Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7429–7432, October 1990
Cell Biology

Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels

(factor VIII/von Willebrand factor)

ANDREW J. DORNER*, LOUISE C. WASLEY, AND RANDAL J. KAUFMAN

Genetics Institute, 87 Cambridge Park Drive, Cambridge, MA 02140

Communicated by Harvey Lodish, July 11, 1990

ABSTRACT **Secretory proteins expressed in Chinese hamster ovary (CHO) cells interact to various degrees with glucose-regulated protein 78 (GRP78), a resident protein of the endoplasmic reticulum. von Willebrand factor (vWF) and wild-type tissue plasminogen activator (tPA) are efficiently secreted and exhibit a slight transient association with GRP78. Factor VIII and unglycosylated tPA are inefficiently secreted and display a more stable association with GRP78. We have studied the effect of ATP depletion mediated by carbonyl cyanide 3-chlorophenylhydrazone (CCCP) treatment on GRP78 association and secretion of factor VIII and vWF that are coexpressed in CHO cells. Low concentrations of CCCP in the medium prevented disassociation of factor VIII from GRP78 and blocked its secretion. In the same cells, higher concentrations of CCCP were required to block secretion of vWF. Thus, the block to factor VIII secretion at low CCCP concentrations did not result from a general defect in secretion. Secretion of unglycosylated tPA but not wild-type tPA from CHO cells was also blocked by low concentrations of CCCP. The increased sensitivity to CCCP concentration observed for secretion of factor VIII and unglycosylated tPA compared to wild-type tPA and vWF correlates with their degree of interaction with GRP78. *In vivo*, dissociation from GRP78 may be a primary ATP-dependent step in transport from the endoplasmic reticulum. ATP requirements for secretion of various proteins may vary.**

Some proteins transiting the endoplasmic reticulum (ER) interact with a resident protein, glucose-regulated protein 78 (GRP78), or immunoglobulin binding protein (BiP). Improperly glycosylated, misfolded, incompletely folded, or incompletely assembled proteins are detected in association with GRP78 (1–4). Stable association with GRP78 correlates with retention in the ER and may serve to target secretion-incompetent protein for degradation. For some proteins bypassing stable association by deletion of peptide sequences that interact with GRP78 (5) or by reduction of constitutive GRP78 levels (6) results in increased secretion of proteins that are normally retained in the ER. Other proteins that transit the ER are detected in transient association with GRP78 (7, 8). In this case, interaction with GRP78 may be part of the normal pathway leading to secretion. *In vitro*, dissociation from GRP78 requires ATP (9). ATP is also required for transport of proteins from the ER to the Golgi complex (10). Depletion of intracellular ATP levels by treatment with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation (11), reversibly inhibits transport from the ER (10, 12).

The role of GRP78 association in secretion has been studied in a model system utilizing CHO cells expressing high levels of complex glycoproteins that display various degrees of association with GRP78. The CHO cell line 10A1C6 was derived to coexpress factor VIII and von Willebrand factor (vWF) (7). A significant proportion of factor VIII synthesized in these cells is detected in both transient and stable association with GRP78 whereas a small percentage of vWF is detected in transient association with GRP78 (7). CHO cells have also been derived that express wild-type tissue plasminogen activator (tPA) (13) or a genetically engineered mutant form in which the three utilized N-linked glycosylation sites have been abolished to yield unglycosylated tPA (14). Wild-type tPA expressed in CHO cells exhibits a slight amount of transient association with GRP78 (3). In contrast, a greater proportion of unglycosylated tPA can be detected in both stable and transient association with GRP78 (3, 6).

We have shown (15) that induction of factor VIII and vWF synthesis in CHO cells results in a large induction of GRP78 and a large proportion of newly synthesized factor VIII, but not vWF, fails to be secreted. Such cells induced to synthesize high levels of factor VIII exhibit a dilated ER juxtaposed to many mitochondria (15). The presence of mitochondria in close proximity to the ER suggested that factor VIII secretion may require high levels of ATP. We have examined the importance of ATP availability for the secretion of GRP78-associated proteins, factor VIII, and unglycosylated tPA, compared to GRP78-unassociated proteins, vWF and wild-type tPA, by depleting intracellular ATP through treatment of CHO cells with CCCP. Low concentrations of CCCP inhibited secretion of factor VIII but not vWF coexpressed in the same cells. This inhibition correlated with a block to dissociation of the GRP78–factor VIII complex. Secretion of unglycosylated tPA was also inhibited by low levels of CCCP compared to wild-type tPA. Our results indicate that secretion of GRP78-associated proteins may be more sensitive to perturbation of intracellular ATP levels by CCCP treatment than unassociated proteins. *In vivo* dissociation from GRP78 may be a primary ATP-dependent step in transport from the ER.

