The endoplasmic reticulum as a protein-folding compartment

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The lumen of the endoplasmic reticulum (ER) provides a dynamic and efficient environment for the folding of proteins destined for secretion and for a variety of cellular compartments and membranes. Usually, the folding process begins on the nascent chains and is completed minutes or hours later during assembly of oligomers. It is assisted by molecular chaperones and folding enzymes, some of which are unique to the ER. Quality control and selective degradation systems ensure only conformationally mature proteins are transported from the ER.

Nearly all polypeptides in a eukaryotic cell are translated in the cytosol, but many fold and assemble into functional, mature proteins only after transport to other compartments. These compartments include mitochondria, chloroplasts, peroxisomes and the ER. As a rule, folding takes place in the compartment of final residence. In this respect, the ER is an exception. It is responsible for the biosynthesis and maturation of proteins and glycoproteins that are destined for secretion, for the plasma membrane, and for transport to the various organelles of the endocytic and exocytic pathways.

In some cell types, the daily output of protein from the ER can exceed the cell's own mass. The repertoire of products is prodigious. It ranges from surface receptors and lysosomal hydrolases to hormones and extracellular matrix components. Many of the proteins are complex multimers whose expression is under sensitive post-translational control, and some have a molecular mass of several hundred thousand Da. The ER is not only responsible for synthesizing and translocating these proteins but also for folding, assembling, modifying, sorting, dispatching and degrading them, as well as regulating their level of expression. Recent progress in protein folding and sorting has increased our understanding of this dynamic organelle and the way by which it deals with some of these tasks.

In this review, we focus on cell biological aspects of protein folding within the ER lumen. In particular, we emphasize the stepwise maturation process in living cells from nascent chains to functional proteins or assemblies (Fig. 1). Other aspects of folding, as studied both *in vitro* and *in vivo*, have been extensively reviewed in the last few years; see Refs 1–4 for a general background on protein folding, protein refolding *in vitro*, and the specific role of different chaperones.

Conditions inside the ER

The ER lumen provides a milieu that is intermediate between the cytosol and the extracellular space. The Ca^{2+} concentration, which is in the millimolar range, approaches values measured extracellularly[5]. The maturation of many proteins in the ER is dependent on Ca^{2+} (Refs 6, 7). The concentrations of other electrolytes are similar to those in the cytosol. The pH in the lumen is probably close to neutrality.

The redox conditions inside the ER are of particular interest in relation to disulphide bond formation. Unlike proteins in the cytosol, those generated in the ER are rich in cysteines and usually acquire multiple disulphide bonds[8]. The orderly formation of these bonds is not only a prerequisite for the stability of the final protein products but also for the progress of the newly synthesized polypeptides along the folding pathway.

Glutathione probably provides the main redox buffer inside the ER. Recent studies by Lodish and colleagues (pers. commun.) suggest that, in contrast to the ratio of reduced to oxidized glutathione of ~100:1 in the cytosol, the ratio inside the ER is ~10:1. Experiments *in vitro* have shown that this ratio is optimal for spontaneous disulphide formation during protein refolding[8]. How glutathione enters the ER lumen and how the redox gradient over the ER membrane is maintained remain unclear. An ATP-independent translocation system selective for oxidized glutathione is a likely possibility.

When reducing agents such as 2-mercaptoethanol or dithiothreitol (DTT), or oxidizing agents such as diamide, are added to cells, the redox conditions in the ER change and the folding, matu-

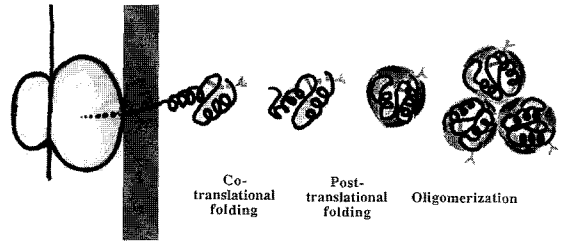

FIGURE 1

Co- and post-translational folding in the ER. The folding and maturation of a typical glycoprotein in the lumen of the ER proceeds in three main phases. The N-terminal folding domains begin to fold on the nascent chain and acquire some intrachain disulphide bonds. After chain termination, folding continues. The folded, fully oxidized monomers frequently assemble into homo- or hetero-oligomers, which are transported from the ER to the Golgi complex.

