutionarily conserved proteins that are expressed when cells are heated or exposed to various other stresses. Thanks to the pioneering work of Alfred Tissieres, the *Drosophila* heat shock genes were amongst the first protein-coding genes to be cloned (Schedl *et al.*, 1978), and they were well characterized by 1981. More importantly, they were available: Mariann Bienz, who had started working in the Cell Biology Division with John Gurdon, had tried to express the *Drosophila* genes in *Xenopus* oocytes, but after some unpromising results had turned her attention instead to the heat shock response of the oocytes themselves. So I tried expressing the genes in COS cells, and after a few attempts found that they were indeed heat-inducible.

Deletion analysis of the *hsp70* promoter was an obvious approach. To my amazement, I found that I had picked an extremely simple eukaryotic promoter: when assayed in animal cells, heat-activation of the *Drosophila* gene required only a TATA box and a short regulatory sequence about 20 base pairs further upstream (Pelham, 1982). After gazing at the sequences of other heat shock promoters, I guessed a symmetric consensus for this regulatory sequence and begged a synthetic decameric oligonucleotide from the chemists in the Structural Studies Division of the laboratory. Synthesis was a laborious manual process at that time, and I was worried that the guess might be wrong, so I picked a sequence that consisted of two overlapping restriction sites, so that if it didn't work as a promoter element it could still be used as a cloning adaptor. In fact, when placed upstream of the TATA box of the herpes thymidine kinase gene, the synthetic sequence did confer heat inducibility, much to my relief and excitement (Pelham and Bienz, 1982). Such an experiment now seems commonplace, but it was the first time a promoter element had been identified in this way.

Over the next few years, we and others analysed a number of heat shock promoters from a variety of species, and several principles emerged (for review see Bienz and Pelham, 1987). Remarkably, the same regulatory sequence is functional in numerous species, including yeast, flies and humans. However, many heat shock promoters contain additional regulatory sequences, allowing complex patterns of expression (e.g. Cohen and Meselson, 1985; Bienz, 1986; Riddihough and Pelham, 1986; Wu *et al.*, 1986). The heat shock elements (HSEs) frequently occur in multiple copies, and can work not only as promoter elements but also as long-range enhancers (Bienz and Pelham, 1986; Riddihough and Pelham, 1986). In many ways, the HSE has proven to be the archetypic example of a eukaryotic regulatory sequence.

Once the HSE had been defined, the question was: how does it work? In 1984, evidence began to accumulate that the HSE is the binding site for a specific transcription factor. Carl Wu, in a series of elegant chromatin digestion experiments, showed that HSEs in *Drosophila* cells are protected from nuclease only when the heat shock genes are active (Wu, 1984a), and that an HSE-binding activity can be detected in heat-shocked cells (Wu, 1984b). Similar results were subsequently obtained with mammalian cells (Kingston *et al.*, 1987; Sorger *et al.*, 1987). Meanwhile, Carl Parker had identified an activity which specifically stimulated the transcription of heat shock genes *in vitro*, and showed that it copurified with an HSE-binding activity (Parker and Topol, 1984). As the years passed, techniques for the purification of DNA-binding proteins improved, and in 1986 Peter Sorger, then a student in my lab, pulled the original hand-made HSE oligonucleotides from the freezer and joined the rush to affinity-purify the heat shock factor (HSF). After a period of immense confusion over their true molecular weights, the *Drosophila* and yeast factors were finally identified (Wu *et al.*, 1987; Sorger and Pelham, 1987), and the gene encoding yeast HSE was cloned both here at the MRC and at CalTech (Sorger and Pelham, 1988; Wiederrecht *et al.*, 1988).

We had chosen to work with yeast largely because it was cheap and easy to grow, but it proved a lucky choice. It turned out that in yeast, unlike in *Drosophila* and mammalian cells, HSF binds to DNA even at normal temperatures (Sorger *et al.*, 1987; Jakobsen and Pelham, 1988). This meant that it could be detected in, and purified from, crude extracts of both control and heat-shocked cells. Comparison of HSF from these two sources showed that heat shock causes it to become highly phosphorylated (Sorger *et al.*, 1987; Sorger and Pelham, 1988). Presumably, the ability of bound factor to stimulate transcription is modulated by phosphorylation (see Figure 1). Subsequent studies showed that the mammalian factor is also phosphorylated after heat shock (Larson *et al.*, 1988).

