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Binding to membrane proteins within the endoplasmic reticulum cannot explain the retention of the glucose-regulated protein GRP78 in *Xenopus* oocytes

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We have studied the compartmentation and movement of the rat 78-kd glucose-regulated protein (GRP78) and other secretory and membrane proteins in *Xenopus* oocytes. Full length GRP78, normally found in the lumen of rat endoplasmic reticulum (ER), is localized to a membraneous compartment in oocytes and is not secreted. A truncated GRP78 lacking the C-terminal (KDEL) ER retention signal is secreted, although at a slow rate. When the synthesis of radioactive GRP78 is confined to a polar (animal or vegetal) region of the oocyte and the subsequent movement across the oocyte monitored, we find that both full-length and truncated GRP78 move at similar rates and only slightly slower than a secretory protein, chick ovalbumin. In contrast, a plasma membrane protein (influenza haemagglutinin) and two ER membrane proteins (rotavirus VP10 and a mutant haemagglutinin) remained confined to their site of synthesis. We conclude that the retention of GRP78 in the ER is not due to its tight binding to a membrane-bound receptor.

Key words: diffusion/glucose-regulated protein/oocytes

Introduction

There has been considerable debate over the last few years regarding the nature and function of sorting signals involved in the translocation of proteins from the endoplasmic reticulum (ER) to the cell surface of eukaryotic cells (for reviews see Garoff, 1985; Kelly, 1985; Rothman, 1987; Warren, 1987). Three lines of indirect evidence have emerged which favour the view that signals exist for ER retention and that secretion occurs by default. First, a bacterial secretory protein β lactamase can be secreted from a eukaryotic cell, the *Xenopus* oocyte, despite the morphological and functional dissimilarities between the eukaryotic and prokaryotic translocation machinery (Weidmann *et al.*, 1984). Second, studies with deletion mutants of resident ER membrane proteins have shown that they can be rerouted to the cell surface by removal of particular amino acid segments (Poruchynsky *et al.*, 1985; Paabo *et al.*, 1987). Finally the rate of bulk flow (i.e. unselected) movement from the ER to the cell surface is sufficiently rapid to account for the movement of all the secretory proteins for which measurements are available (Wieland *et al.*, 1987). Recently direct evidence for the existence of an ER retention signal was provided by Munro and Pelham (1986, 1987) who identified the C-terminal sequence Lys-Asp-Glu-Leu (KDEL) of the 78-kd glucose-regulated protein GRP78 [otherwise known as the immunoglobulin heavy chain binding protein, BiP (Haas and Wabl, 1983)] as responsible for its ER localization. Significantly this sequence also occurs in the same position in two other luminal proteins, the glucose regulated 94-kd protein (Munro and Pelham, 1987) and protein disulphide isomerase (Edman *et al.*, 1985). One explanation for the retention of all these proteins is that the sequence KDEL is recognized and bound by a membrane-associated receptor which is itself exclusive to the ER membrane.

Previously we have shown that the synthesis and subsequent translocation of secretory proteins can be spatially distinguished within large (> 1.2 mm diameter) *Xenopus* oocytes (Drummond *et al.*, 1985a). This is achieved by first radiolabelling oocytes for short periods after mRNA injection; by this means the production of newly synthesized proteins of interest can be restricted to the injection zone. The movement of these proteins away from the site of synthesis can then be assessed by biochemically analysing sections of such oocytes and monitoring protein migration as a function of time after synthesis. In this way, we have been able to examine the lateral diffusion of secretory and cytosolic proteins within the oocyte. We have now utilized this approach to observe the diffusional behavior of GRP78 within oocytes. We find that rat GRP78, synthesized in localized regions of the *Xenopus* oocyte ER, diffuses laterally throughout the oocyte although it is not secreted. Its intracellular behaviour is similar to that of a truncated GRP78 lacking the retention signal and to that of chick ovalbumin, both secretory proteins, but different from that of two ER membrane proteins, rotavirus VP10 and a mutant influenza haemagglutinin, where significant lateral movement was not detected. We conclude that binding within the ER to a membrane receptor is not an explanation for GRP78 retention.