MATERIALS AND METHODS

Cell Lines. CHO cells coexpressing factor VIII and vWF (10A1C6), wild-type tPA (clone 5), or unglycosylated tPA (tPA3x) have been described (3, 7, 13, 14).

Radiolabeling and Immunoprecipitation. Cells were labeled with a 15-min pulse of [^{35}S]methionine (Amersham) followed by a chase with unlabeled complete medium as described (16). Conditioned medium was harvested and cell extracts were prepared in Nonidet P-40 lysis buffer (16). To study secretion, equal trichloroacetic acid-precipitable counts of cell extracts and corresponding volumes of conditioned medium were immunoprecipitated with anti-factor VIII mono-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GRP78, glucose-regulated protein 78; vWF, von Willebrand factor; tPA, tissue plasminogen activator; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; ER, endoplasmic reticulum; BiP, immunoglobulin binding protein.

*To whom reprint requests should be addressed.

clonal antibody (Genetics Institute), rabbit anti-vWF antiserum (Behring Diagnostics), or goat anti-tPA antiserum (American Diagnostica, Greenwich, CT). For coprecipitation studies, equal volumes of cell extracts were immunoprecipitated with anti-BiP monoclonal antibody culture supernatant (1), anti-ERp72 rabbit antiserum (17), or anti-ERp99 rabbit antiserum (17). BiP is identical to GRP78 (1, 9). ERp99 is identical to GRP94 (18). Immunoprecipitated protein was visualized on 6% or 10% polyacrylamide gels containing SDS after treatment with EN^{3}HANCE (DuPont). CCCP (Boehringer Mannheim) was prepared in dimethyl sulfoxide and used at the indicated concentrations in the chase medium.

RESULTS

Factor VIII- and vWF-coexpressing cells (10A1C6) were pulse-labeled with [^{35}S]methionine followed by a 9-hr chase in unlabeled medium containing various concentrations of CCCP. Immunoprecipitation of cell extracts with an anti-factor VIII antibody detected the 300-kDa factor VIII precursor protein and a 78-kDa protein corresponding to GRP78 (Fig. 1, lanes 1–4). Immunoprecipitation of conditioned medium from untreated cells revealed the heterogenous 200-kDa heavy chain and 80-kDa light chain of factor VIII (Fig. 1, lane 5). Factor VIII was not detected in the conditioned medium of cells treated with 50 μM or greater CCCP (Fig. 1, lanes 6–8). In further experiments, secretion of factor VIII was inhibited by CCCP concentrations as low as 10 μM (data not shown). Immunoprecipitation of the same cell extracts with anti-vWF antiserum detected the 330-kDa vWF precursor protein (Fig. 1, lanes 9–12). Coprecipitation of GRP78 was not detected. Immunoprecipitation of vWF in the conditioned medium showed that higher concentrations (100 μM or more) of CCCP were required to markedly reduce secretion (Fig. 1, lanes 13–16). Quantitation of the level of secretion of vWF and factor VIII is presented in Table 1. Similar results were obtained for vWF when the pulse-labeling period was reduced to 5 min (data not shown). Thus, in the same cells,