It is likely that the activity of many transcription factors will prove to be regulated by phosphorylation. In some cases the affinity of the factors for their binding sites may be

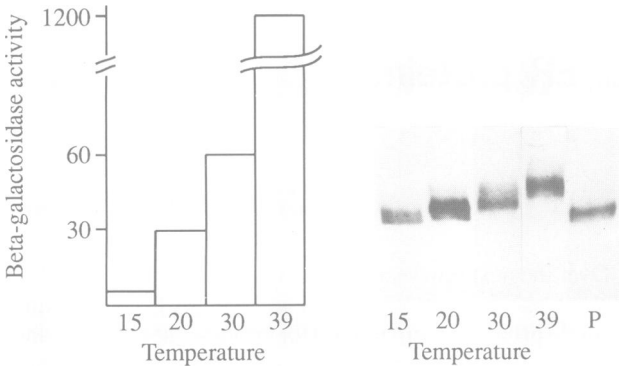

Fig. 1. Phosphorylation of yeast HSF at different temperatures. The graph shows β-galactosidase activity expressed from a promoter containing a synthetic HSE sequence, in yeast grown at 15, 20 or 30°C, or heat-shocked briefly at 39°C. The right hand panel shows an immunoblot of HSF in cells grown under the same conditions. The changes in mobility of the factor are due to phosphorylation; the final lane (P) contains purified, dephosphorylated HSF. In general, higher levels of phosphorylation correlate with higher levels of β-galactosidase expression (see Sorger and Pelham, 1988).

altered, but in the case of yeast HSF phosphorylation clearly affects a later step in transcriptional activation. The availability of the HSF gene will allow detailed genetic and biochemical studies of the activation mechanism, which may reveal features that are common to other regulatory systems.

Functions of the *hsp70* family

Analysis of transcriptional factors is one approach to understanding the heat shock response, but another major question concerns the way in which a rise in temperature, or other stress, is sensed by the cell. As early as 1982, Susan Lindquist had compiled indirect but rather compelling evidence in favour of a feedback regulation model in which the levels of hsp70 protein control the heat shock response in *Drosophila* cells (DiDomenico *et al.*, 1982), and there is genetic evidence from yeast (Chappell *et al.*, 1986) and even *Escherichia coli* (Tilly *et al.*, 1983) to support this idea. Clearly, if this is so, we have to understand the function of hsp70 before a complete picture of the regulatory circuits can be drawn.

The summer of 1983 was long and hot. The transcription work was going slowly. In September, Susan Lindquist visited the lab, bringing monoclonal antibodies specific for *Drosophila* hsp70. I learned to do immunofluorescence microscopy for the first time, and suddenly cell biology seemed like fun. So when Sean Munro joined the lab in October, ostensibly to work on transcription, I persuaded him to study the function of hsp70.

It was widely assumed that heat shock proteins help to protect cells from thermal damage. In 1980, Larry Hightower noted that most inducers of the stress response create denatured or abnormal proteins within cells, and postulated that heat shock proteins somehow aid their degradation (Hightower, 1980); unfortunately, this prescient proposal was rather lost amongst the many other ideas in the literature. Early studies had shown that hsp70 was associated with many different cellular components after heat shock; the most striking observation being that in *Drosophila* cells it moved to the nucleus, returning to the cytoplasm as the cells recovered (Arrigo *et al.*, 1980; Velazquez and Lindquist, 1984). In animal cells, we and others found a similar phenomenon, except that it was in nucleoli that the protein concentrated most spectacularly after heat shock (Pelham, 1984; Welch and Feramisco, 1984)—nucleoli have been known since the 1960s to be very sensitive to thermal damage, but the damage is reversible during subsequent incubation at 37°C (Simard and Bernhard, 1967). A key discovery was that when COS cells were transfected with a plasmid that constitutively expressed hsp70, their nucleoli recovered from heat shock more rapidly than usual, even when synthesis of other heat shock proteins was blocked (Pelham, 1984). This was the first indication that hsp70 might actively promote repair processes in damaged cells.

Mike Lewis, who had joined me in 1982, and I, began to study the interaction of hsp70 with heat-shocked nuclei and nucleoli, but it was a frustrating task because nothing seemed to release the protein from them. In the Spring of 1985 I went to a UCLA meeting and learnt that hsp70 bound extremely tightly to ATP−agarose, a discovery that was interpreted as evidence that the protein had an affinity for RNA (Welch and Feramisco, 1985). I thought it more likely that hsp70 was an ATPase—indeed, the *E.coli* version of hsp70, the dnaK protein, had been shown two years earlier to have ATPase activity (Zylicz *et al.*, 1983), but this activity was so weak that I had doubted its significance. On my return, we added ATP to our heat-shocked nuclei and found that hsp70 was rapidly and completely released from them (see Figure 2).