Results

Compartmentation of GRP78 within oocytes

Xenopus oocytes have demonstrated great fidelity in the targeting of foreign proteins introduced either by direct microinjection or via RNA or DNA injections (Colman, 1984a,b). We first wished to establish that this fidelity extended to GRP78 processing. Oocytes were microinjected with synthetic GRP78 mRNA and radiolabelled for 24 h with [^{35}S]methionine. Incubation media, oocyte membrane and cytosol fractions were then immunoprecipitated for electrophoresis. It is clear from Figure 1a that GRP78 was localized to the oocyte membrane fraction and was not secreted; in contrast chicken ovalbumin was efficiently secreted as has been previously shown (Colman *et al.*, 1981).

The specificity of GRP78 retention in the oocyte was further substantiated by the demonstration in Figure 1b that a C-terminally truncated GRP78 protein (GRP78$_t$) is

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Fig. 1. Synthesis and compartmentation of GRP78 in oocytes. Oocytes were injected with RNA, incubated in [^{35}S]methionine for 24 h, and immunoprecipitates of incubation media (S), cytosol (C), membranes (M) or total oocyte homogenates (O) were electrophoresed. Each track contains the equivalent of 0.25 (O), 1.0 (C and M) and 5 (S) oocytes. **Panel a**. Coinjection of oviduct and GRP78 mRNAs. The positions in the gels of GRP78 (GRP) and ovalbumin (OV) are indicated. Track W contains a full length GRP78 made in a wheat-germ cell-free translation assay from GRP78 RNA. **Panel b**. Coinjection of GRP and GRP_t RNAs. The positions in the gels of full length (GRP) and truncated (GRP_t) GRP78 are indicated. Two different exposures of the membrane track are included. The control track (i.e. no RNA injection) contains endogenous GRP78 which comigrates with the rat GRP78 but is made at a much lower rate (cf. panel a). Comparison of ovalbumin and GRP_t contents in the membrane and media tracks by band excision and scintillation counting revealed that the proportion of total ovalbumin secreted was at least five times greater than the corresponding value for GRP_t.

Fig. 2. Protein movement after localized translation. **Panel a**. pSP6B, oviduct (OVI) and pSP64VP10 (VP10) mRNAs were injected as indicated into either the animal (Ap) or vegetal (Vp) poles of oocytes and the oocytes immediately labelled for 3 h in 1 mCi/ml [^{35}S]methionine before a 21-h chase (see Materials and methods). Frozen oocytes were bisected and pooled animal (A) and vegetal (V) halves were homogenized and immunoprecipitated with the appropriate antisera before electrophoresis. Each track contains the equivalent of 2.5 oocytes. The positions in the gels of GRP78 (GRP), ovalbumin (OV) and VP10 (VP10) are indicated. The small amount of VP10 found in the 'opposite' half may reflect real movement but could also be generated by inaccuracies in sectioning or mRNA deposition. **Panel b**. pSP64VP10 (VP10) and $pSP64HA_{WT}$ (HA) RNAs or pSP64VP10 and $pSP64HA_{ENV}$ (HA_{ENV}) were coinjected at the vegetal pole of oocytes which were then cultured and processed as in panel a above.

secreted by oocytes even when both wild-type and mutant proteins are co-expressed in the same oocytes. Munro and Pelham (1987) have previously demonstrated the secretion of this protein from transfected monkey COS cells. It is notable (see later) that in comparison with ovalbumin very little truncated GRP78 is secreted (Figure 1b, legend), indicating a slow rate of secretion as previously shown by Munro and Pelham (1986).