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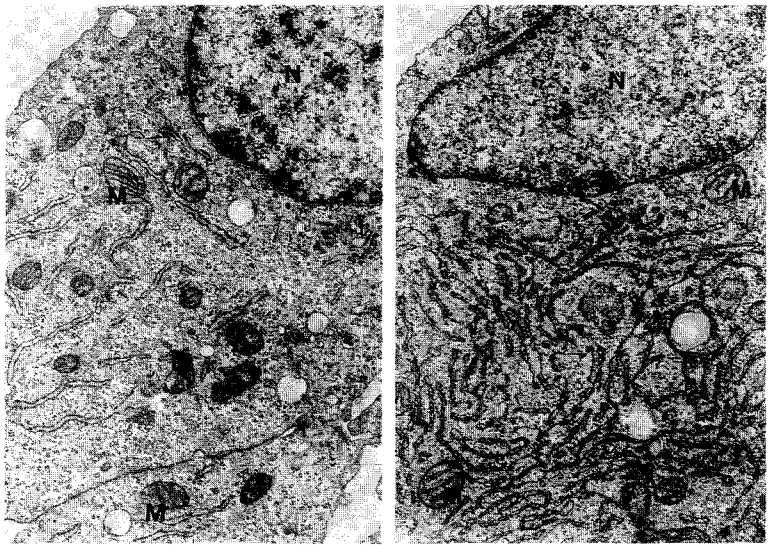

FIGURE 2

The effects of DTT on the ER. When added to cells, DTT prevents protein folding in the ER by inhibiting the formation of disulphide bonds. This leads to the accumulation of transport-incompetent proteins, and to the swelling of the ER compartment. (right) CHO cells treated with DTT for 4 h. (left) Control CHO cells. Magnification: 1000 ×. Photo by Jürgen Kartenbeck.

ration and transport of proteins are affected[9,10]. DTT, for example, reduces partially and fully oxidized monomers of viral membrane glycoproteins (influenza haemagglutinin (HA), vesicular stomatitis virus (VSV) G protein and Semliki Forest virus glycoproteins) inside the ER, although it does not affect most proteins in other compartments[10] (A. de Silva, I. Braakman, T. Marquardt and A. Helenius, unpublished). DTT does not inhibit translation or translocation of proteins, but it prevents the formation of disulphides and folding. After removal of DTT, normal redox conditions are rapidly restored, and this results in normal folding of the unfolded chains post-translationally. Since the addition of DTT prevents folding of disulphide-containing proteins without blocking their synthesis, a dramatic accumulation of products that cannot be transported takes place in the ER. This leads to a rapid expansion of the compartment, as seen in Fig. 2.

Recent studies by Clairmont and colleagues have shown that the ER contains ATP (Ref. 11). It is transported from the cytosol through the ER membrane by a specific ATP translocator. The same translocator may also be responsible for the extrusion of ADP or AMP. The presence of ATP in the lumen is important because some of the proteins that facilitate folding, such as BiP/GRP78, not only associate with ATP but also depend on it for their functions[12].

When cells are depleted of ATP, the folding process in the ER is inhibited. Instead of folding normally, influenza HA and VSV G protein form misfolded, disulphide-crosslinked aggregates with stably associated BiP/GRP78 (Ref. 13; A. de Silva, I. Braakman and A. Helenius, unpublished). The fidelity of folding is thus severely disturbed. ATP is also needed to maintain some proteins in their folded state inside the ER, and for the rescue and refolding of misfolded proteins generated after ATP depletion. Some of the effects of DTT addition and ATP depletion on HA folding are shown in Fig. 3.