On the basis of this finding, we proposed an 'ATP-driven detergent' model of hsp70 action (Lewis and Pelham, 1985). The basic idea was that hsp70 binds to denatured, aggregated proteins and solubilizes them, and then uses the energy of ATP hydrolysis to release itself, thereby giving the proteins a chance to fold. A refinement of this model envisages distortion of the substrates as a result of conformational changes in hsp70, thus facilitating their extraction from aggregates or misfolded states.

Denatured nucleoli are not the best substrates for biochemistry, so we began to look for a more tractable system. Even in the absence of stress, cells contain abundant hsp70-like proteins which appear to have an essential function (for review see Lindquist, 1986). Sean Munro began to isolate cDNA clones for these proteins from a rat liver library, against the advice of many of our colleagues who thought this a particularly uninteresting project. The first clones he sequenced encoded a cytoplasmic member of the hsp70 family and were not very instructive. However, to our complete surprise, he also found clones that encoded an hsp70 homologue with a signal peptide (Munro and Pelham, 1986). This protein seemed to be in the endoplasmic reticulum (ER), and after scouring the literature we were able to show that it had been previously identified not only as a 'glucose-regulated protein' (see Lee, 1987 for review) but also as 'immunoglobulin heavy chain binding protein' (BiP for short), a protein that had first been found in pre-B cells and was thought to be involved in the allelic exclusion of immunoglobulin genes (Haas and Wabl, 1983). It later turned out to be a major component of the ER in most cell types.

Things rapidly began to make sense. Just as we imagined hsp70 binding only to partially denatured or disassembled proteins in heat-shocked nuclei, so BiP was found to bind only to incompletely folded or assembled proteins: immuno-

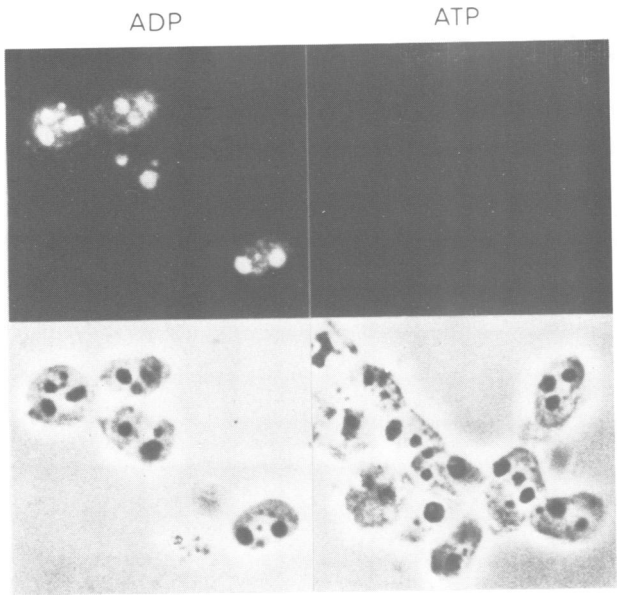

Fig. 2. Release of hsp70 from isolated nuclei by ATP. Nuclei were isolated from heat-shocked cells, at a time when hsp70 was principally concentrated in nucleoli, and incubated with 1 mM ADP or ATP. Immunofluorescent staining (upper panels) shows that ATP released the hsp70 from the nuclei, whereas ADP did not (taken from Lewis and Pelham, 1985).

globulin molecules lacking at least one light chain (Bole *et al.*, 1986), mutant or unglycosylated forms of influenza haemagglutinin (Gething *et al.*, 1986), and, as shown later, to non-disulphide-bonded forms of prolactin (Kassenbrock *et al.*, 1988). Moreover, BiP could be released from its substrates *in vitro* by the addition of ATP (Munro and Pelham, 1986). It seemed to do much the same in the ER as did hsp70 in heat-shocked nuclei.

We were so convinced of their role in protein folding that in 1986 we filed a preliminary patent on the entire family of hsp70-like proteins, hoping that they would allow the efficient *in vitro* refolding of proteins produced in *E.coli*, or that when overproduced *in vivo* they would improve the yield of active genetically engineered products. We happily mixed denatured proteins with hsp70, but despite a few promising results no great breakthrough came, and our hopes of becoming millionaires faded.