Diffusion of GRP78 within oocyte membranes

Both synthetic and natural mRNAs diffuse slowly away from the site of their deposition in large *Xenopus* oocytes (>1.2 mm diameter) and even 6 h after animal or vegetal pole injection no RNA can be detected in the opposite hemisphere (Drummond *et al.*, 1985a,b). However, protein encoded on the injected mRNA moves much more rapidly within the oocyte and we have shown that both cytosolic and secretory proteins of similar size can achieve a near equilibrium distribution within 24 h of polar mRNA injection (Drummond *et al.*, 1985a). In Figure 2a we show an experiment where GRP78 mRNA was coinjected with either bovine rotavirus VP10 mRNA or chick oviduct mRNA at either the animal or vegetal pole of oocytes. The oocytes were immediately radiolabelled for 3 h whereupon further incorporation of label was abolished by addition of excess, unlabelled methionine. The incubation was continued for 21 h and the oocytes were first frozen, and then bisected into animal and vegetal halves. Pooled halves were homogenized and their protein contents analysed. Within the 24-h period ovalbumin appears in both oocyte hemispheres. However, the distribution is skewed to the animal segment irrespective of the injection site, a result which indicates the attainment of an equilibrium distribution. GRP78 also is found in both halves and again vegetal-to-animal movement is greater than animal-to-vegetal although in this case an equilibrium distribution is not attained. In contrast, very little (if any, see Figure 2 legend) VP10 has moved from the injected hemisphere. This result serves as an important control on both conceptual and technical aspects of this experiment since rotavirus VP10 protein has been shown to localize to the ER membrane of cultured mammalian cells, whether it is expressed after viral infection (Kabancell and Atkinson, 1985) or after injection of synthetic VP10 mRNA (Armstrong *et al.*, 1987). Although it could be argued that VP10 is atypical and other membrane proteins (e.g. a putative GRP78 receptor) might diffuse more rapidly in the plane of the ER membrane, we consider this to be unlikely since two other membrane proteins behave similarly; as shown in Figure 2b, both influenza haemagglutinin, a plasma membrane protein, which moves to the oocyte Golgi from the ER with a $T_{1/2}$ of between 3 and 4 h (A.Colman and A.Ceriotti, unpublished), and a mutant haemagglutinin (HA_{ENV}) which never leaves the ER in cultured cells (Gething *et al.*, 1986) or oocytes (A.Ceriotti and A.Colman, unpublished), remain localized to the site of their synthesis.

Compared with the amounts of newly synthesized VP10 and ovalbumin, very little rat GRP78 was detected in the experiment shown in Figure 2. We attribute this to the short labelling period used (to minimize complications from mRNA diffusion) which would not allow efficient translational recruitment of the large GRP78 mRNA (Berridge and Lane, 1976), to the method of labelling (but see Figure 4, legend), and also to inefficient immunoprecipitation with this antiserum (data not shown). Nonetheless, it is clear that in contrast to an ER membrane protein, GRP78 is able to diffuse away from its site of synthesis.

The data in Figure 2 reveal differences in the 24-h distribution of GRP78, ovalbumin and VP10 although the incorporation of label into GRP78 in Figure 2a was very low. In Figure 3 we have sought to confirm this differential movement by monitoring the distribution of each protein as a function of time after the coinjection of all three RNAs into the vegetal pole of the same oocytes. Clearly for both GRP78 and ovalbumin it takes between 6 and 24 h for at least 50% of the protein to enter the opposite half with, again, a bias in the ovalbumin distribution towards the animal half; as expected little movement of VP10 occurred. Unfortunately the low signal to noise ratios in the GRP78 tracks precluded a more quantitative study of GRP movement (but see below).

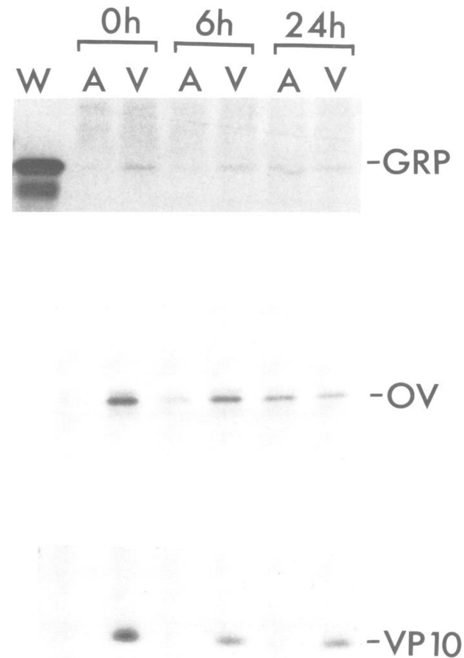

Fig. 3. Time-dependence of protein movement from localized translation sites. pSP6B (GRP78), oviduct and pSP64VP10 (VP10) mRNAs were coinjected into the vegetal pole of oocytes which were then labelled for 3 h as in Figure 2. After the addition of 5 mM methionine, oocytes were frozen at 0, 6 and 24 h. Processing of oocytes was then performed exactly as in Figure 2, except that 60, 20 and 10 µl of homogenate were immunoprecipitated with anti-GRP, anti-VP10 and anti-chick egg white antisera respectively. Abbreviations: GRP, GRP78; OV, ovalbumin; VP10, rotavirus VP10; A, animal half; V, vegetal half; track W contains a full-length GRP78 protein made in the wheat-germ-cell-free translation system from GRP78 RNA.