Folding factors in the ER

Protein folding in aqueous solution has been extensively analysed by following the refolding of denatured proteins *in vitro* after removal of denaturant. Several important generalizations have emerged (see Refs 1, 3 and 14 for reviews): (1) the three-dimensional structure of proteins is determined by their amino acid sequence; (2) proteins can fold spontaneously, i.e. they do not require, *a priori*, the assistance of other macromolecules; (3) folding occurs along specific pathways with defined intermediates; (4) larger proteins possess separate folding domains that fold independently of each other; (5) folding begins with rapid reactions leading to native-like compact intermediates with native-like secondary structure but without well-defined tertiary structure (this is the so-called 'molten globule' state or collapsed intermediate), and these are followed by slower reactions that involve proline isomerization, disulphide bond formation, and conformational adaptation between domains.

While these principles are highly relevant for folding in the living cell, they do not alone explain the entire process. Most importantly, it is evident from a variety of studies that folding in the cell is not unassisted. Rapid and successful navigation of polypeptides through the folding pathways depends on the action of cellular proteins called molecular chaperones and on a variety of folding enzymes (see Refs 2, 4, 13 and 15 for reviews). These are needed to enhance the efficiency of folding, particularly during the late reactions that lead from the 'molten globule' state to the finally folded and oligomerized protein. The role of chaperones is not to impart folding information, but to prevent inappropriate intra- and intermolecular interactions during the folding process.

Consistent with its role as a central site of protein biogenesis, the ER lumen is rich in chaperones and folding factors (Table 1). In fact, it seems that nearly all major resident proteins in the ER lumen have functions related to folding and assembly. Most of the chaperones present are members of families that are also represented in the cytosol, mitochondria and chloroplasts. The most abundant are BiP/GRP78 and GRP94, which are homologous to cytosolic HSP70 and HSP90, respectively. There is no evidence yet for an HSP60-like chaperone in the ER, but an ER membrane protein with partial homology to DnaJ has been identified in yeast[16]. In the bacterial cytoplasm, DnaJ is known to serve as an activator and cofactor for HSP70 during protein folding[17].

In addition, the ER contains proline *cis–trans* isomerases similar to those observed in the cytosol. While their role in folding inside the ER has been unambiguously demonstrated only for a subset of *Drosophila* rhodopsin molecules (the *ninaA* gene

product)[18], studies using the inhibitor cyclosporin suggest that they are more generally deployed as folding factors[19,20]. As more is learned about the folding of individual proteins, it is likely that additional cases where specific folding factors are required will emerge.

There are also folding enzymes that are unique to the ER (Table 1). Protein disulphide isomerase (PDI) is the best-characterized redox enzyme[21,22]. It is involved in the formation and reshuffling of disulphide bonds during folding. Studies of the biochemistry of PDI have shown that it can serve as a reductase, an isomerase or an oxidase, depending on redox conditions. Moreover, folding experiments in microsomes have confirmed that PDI is required for folding and disulphide formation[23]. Disruption of the gene encoding PDI in yeast is lethal[24]. The functions of two other abundant proteins, called ERp72 and ERp61, that, like PDI, have thioredoxin-like active sites, remain unclear at present[25]. It is likely that they too play a role in disulphide bond formation and in folding.

While the oligosaccharide-transferase enzyme that adds *N*-linked core sugar moieties cannot be categorized as a *bona fide* folding enzyme, it plays an important role in facilitating the folding of many proteins in the ER. Without the addition of *N*-linked sugars, numerous proteins fail to fold correctly[26]. The bulky polar *N*-linked oligosaccharides added cotranslationally have three potential functions in the folding process: they may ensure the correct local positioning of the peptide segments to which they are bound, for example by directing them to the surface of the molten globule; they may discourage the binding of chaperones to specific sites; and, through their hydrophilic nature, they may render the folding intermediates more soluble, thus preventing their irreversible aggregation[27]. If the main function of *N*-linked oligosaccharides were, indeed, to facilitate the folding process, it would explain why cells add oligosaccharides to nascent chains before they fold. This would also explain why cells have evolved an elaborate glycosylation machinery in the ER in addition to the systems present in the Golgi complex.