The concept, however, proved more durable. In a simple extension of our working model, I suggested that the real purpose of the hsp70 family under normal circumstances is to perform what has become known as a 'chaperoning' role for newly synthesized proteins; that is, to prevent or disrupt inappropriate (primarily hydrophobic) interactions of proteins that have not yet achieved their final state of assembly, and thus catalyse folding and assembly processes both in the cytoplasm and in the ER (Pelham, 1986). This idea led in due course to the discovery that hsp70 is required for the maintenance of some secretory and mitochondrial proteins in a loosely folded state prior to their translocation across the appropriate membrane (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Zimmermann *et al.*, 1988). There is also increasing evidence (mostly still unpublished) that normal membrane and secretory proteins, when they emerge into the ER, transiently associate with BiP prior to achieving their final folded state. Indeed, yeast BiP is essential for normal secretion and hence for cell viability (Normington *et al.*, 1989; Rose *et al.*, 1989; our observations). Other proteins, notably relatives of the *E.coli* groE heat shock protein, also act as chaperones during protein folding and assembly in bacteria, chloroplasts and mitochondria (Hemmingsen *et al.*, 1988; Cheng *et al.*, 1989) or prior to membrane translocation in *E.coli* (reviewed by Meyer, 1988).

More esoteric functions of hsp70 have also been discovered: Jim Rothman's group isolated hsp70 as an 'uncoating ATPase' capable of removing clathrin coats from vesicles (Chappell *et al.*, 1986), and the *E.coli* dnaK protein catalyses a specific step in the initiation of phage lambda DNA synthesis (Dodson *et al.*, 1986; Liberek *et al.*, 1988). Both of these functions involve the disruption of protein–protein interactions, one of the roles of a chaperone.

There now seems to be general agreement about the kinds of reactions catalysed by hsp70, if not their mechanistic details. However, it remains unclear how the properties of hsp70 relate to the induction of the heat shock response. A shortage of hsp70 in yeast cells is sufficient to activate synthesis of heat shock proteins (Craig and Jacobsen, 1984; Deshaies *et al.*, 1988), and it seems likely that thermally denatured proteins will bind to hsp70 and reduce its effective concentration. But is it the absence of hsp70 or the presence of unfolded proteins that triggers the heat shock response? How is phosphorylation of the transcription factor regulated? Clearly, there is still a gap to be filled in our understanding of the regulatory circuit, but with our current knowledge a biochemical approach to this problem seems feasible.

Retention of proteins in the ER

The unexpected cloning of BiP abruptly introduced us to a completely new field, namely the intracellular sorting of proteins. BiP was predicted to be a soluble ER protein, which made sense because its substrates (such as immunoglobulin chains) were often soluble constituents of the ER lumen. But why was BiP not secreted from cells? At the time, conventional wisdom held that exit of secretory proteins from the ER was a receptor-mediated process, such proteins possessing some kind of specific transport signal (Lodish *et al.*, 1983). Proteins that stayed behind would lack such a signal. We, however, were blissfully ignorant of the field, and assumed without any serious thought that secretion was the natural fate of proteins in the ER. Thus, the non-secretion of BiP was puzzling.

Luckily, BiP was the second luminal ER protein to be identified and the sequence of the first one, protein disulphide isomerase, was available (Edman *et al.*, 1985). We looked for signs of some common feature that could account for the localization of these proteins, and noticed that they both had the C-terminal sequence Lys-Asp-Glu-Leu (KDEL). Within a few days, Sean Munro found that deletion of this sequence resulted in the secretion of BiP from COS cells. We later showed that addition of the sequence SEKDEL to the secretory protein lysozyme was sufficient to keep the lysozyme in the ER (Munro and Pelham, 1987; for another example see Figure 3). Subsequent cloning and sequence analysis of soluble ER proteins from a variety of species has confirmed that KDEL, or a closely related sequence, is almost universally present at the C terminus (for review see Pelham, 1989).

The conclusion from these studies was clear: retention of resident soluble proteins in the ER is a specific process that

is mediated by the KDEL signal. Conversely, the fact that truncated BiP can be secreted even though it has no reason to contain specific transport signals implies that secretion occurs by default: that is, entry into transport vesicles is a non-selective process. A number of other studies had arrived at a similar conclusion (Wiedmann *et al.*, 1984; Poruchynsky *et al.*, 1985; Wieland *et al.*, 1987), but the discovery of the KDEL system was perhaps the strongest argument in favour of this concept.