The KDEL retention signal does not influence GRP78 lateral movement

The above data indicate that GRP78 is able to diffuse away from its site of synthesis. Whilst this movement would appear to exclude a tight, permanent anchoring of the protein to membrane-bound receptors, it might be argued that the observed rate of movement could be satisfied if GRP78 was bound tightly most of the time yet could diffuse extremely rapidly when in the 'off' mode. We have attempted to exclude this unlikely possibility by comparing the lateral movement of wild-type GRP78 with that of the truncated derivative ($GRP78_t$) described earlier (see Figure 1b). If the above scenario regarding GRP78 binding is correct, $GRP78_t$ which lacks the KDEL signal and is secreted, would not be expected to participate in this type of binding and would therefore diffuse more rapidly than wild-type GRP78. We have compared (Figure 4) and measured (Figure 5) the rates of movement of wild-type and truncated GRP78 either in separate oocytes (Figure 4a) or within the same

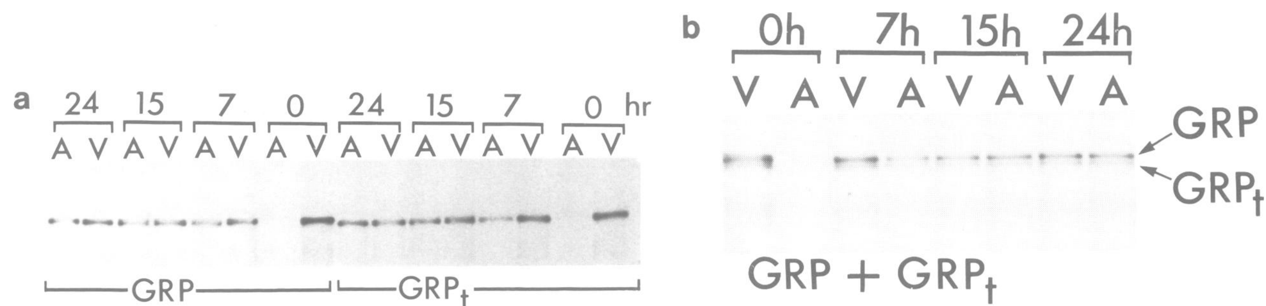

Fig. 4. Wild-type and truncated GRP78 proteins move at similar rates. pSP6B (encoding GRP78) and pSP64GRP_t (encoding truncated GRP78) RNAs were injected either separately along with HA_{ENV} and oviduct RNAs (**panel a**) or together (**panel b**) into the vegetal pole of oocytes; in each case [^{35}S]methionine (at 7.5 mCi/ml) was included in the injection mixture. Oocytes were labelled, chased for various times and processed as in Figure 3 except that protein A−sepharose (200 μl of a 10% suspension) was used during immunoprecipitation using either the anti-GRP, anti-HA or anti-chick egg white antibodies. Only the GRP78 data are shown. GRP, full length GRP78; GRP_t, truncated GRP78; V, vegetal half; A, animal half.