Although the data are few, it is apparent that removal of the signal sequence in the ER may also be required for correct folding[28]. For secretory proteins and type I membrane proteins this is not surprising since the signal peptide is hydrophobic and thought to remain associated with the membrane; its presence would severely restrict the mobility of N-terminal sequences during folding.

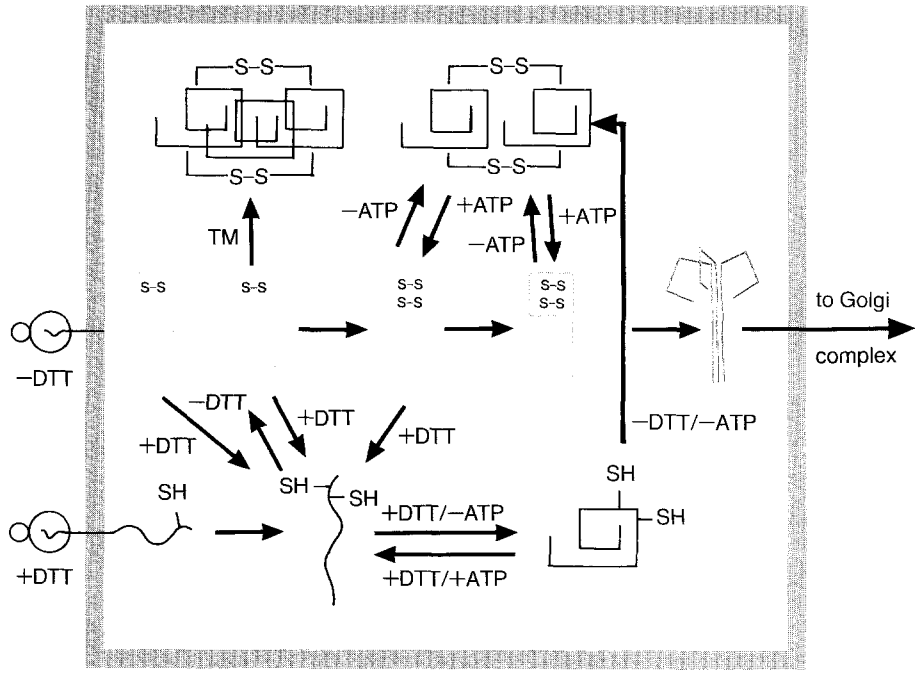

FIGURE 3

Normal and inhibitor-induced conformational modifications in influenza HA. The pathway drawn in blue shows the normal steps of influenza HA maturation in the ER; various aberrant forms induced by DTT and ATP-synthesis inhibitors alone or in combination are shown above and below. Normally, the HA subunits undergo stepwise folding and oxidation, acquiring six intrachain disulphide bonds. The folded monomers trimerize and the trimers are selectively transported to the Golgi complex. The addition of DTT during or after synthesis causes reduction of disulphide bonds. The addition of ATP-synthesis inhibitors causes misfolding. In contrast to tunicamycin (TM)-induced aggregates, the aggregates formed during ATP depletion are fully reversible. The scheme is based on Refs 10, 13 and 39, as well as unpublished data of I. Braakman, U. Tatu and A. Helenius.

Co- and post-translational folding

The folding of proteins translocated into the ER lumen usually begins on the nascent chain[29] (Fig. 1). This is possible because most proteins are cotranslationally translocated and the rate of folding is much higher than the elongation rate. Whereas the formation of molten globules occurs within milliseconds, elongation in mammalian cells occurs at about four to five residues per second. However, the extent of cotranslational folding obviously depends on the size of a protein and on the number of folding domains. Single domain proteins may not be able to fold extensively until chain termination has occurred.