The retention mechanism

Once the principle of non-selective bulk flow from the ER to the Golgi was established, a conceptual problem arose. The only way for KDEL-containing proteins to avoid this flow would be for them to be physically held in the ER, for example by binding to a membrane associated receptor; but there was no obvious candidate for a receptor protein of sufficient abundance, and immunogold labelling has now confirmed that the luminal proteins are not associated with membranes (Koch *et al.*, 1988; Tooze *et al.*, 1989). Most convincingly, Alan Colman found that BiP could diffuse within the ER of *Xenopus* oocytes, albeit slowly, and that the rate of diffusion was unaffected by the presence or absence of KDEL (Ceriotti and Colman, 1988). We therefore believed that the most likely explanation for KDEL-dependent retention was that the proteins were continuously retrieved from a post-ER compartment and returned to the ER (Munro and Pelham, 1987). Only when I said this in seminars did I discover that it was considered a heretical view. In fact, Jim Rothman had proposed a similar model for the sorting of ER membrane proteins some years earlier (Rothman, 1981), but this was based on the detection of low levels of ER proteins in purified Golgi fractions, and when immunoelectron microscopy failed to confirm the results, the model was discounted (Yamamoto *et al.*, 1985; Brands *et al.*, 1985).

One argument against the recycling of ER glycoproteins through the Golgi is that such proteins, including the KDEL-containing protein GRP94, do not have the oligosaccharide modifications that would be expected if they were exposed to the enzymes present in the *cis*-Golgi (Lewis *et al.*, 1985). A possible explanation for this was that retrieval of ER proteins occurs from a compartment that lies in the secretory pathway between the ER and the conventionally defined *cis*-Golgi. During a visit I made to the EMBL, Gareth Griffiths pointed out that the addition of mannose-6-phosphate to lysosomal enzymes was one of the earliest post-ER events (Kornfeld and Kornfeld, 1985), and suggested that I add KDEL to cathepsin D, a lysosomal enzyme, to see whether it could still be modified. I took his advice. KDEL-tagged cathepsin D accumulated in the ER (see Figure 3), but was still a substrate for the first of the enzymes involved in mannose-6-phosphate addition (Pelham, 1988). The next enzymatic step, which is thought to occur in a slightly later compartment, was not observed. Thus, this experiment provided strong evidence for retrieval from an early post-ER compartment.

We have recently obtained similar evidence for recycling in yeast: proteins that bear HDEL (the *Saccharomyces cerevisiae* version of KDEL) are retained within cells, presumably in the ER, but are still subjected to some post-ER oligosaccharide modifications (Pelham *et al.*, 1988; our

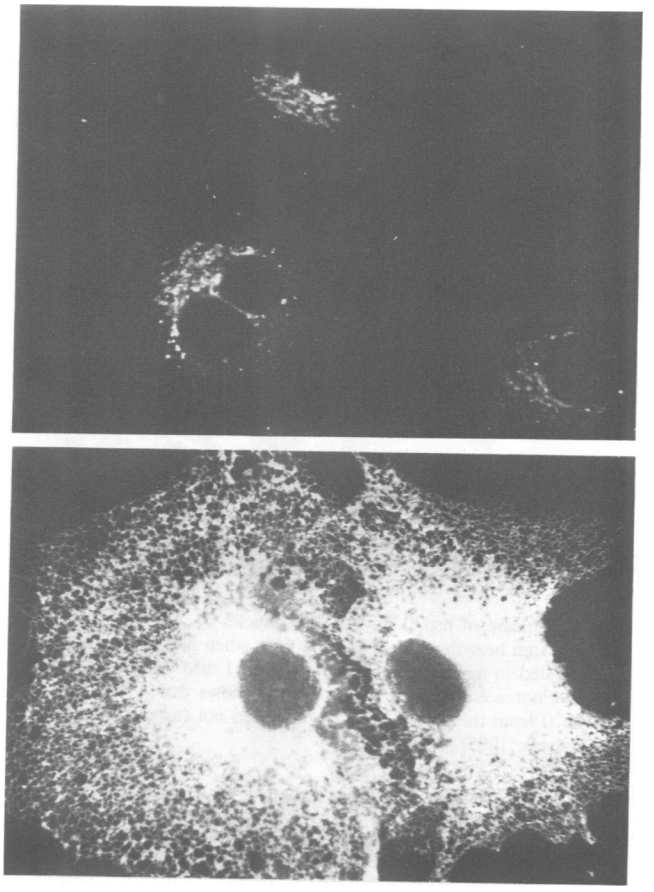

Fig. 3. Retention of KDEL-tagged cathepsin D in the ER. COS cells were transfected with plasmids expressing cathepsin D without (**top**) or with (**bottom**) KDEL, and the expressed protein detected by immunofluorescence. Without the retention sequence, the cathepsin is mainly in the Golgi, and in nearby vesicles; it is not seen in lysosomes because the epitope recognized by the antibody is removed by proteolysis. With KDEL, the protein accumulates to high levels in the ER (taken from Pelham, 1988).