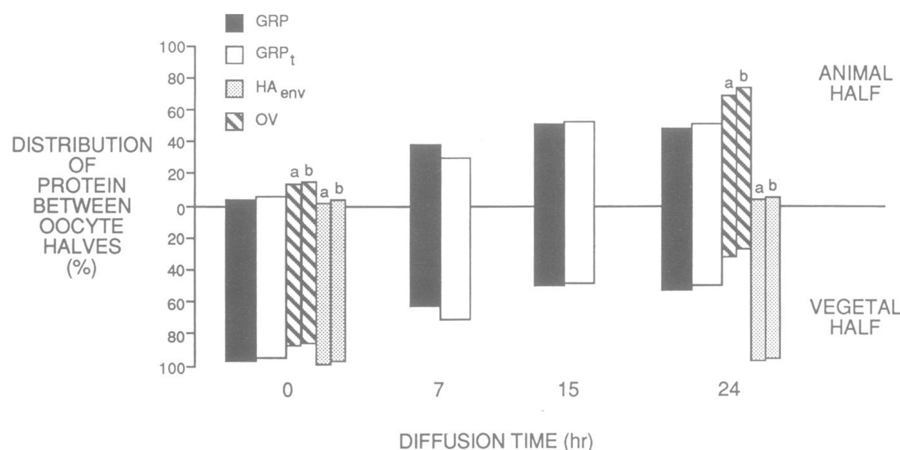

Fig. 5. Quantitation of protein movement in oocytes. Dried-down gel slices containing radioactive GRP78 (GRP) and truncated GRP78 (GRP_t) were excised from the gels shown in Figure 4a and counted in scintillant. Data for ovalbumin (OV) and the mutant haemagglutinin (HA_{ENV}) were obtained in a similar manner after the appropriate immunoprecipitation and electrophoresis (not shown). The superscripts a and b indicate HA_{ENV} and OV precipitated from HA_{ENV}- and OV mRNA-injected oocytes coinjected with GRP or GRP_t RNAs respectively. The figure shows that 50% of GRP has entered the animal half within 15 h. We have estimated that if GRP were bound to a membrane-bound receptor with a diffusion coefficient of 5×10^{-9} cm^2/s [= vertebrate rhodopsin (Poo and Cane, 1974)] it would take over 250 h for this degree of movement to occur and this estimate assumes the ER membrane both to be an unbending conduit aligned along the animal/vegetal axis and also to fill the oocyte entirely; it is therefore clearly a gross underestimate of the time required.

oocyte (Figure 4b); in these same oocytes (Figure 4a), whilst movement of co-expressed ovalbumin was rapid (Figure 5), co-expressed haemagglutinin did not move significantly (Figure 5). Clearly for both forms of GRP78, equilibration within the oocyte is incomplete 7 h after the end of the labelling period but complete within 15 h. Most significantly the rates of movement of the two proteins appear indistinguishable. We conclude that the presence or absence of the KDEL retention signal does not influence movement of GRP78 within oocytes.

Discussion

There is a growing consensus (see Rothman, 1987; Warren, 1987) that no specific sorting signal is required to route secretory proteins from the ER to the cell surface in eukaryotic cells. If true, it follows that resident, soluble proteins of the ER lumen must contain specific retention signals. Recently Munro and Pelham (1986, 1987) identified the C-terminal sequence Lys-Asp-Glu-Leu (KDEL) GRP78 as responsible for its ER localization. However, the retention of GRP78 within the ER lumen remains an enigma. Clearly the autonomy of the retention signal, KDEL, implies that it interacts with a receptor rather than acting indirectly to influence protein conformation. It is difficult to explain the retention of GRP78, a major ER protein, by its stoichiometric attachment to a membrane-bound receptor since no suitably abundant candidate is evident. We might therefore expect GRP78 to be free to move within the ER. Munro and Pelham (1987) have speculated that a membrane-bound receptor exists which shuttles between ER and *cis* Golgi but can bind to GRP78 only in the Golgi. The receptor would then be responsible in a catalytic way for retrieval of GRP78 from the Golgi whilst passive secretion would account for forward movement. If this were true, the ER to Golgi movement of GRP78 would have to be considerably slower than the reverse movement of its receptor in order to avoid receptor saturation and further movement along the secretory pathway. Indeed the low rate of secretion of the KDEL-minus GRP78 (Munro and Pelham, 1987) is consistent with this prediction. But does GRP78 enter the *cis* Golgi? It has been found that the oligosaccharide side chains of GRP94, another luminal ER protein, retain their high mannose configuration (Lewis *et al.*, 1985). This would seem to argue against the shuttling of these ER proteins between ER and Golgi. However, this point is not conclusively

proved since several proteins are known to cross the Golgi without significant mannose trimming (Hsieh *et al.*, 1983; Faye *et al.*, 1986). If GRP78 truly never enters the Golgi, then it is difficult to reconcile this behaviour and free GRP78 movement, without invoking the existence of new compartments either within the ER or between the ER and Golgi (Warren, 1987) where the type of receptor cycling envisaged above by Munro and Pelham (1987) can be encouraged.