Most secretory proteins, extracellular matrix proteins and membrane glycoproteins contain several structural domains. These domains constitute the basic folding unit. The modular nature of protein structure, and the domain-by-domain nature of folding presumably explains why proteins as large as 500 kDa can fold correctly and efficiently. It also explains why domain exchanges and truncations arising from alternative splicing and *in vitro* mutagenesis can result in correctly folded, transport-competent molecules.

For immunoglobulin, serum albumin and influenza HA, folding in living cells starts cotranslationally from N-terminal domains and proceeds towards the C-terminal domains[29,30,39]. Such vec-

TABLE 1 – ER CHAPERONES AND FOLDING ENZYMES

Protein	Function	Refs
Chaperones		
BiP/GRP78	Folding, retention, oligomeric assembly, translocation	32,41–43
GRP94	Folding?	34,44,45
Folding enzymes		
Protein disulphide isomerase	Disulphide formation	21,23,24
Peptidyl-prolyl *cis–trans* isomerase	Peptide-bond isomerization	18–20
Prolyl hydroxylase	Collagen maturation	46
Lysyl hydroxylase	Collagen maturation	46
Other potential folding factors		
ERp72	Disulphide formation	25
ERp61	Disulphide formation	25
Calnexin	Folding or assembly?	35

torial folding, dictated by the direction of translation, is probably common for multidomain proteins, but whether the vectorial nature of cotranslational folding is necessary for protein folding in the ER remains to be seen. Studies that used DTT to delay folding of influenza HA and other viral proteins have suggested that vectorial cotranslational folding may not necessarily be crucial for multidomain proteins of intermediate size[10].

There appear to be no major differences between the folding of soluble proteins and the luminal domains of type I and II membrane proteins. It is not clear how the cytosolic and transmembrane domains of membrane proteins fold, but their folding is generally independent of the luminal moiety. Presumably, the cytosolic domains follow the folding rules of normal cytosolic proteins. How the transmembrane domains acquire their three-dimensional structure and how multispanning membrane proteins fold within the membrane are interesting problems which are being addressed in several systems. This work has recently been reviewed by Popot and colleagues[31].

The cotranslational folding phase is followed by post-translational folding. The full-length polypeptides released from ribosomes continue to fold and acquire intrachain disulphide bonds. Folding intermediates of many proteins associate with BiP/GRP78 during the post-translational phase of folding[23,24]. The association is transient, and is usually not detectable once proteins have become fully oxidized; however, association of BiP/GRP78 with the CH1 domain of the IgG heavy chain persists until the immunoglobulin molecules have assembled[32]. Some proteins have been shown to associate transiently with PDI (Ref. 33), GRP94 (Ref. 34) and an 88 kDa membrane protein called calnexin[35]. There is also increasing evidence that transient complex formation between folding intermediates themselves may take place at this time (Ref. 36; A. de Silva, I. Braakman and A. Helenius, unpublished).

Post-translational folding usually takes only a few minutes, but sometimes it can go on for much longer. Initial folding of individual glycopolypeptide chains is often followed by oligomerization[37]. Oligomer assembly is a concentration-dependent process, and is likely to involve additional conformational alterations in the subunits. In some cases, assembly of subunits leads to the formation of interchain disulphide bonds.

Quality control

In general, folding intermediates, incompletely assembled proteins, misfolded proteins, and aggregated side products are selectively retained in the ER. Transport to the Golgi complex (and beyond) occurs only when a protein has folded, and (if applicable) assembled into oligomers (see Fig. 3). This important phenomenon, which secures the functional integrity of proteins leaving the ER, has been called 'quality control'[37] or 'architectural editing'[38].

The time needed for a protein to reach competence for transport ranges from a few minutes to several hours. It is determined by the rate of folding and the rate of assembly of oligomers[39]. For the same protein it can vary between cell types and with physiological conditions, and since oligomerization events are concentration dependent, it can be affected by the expression levels of the polypeptides. The molecular signals and mechanisms that lead to retention and/or transport during quality control are incompletely understood. It is apparent that proteins that are either aggregated or associated with BiP/GRP78 are not transported. Free sulphydryl groups may also be involved in the retention of proteins[9].