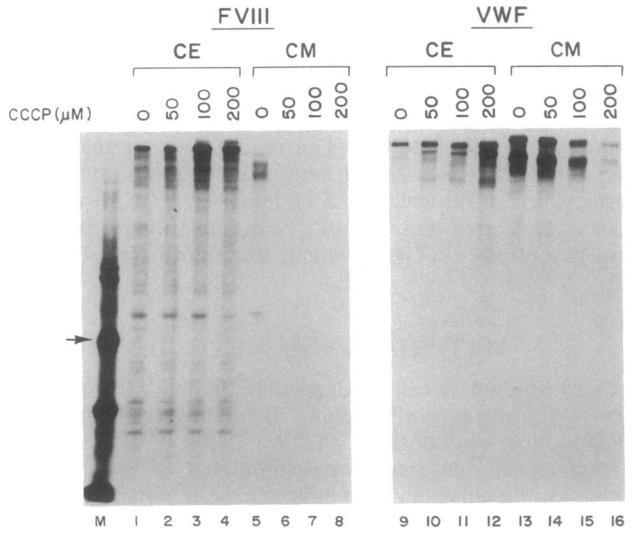

FIG. 1. Effect of CCCP concentration on secretion of factor VIII (FVIII) and vWF. 10A1C6 cells coexpressing factor VIII and vWF were labeled with a 15-min pulse of [^{35}S]methionine followed by a 9-hr chase with unlabeled complete medium containing CCCP, as indicated. Autoradiogram of a 6% polyacrylamide gel containing SDS. Lanes: 1–4, cell extracts (CE); 5–8, conditioned medium (CM) immunoprecipitated with anti-factor VIII monoclonal antibody; 9–12, cell extracts (CE); 13–16, conditioned medium (CM) immunoprecipitated with anti-vWF antiserum; M, molecular mass markers, in kDa, 200, 100, 92, 69, 46, and 30. Position of 69-kDa marker is indicated by arrow.

Table 1. Inhibition of secretion by CCCP

Protein	Protein in conditioned medium, % of control			
	0	50	100	200
Factor VIII	100	4	1	ND
vWF	100	100	33	9
wt tPA	100	79	37	0.5
tPA3x	100	0.8	0.8	0.8

Amount of secreted protein was determined by densitometer scan of appropriate exposures of the autoradiograms of immunoprecipitated conditioned medium shown in Figs. 1 and 3. The amount of the specific protein in medium without CCCP (control) was assigned the value of 100%. The amount of secreted protein in the presence of CCCP at 50, 100, or 200 μM was determined as a proportion of this number. For factor VIII, the level of heavy chain was quantitated. For vWF the levels of mature and pro-vWF in conditioned medium were combined. wt tPA, wild-type tPA; tPA3x, unglycosylated tPA produced from the tPA3x line; ND, not detectable.

secretion of factor VIII and vWF displays different sensitivities to inhibition by CCCP. The finding that secretion of factor VIII but not vWF was inhibited at low CCCP concentration precludes a general disruption of secretion.

The state of association of factor VIII and vWF with GRP78 in CCCP-treated cells was studied by coprecipitation with an anti-BiP antibody (1). 10A1C6 cells were pulse-labeled followed by a 7-hr chase in the presence or absence of 50 μM CCCP. Immunoprecipitation of cell extracts at the pulse time point with anti-vWF antiserum (Fig. 2, lane 1) or anti-factor VIII antibody (Fig. 2, lane 2) detected the appropriate precursor proteins. Immunoprecipitation with anti-BiP antibody detected GRP78 and coprecipitated the majority of factor VIII but only a small proportion of vWF (Fig. 2, lane 3). The specificity of these associations was demonstrated by