In this study lateral movement of membrane and secretory proteins has been equated with movement within the ER cisternae. This assumption is based on knowledge of the normal intracellular phenotype of VP10, HA_{ENV} and GRP78, and the fact that the rate-limiting step in the translocation of many secretory proteins in cultured cells (Fitting and Kabat, 1982; Lodish *et al.*, 1983) and of ovalbumin in oocytes (Colman *et al.*, 1985) is the movement from ER to Golgi, so that most intracellular protein is pre-Golgi. Additionally our previous studies (Drummond *et al.*, 1985a) on chick lysozyme, whose movement from the ER does not appear to be rate limiting for secretion in transfected mammalian cells (Munro and Pelham, 1987; Krieg *et al.*, 1984), or oocytes (Cutler *et al.*, 1981; Krieg *et al.*, 1984) also support our contention that lateral diffusion is occurring in the ER compartment rather than the Golgi; for in this case intracellular lysozyme, most of which is in the Golgi, is confined to the oocyte half into which mRNA was injected. Unfortunately the combination of low resolution of different membraneous compartments using light or electronmicroscopy and the dilution of newly synthesized proteins amongst the oocyte's large protein reserves has prevented a more direct examination of protein localization.

A further assumption, implicit to our main conclusions, is that VP10 and HA_{ENV} are legitimate models for the behaviour of endogenous ER membrane proteins. In the case of VP10 we believe this to be the case since all wild-type viral membrane proteins (like VP10) have behaved appropriately after their individual expression in eukaryotic cells (Garoff, 1985); in the case of HA_{ENV} it is unlikely that ER retention is due to aggregation since, in cultured cells, it is found in the form of non-aggregated trimers (Gething *et al.*, 1986).

Although our observations cannot resolve the mechanism of GRP78 retention, they do present *in vivo* evidence for the free movement of GRP78 within the ER. This conclusion is based on the comparison of normal GRP78 movement with that of its truncated derivative and that of several viral membrane proteins. For a membrane-bound GRP receptor to satisfy the observed trans-oocyte GRP movement it would have to possess a diffusion coefficient considerably greater than that of any membrane protein for which measurements are available (see Figure 5, legend). Previous evidence for the lack of membrane attachment of GRP78 (Bole *et al.*, 1986) and other luminal proteins (Freedman, 1984) is based on experiments involving their release from disrupted microsomes and is subject to the limitations of such *in vitro* experiments.

Finally, what significance can be derived from comparing the rates of movements of proteins within the oocyte ER? It is generally accepted (Pfeffer and Rothman, 1987) that the rate-limiting step in secretion is ER to Golgi movement and that different proteins make this journey at vastly different rates (Fitting and Kabat, 1982; Lodish *et al.*, 1983). It is not clear whether such variation reflects the relative translational movement of different proteins within the ER or constraints on their access to hypothetical 'assembly points' for packaging into transit vesicles. It may prove possible to resolve these alternatives using this type of oocyte assay, but only when comparing soluble proteins whose exit rate from the ER is slow compared to the time necessary to monitor lateral diffusion. These conditions would seem to apply to ovalbumin and $GRP78_t$ and the discrepancy noted between the relative rates of diffusion and rates of secretion of these proteins might indicate that it is packaging and not movement within the ER which is limiting for secretion. Clearly a more comprehensive study needs to be done to confirm the significance of these comparisons. However, this does not alter the main conclusion of this study that GRP78 movement within the ER is unhindered by the close attentions of a membrane-bound receptor.

Materials and methods

Microinjection and processing of oocytes

Oocytes from large females of *Xenopus laevis* were obtained, maintained and microinjected as described by Colman (1984a). For microinjection chick oviduct mRNA, prepared as described by Cutler *et al.* (1981) was dissolved in distilled water at 100 μg/ml whilst *in vitro* transcripts (see below) were injected in transcription mix at 40–100 μg/ml. Injections were performed as follows.