Although folding, assembly and exit from the ER are remarkably efficient for many proteins, they may in other cases represent the least efficient steps in the entire chain of events leading from the gene to the mature, active protein. Losses in the form of misfolded side products in the ER can sometimes exceed the amount of correctly folded protein. Together with excess unassembled subunits and incompletely assembled oligomers, the misfolded proteins are usually slowly degraded by nonlysosomal degradation pathways, although some undergo more rapid degradation[40].

Why a separate folding compartment for export?

It is interesting to speculate about the advantages that eukaryotic cells may derive from maintaining a separate, highly specialized organelle for the synthesis and maturation of secretory and membrane proteins. Why not secrete the proteins directly through the plasma membrane?

As a closed compartment, the ER obviously enables the cell to maintain a controlled environment optimized for folding and maturation. A complement of resident chaperones and ATP can

be maintained at high concentrations, the redox state can be regulated, and the ionic conditions can be controlled. The closed nature of the ER also permits easy assembly of complex oligomers. Its limited volume allows the subunits of oligomers to reach high enough concentrations to permit efficient assembly even when the subunits are expressed at low levels. The generation of complicated assemblies with numerous polypeptide components is thus feasible.

The closed nature of the ER also allows the cell to control the quality of proteins produced, by exporting only the correctly folded ones. Since the folding and assembly processes are seldom 100% efficient, misfolded proteins and unassembled subunits are continuously generated. If transported out of the ER these products could have deleterious effects on cell function. As an important side benefit, the quality control systems also provide a means for the regulation of protein expression.

Most importantly, the high secretion rates of many eukaryotic cell types would be difficult to achieve unless protein translocation and maturation were separated from the final secretion step at the plasma membrane. As a separate organelle, the ER can be expanded and elaborated without increasing the cell surface. Indeed, in most cells, it is by far the largest organelle.