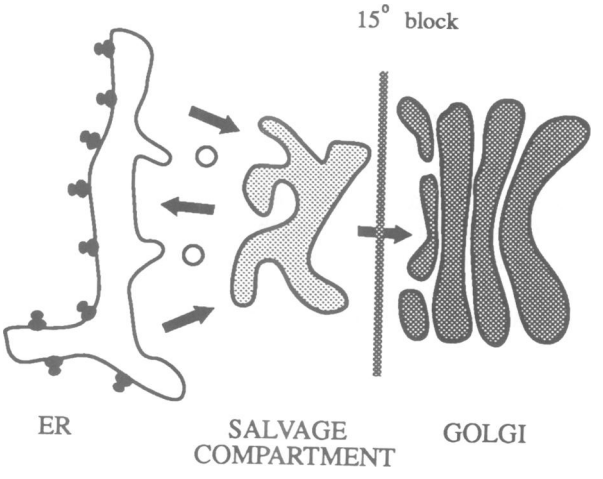

Fig. 4. Proposed sorting pathway for luminal ER proteins. ER proteins and secretory proteins travel together to the salvage compartment. ER proteins are then returned to the ER by receptor-mediated vesicular transport. Secretory proteins move to the Golgi, becoming concentrated in the process. Incubation of cells at 15°C appears to block transport between the salvage compartment and the Golgi (see Pelham, 1989).

unpublished observations). It is easy to prove that these modifications occur outside the ER, because they are blocked in a *sec18* ts mutant, which is defective in vesicular transport at the non-permissive temperature (Novick *et al.*, 1980; Wilson *et al.*, 1989).

Our current model for the retention system postulates a receptor that shuttles between the ER and a 'salvage compartment', binding KDEL in the salvage compartment and releasing it in the ER (see Figure 4). We assume that this is made possible by a difference in the ionic environment of the two compartments. The identity of the salvage compartment in animal cells remains somewhat controversial, but it may correspond to the structures that hypertrophy when cells are incubated at 15°C (Saraste and Kuismanen, 1984; see Pelham, 1989 for further discussion).

One conceptual problem that remains is that of how a unidirectional flow of secretory proteins is maintained. As pointed out by Wieland *et al.* (1987), non-selective transport implies a massive flow of membrane, in the form of vesicles, out of the ER. The membrane lipid must be returned to the ER, most likely from the Golgi or salvage compartment. I have argued for a return vesicle flow, but this creates as many problems as it solves: proteins would be just as likely to travel back to the ER as they were to leave it in the first place. In the steady state, many newly synthesized membrane and secretory proteins are more concentrated in the Golgi than in the ER (Quinn *et al.*, 1984; Munro and Pelham, 1987), which implies a filtration mechanism that prevents their reverse transport. The nature of that mechanism remains a problem for the future.

A further understanding of the sorting events that occur on the *cis* side of the Golgi requires the identification of proteins that are involved in the process. One way to achieve this without prejudice as to the mechanisms involved is to isolate yeast mutants that are defective in the sorting process and clone the corresponding genes. Recently, we have identified at least two genes that are required for retention of HDEL-containing proteins in the ER (Pelham *et al.*, 1988 and unpublished data). Further study of these genes may help to explain how errant ER proteins are incorporated into vesicles, and how those vesicles are directed to the ER while other proteins in the salvage compartment go forwards along the secretory pathway. Lessons learnt from this system may in turn help to explain other sorting events that occur within the secretory and endocytic pathways.

Eight years ago, I could never have predicted that I would now be addressing such problems. I am lucky to have had the freedom to follow experiments wherever they lead, and the colleagues to teach me new fields. I am particularly grateful to those who have worked with me, especially Mariann Bienz, Mike Lewis, Sean Munro, Peter Sorger, Guy Riddihough and, more recently, Bent Jakobsen, Kevin Hardwick, Jan Semenza and Neta Dean.

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