Compartmentation experiments. Following equatorial injection, oocytes were cultured overnight before incubation at 20°C in 1 mCi/ml [^{35}S]methionine (800 Ci/mmol, Amersham, UK) in modified Barths' saline (MBS) at 6 μCi/oocyte for 24 h. Incubation media were then retained for analysis whilst oocytes were fractionated on sucrose step gradients to give cytosol and membrane fractions, or homogenized directly in buffer containing Triton X-100 (Colman, 1984a). Aliquots (100 μl) of homogenates, cytosol, membrane and media samples were diluted with 450 μl immunoprecipitation buffer (Colman, 1984a) before the addition of either 3 μl rabbit anti-rat GRP78 antisera (a gift of H.Pelham, Cambridge, UK) or 1 μl rabbit anti-chick egg white antisera (Cutler *et al.*, 1981). Immunoprecipitations were conducted, and the immunoprecipitates analysed on 12.5% SDS polyacrylamide gels, as described by Colman (1984a); in all cases control experiments showed that all available antigen was immunoprecipitated. Gels were fixed and fluorographed as described by Bonner and Laskey (1974).

Diffusion experiments. Oocytes were injected either at the animal (pigmented) or vegetal pole and labelled immediately as above. In later experiments, [^{35}S]methionine at 7.5 μCi/μl was included in the injection mixture. After 3 h labelling, oocytes were washed in unlabelled media before transfer to media containing 5 mM methionine (Chase) and continued incubation. After various times in chase media, oocytes were placed on used X-ray film, aligned with their longitudinal axis parallel to the plate, drained of excess liquid, and then frozen on a bed of dry ice. After storage at −70°C, oocytes were then bisected along their animal/vegetal equator with a hand-held razor blade. Pooled halves were then homogenized in 40 μl per oocyte half of homogenization buffer and processed as described above, except that in some experiments 5 μl rabbit anti-bovine rotavirus antisera (a gift of M.McCrea, Warwick, UK) or 1 μl rabbit anti-haemagglutinin antisera (a gift of M.-J.Gething, Dallas, USA) were used per 100 μl homogenate.

DNA constructs

$pSP64GRP_t$, a plasmid encoding a C-terminally truncated GRP78 was made by first end-filling the *Hin*dIII–*Sma*I coding fragment of SAG4 DNA (Munro and Pelham, 1987) into the end-filled, phosphatased *Bgl*II site of the plasmid pSP64T (Krieg and Melton, 1984). $pSP64HA_{WT}$, a plasmid containing full-length haemagglutinin (HA) from the A/Japan/305/57 strain of influenza virus (Gething and Sambrook, 1981), was prepared by removing the *Hin*dIII coding fragment from the plasmid pJHH (a gift from M.-J.Gething, Dallas), end-filling and inserting into the *Bgl*II site of pSP64T as described above. $pSP64HA_{ENV}$ is a plasmid containing a mutant HA where the N-terminal hydrophobic signal and first two amino acids of mature HA have been replaced by the signal and six amino acids of the Rous sarcoma virus envelope protein (Gething *et al.*, 1986). It was made by inser-

ting an end-filled *Asp*718−*Bam*HI coding fragment from $pSV_{ENV}HA12$ into pSP64T as described for HA above.

The construction of pSP64VP10 a plasmid containing the complete rotavirus VP10 sequence, and pSP6B, a plasmid encoding full-length GRP78, have been described previously (Munro and Pelham, 1986; Armstrong *et al.*, 1987). pSP6B was a kind gift of H.Pelham.

Preparation of synthetic RNAs

Plasmid DNAs were linearized using *Bam*HI (pSP6B), *Sac*I ($pSP64GRP_t$, $pSP64HA_{WT}$ and $pSP64HA_{ENV}$) and *Eco*RI (pSP64VP10) before transcription by SP6 RNA polymerase (New England Nuclear, Boston, USA) in the presence of the capping dinucleotide G(5')ppp(5')G (Pharmacia, Uppsala, Sweden) and [^{32}P]UTP (Amersham, UK) as described by Krieg and Melton (1984). The transcription mix which usually contained 40−100 μg/ml RNA, was injected without further processing.

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