References

1 JAENICKE, R. (1991) *Biochemistry* 30, 3147–3161
2 ELLIS, J. and VAN DER VIES, S. M. (1991) *Annu. Rev. Biochem.* 60, 321–347
3 CREIGHTON, T. E. (1986) *Methods Enzymol.* 131, 83–106
4 GETHING, M-J. and SAMBROOK, J. (1992) *Nature* 355, 33–45
5 BAUMANN, O., WALZ, B., SOMLYO, A. V. and SOMLYO, A. P. (1991) *Proc. Natl Acad. Sci. USA* 88, 741–744
6 SUZUKI, C. K., BONIFACINO, J. S., LIN, A. Y., DAVIS, M. M. and KLAUSNER, R. D. (1991) *J. Cell Biol.* 114, 189–205
7 LODISH, H. F. and KONG, N. (1990) *J. Biol. Chem.* 265, 10893–10899
8 GILBERT, H. F. (1990) *Adv. Enzymol.* 63, 69–172
9 SITIA, R. *et al.* (1990) *Cell* 60, 781–790
10 BRAAKMAN, I., HELENIUS, J. and HELENIUS, A. (1992) *EMBO J.* 11, 1717–1722
11 CLAIRMONT, C. A., DE MAIO, A. and HIRSCHBERG, C. B. (1992) *J. Biol. Chem.* 267, 3983–3990
12 ROTHMAN, J. E. (1989) *Cell* 59, 591–601
13 BRAAKMAN, I., HELENIUS, J. and HELENIUS, A. (1992) *Nature* 356, 260–262
14 ANFINSEN, C. B. and SCHERAGA, H. A. (1975) *Adv. Protein Chem.* 29, 205–300
15 PELHAM, H. R. B. (1986) *Cell* 46, 959–961
16 SADLER, I., CHIANG, A., KURIHARA, T., ROTHBLATT, J., WAY, J. and SILVER, P. (1989) *J. Cell Biol.* 109, 2665–2675
17 LANGER, T., LU, C., ECHOLS, H., FLANAGAN, J., HAYER, M. K. and HARTL, F. U. (1992) *Nature* 356, 683–689
18 STAMNES, M. A., SHIEH, B-H., CHUMAN, L., HARRIS, G. L. and ZUKER, C. S. (1991) *Cell* 65, 219–227
19 STEINMANN, B., BRUCKNER, P. and SUPERTI-FURGA, A. (1991) *J. Biol. Chem.* 266, 1299–1303
20 LODISH, H. F. and KONG, N. (1991) *J. Biol. Chem.* 266, 14835–14838
21 FREEDMAN, R. B. (1989) *Cell* 57, 1069–1072
22 NOIVA, R. and LENNARZ, W. J. (1992) *J. Biol. Chem.* 267, 3553–3556
23 BULLEID, N. J. and FREEDMAN, R. B. (1988) *Nature* 335, 649–651
24 LAMANTIA, M. L., MIURA, T., TACHIKAWA, H., KAPLAN, H. A., LENNARZ, W. J. and MIZUNAGA, T. (1991) *Proc. Natl Acad. Sci. USA* 88, 4453–4457
25 MAZZARELLA, R. A., SRINIVASAN, M., HAUGEJORDEN, S. M. and GREEN, M. (1990) *J. Biol. Chem.* 265, 1094–1101
26 PAULSON, J. C. (1989) *Trends Biochem. Sci.* 14, 272–276
27 MARQUARDT, T. and HELENIUS, A. (1992) *J. Cell Biol.* 117, 505–513
28 ROTTIER, P. J. M., FLORKIEWICZ, R. Z., SHAW, A. S. and ROSE, J. K. (1987) *J. Biol. Chem.* 262, 8889–8895
29 BERGMAN, L. W. and KUEHL, W. M. (1979) *J. Biol. Chem.* 254, 8869–8876
30 PETERS, T., JR, and DAVIDSON, L. K. (1982) *J. Biol. Chem.* 257, 8847–8853
31 POPOT, J-L., DE VITRY, C. and ATTEIA, A. in *Membrane Protein Structure: Experimental Analysis* (White, S. H., ed.), Oxford University Press (in press)
32 HENDERSHOT, L., BOLE, D., KÖHLER, G. and KEARNEY, J. F. (1987) *J. Cell Biol.* 104, 761–767
33 PERSSON, R. and PETTERSSON, R. F. (1991) *J. Cell Biol.* 112, 257–266
34 MELNICK, J. R., AVIEL, S. and ARGON, Y. (1991) *J. Cell Biol.* 115, 1a
35 BELL, W., DIGNARD, D., THOMAS, D. Y. and BERGERON, J. J. M. (1991) *J. Biol. Chem.* 266, 19599–19610
36 KIM, P. S. and ARVAN, P. (1991) *J. Biol. Chem.* 266, 12412–12418
37 HURTLEY, S. M. and HELENIUS, A. (1989) *Annu. Rev. Cell Biol.* 5, 277–307
38 KLAUSNER, R. D. (1989) *New Biol.* 1, 3–8
39 BRAAKMAN, I., HOOVER-LITTY, H., WAGNER, K. R. and HELENIUS, A. (1991) *J. Cell Biol.* 114, 401–411
40 BONIFACINO, J. S., SUZUKI, C. K. and KLAUSNER, R. D. (1990) *Science* 247, 79–82
41 HAAS, I. G. and WABL, M. (1983) *Nature* 306, 387–389
42 ROSE, M. D., MISRA, L. M. and VOGEL, J. P. (1989) *Cell* 57, 1211–1221
43 VOGEL, J. P., MISRA, L. M. and ROSE, M. D. (1990) *J. Cell Biol.* 110, 1885–1895
44 LEE, A. S., BELL, J. and TING, J. (1984) *J. Biol. Chem.* 259, 4616–4621
45 MAZZARELLA, R. A. and GREEN, M. (1987) *J. Biol. Chem.* 262, 8875–8883
46 KIVIRIKKO, K. I. and MYLLYLÄ, R. (1986) *Ann. NY Acad. Sci.* 460, 187–201