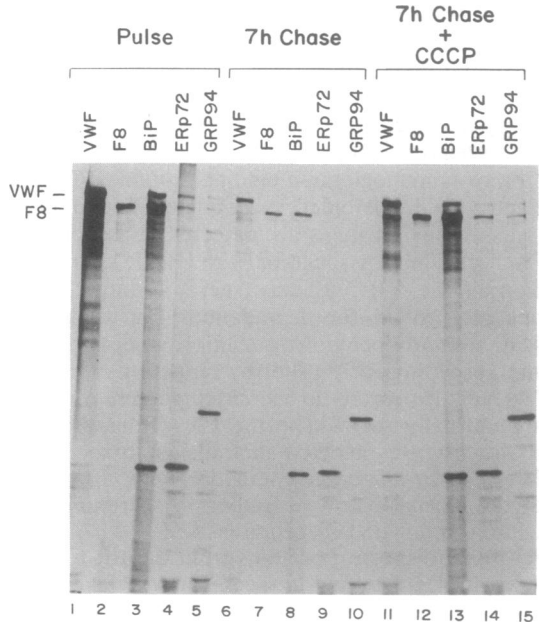

FIG. 2. Association of factor VIII and vWF with GRP78 in CCCP-treated cells. 10A1C6 cells were labeled with a 15-min pulse of [^{35}S]methionine followed by a 7-hr chase with unlabeled complete medium in the presence or absence of 50 μM CCCP. Autoradiogram of a 6% polyacrylamide gel containing SDS. Lanes: 1–4, immunoprecipitated cell extracts after pulse label; 6–10, immunoprecipitated cell extracts after pulse-label and 7-hr chase in unlabeled complete medium; 11–15, immunoprecipitated cell extracts after pulse-label and 7-hr chase in unlabeled complete medium containing 50 μM CCCP. Specificity of antibody is indicated at the top of the lane.

immunoprecipitation with antibodies that recognize two other resident ER proteins, ERp72 and GRP94 (18, 19). Immunoprecipitation with antiserum directed against ERp72 (Fig. 2, lane 4) or GRP94 (Fig. 2, lane 5) detected ERp72 or GRP94 but negligible amounts of vWF or factor VIII were coprecipitated. After a 7-hr chase, most vWF labeled during the pulse disappeared from the cell and little association with GRP78 was detected (Fig. 2, lanes 6 and 8). At the same time point, the majority of the factor VIII also disappeared from the cell. The remaining factor VIII was associated with GRP78 (Fig. 2, lanes 7 and 8). No coprecipitation of vWF or factor VIII with ERp72 or GRP94 was observed (Fig. 2, lanes 9 and 10).

After a 7-hr chase in the presence of 50 μM CCCP, a concentration that effectively blocks factor VIII secretion, the amount of factor VIII remaining in the cell and the extent of association with GRP78 was the same as that observed at the pulse point (Fig. 2, lanes 12 and 13). Thus, inhibition of secretion correlated with retention of factor VIII in association with GRP78. The majority of vWF was not associated with GRP78 and was secreted in 50 μM CCCP. However, the small amount of vWF associated with GRP78 at the pulse time point remained associated in the presence of CCCP (Fig. 2, lanes 11 and 13). Although the amount of intracellular vWF was greatly reduced compared to the pulse time point, a larger percentage was detected compared to untreated cells. Further time course experiments utilizing a shorter chase time point of only 4 hr revealed a reduced level of secreted vWF in the presence of 50 μM CCCP compared to untreated cells (data not shown). However, by 7 hr an amount of vWF comparable to untreated cells was secreted in those experiments. Between 1 hr and 3 hr has been shown to be the earliest time point and between 7 hr and 10 hr has been shown to be the latest time point of vWF secretion from 10A1C6 cells (7). Longer chase times did not result in secretion of factor VIII in the presence of CCCP (data not shown). These results indicate that the rate of secretion of vWF may be slower in the presence of 50 μM CCCP but secretion is not blocked as is secretion of factor VIII. Little vWF or factor VIII coprecipitates with ERp72 or GRP94, indicating that the extended association with GRP78 is not due to nonspecific aggregation with ER proteins (Fig. 2, lanes 14 and 15). Quantitation of the levels of intracellular factor VIII and vWF is presented in Table 2.

To extend our observations beyond the factor VIII/vWF system the effect of CCCP treatment on the secretion of wild-type or unglycosylated tPA was examined. Clone 5 cells expressing wild-type tPA were pulse-labeled for 15 min with [^{35}S]methionine followed by a 4-hr chase in the presence of various concentrations of CCCP. Immunoprecipitation of cell extracts with anti-tPA antibody detected the wild-type tPA migrating at 68 kDa that accumulated intracellularly with increasing CCCP concentration (Fig. 3*A*, lanes 1–4). Immunoprecipitation of conditioned medium revealed that CCCP concentrations of 100 μM and 200 μM blocked tPA secretion (Fig. 3*A*, lanes 5–8). This concentration range was similar to that observed for vWF. tPA3x cells expressing unglycosylated tPA were pulse-labeled for 15 min with [^{35}S]methionine followed by a 6-hr chase with various concentrations of CCCP. Immunoprecipitation of cell extracts with anti-tPA antibody detected unglycosylated tPA migrating at 60 kDa (Fig. 3*B*, lanes 1–3). Immunoprecipitation of conditioned medium revealed that secretion of unglycosylated tPA was blocked at 50 μM CCCP (Fig. 3*B*, lanes 4–6). Quantitation of the level of secretion of wild-type tPA and unglycosylated tPA is presented in Table 1. Inhibition of secretion at 50 μM CCCP was also observed when unglycosylated tPA was generated by tunicamycin treatment of clone 5 cells expressing wild-type tPA (data not shown). Thus, these results do not reflect differences between clone 5 and tPA3x cells but rather structural differences between wild-type and unglycosylated tPA. Secretion of unglycosylated tPA that associates with GRP78 is blocked at low CCCP concentration compared to wild-type tPA that is not detected in significant association with GRP78.

Table 2. Amount of protein detected in cell extracts by immunoprecipitation

Antibody specificity	vWF, % of control			Factor VIII, % of control		
	A	B	C	A	B	C
vWF	100	8	27	ND	ND	60
Factor VIII	ND	ND	ND	100	28	100
GRP78	12	ND	5	>100	28	>100

Protein levels were determined by densitometric quantitation of band intensities from an appropriate exposure of the autoradiogram shown in Fig. 2. The amount of vWF immunoprecipitated with anti-vWF antiserum or factor VIII immunoprecipitated with anti-factor VIII monoclonal antibody at the pulse time point was assigned a value of 100%. All other values were determined by dividing by the pulse time point value. ND, not detectable. A, pulse time point; B, chase time point without CCCP; C, chase time point with 50 μM CCCP.

DISCUSSION

In this study we have examined the effect of ATP depletion mediated by CCCP treatment on secretion and GRP78 association. Our results show that in the same cells secretion of factor VIII, which displays a high degree of association with GRP78, is inhibited at a lower concentration of CCCP than secretion of vWF, which displays little association with GRP78. In the presence of low CCCP concentration, factor VIII did not dissociate from GRP78 and was not secreted. Under the same conditions in the same cells, a small proportion of vWF that can be detected in association with

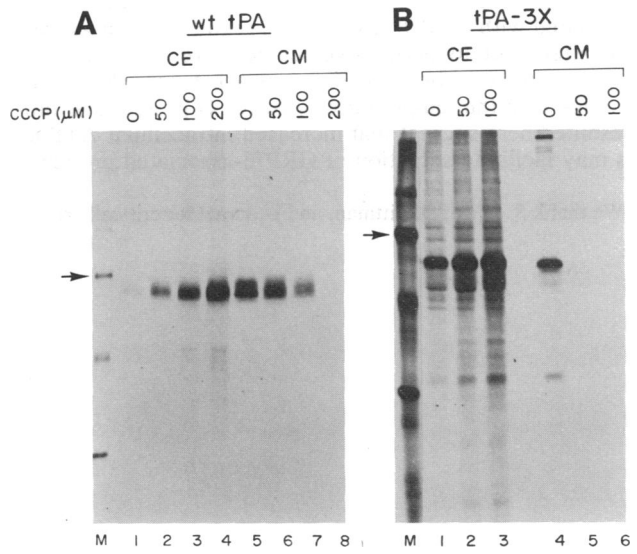

FIG. 3. Effect of CCCP concentration on secretion of wild-type and unglycosylated tPA. Cells were labeled with a 15-min pulse of [^{35}S]methionine followed by a 4-hr chase for wild-type (wt) tPA or a 6-hr chase for unglycosylated tPA (tPA-3x). Autoradiogram of a 10% polyacrylamide gel containing SDS. (*A*) Lanes: 1–4, cell extracts (CE); 5–8, conditioned medium (CM) of wild-type tPA-producing cells immunoprecipitated with anti-tPA antibody. (*B*) Lanes: 1–3, cell extracts (CE); 4–6, conditioned medium (CM) of unglycosylated-tPA-producing cells immunoprecipitated with anti-tPA antibody; M, molecular mass markers, in kDa, 92, 69, 46, and 30. Position of the 69-kDa marker is indicated by arrow.

GRP78 also fails to dissociate, whereas the majority of unassociated vWF is secreted. This suggests that the CCCP-induced block to dissociation is not unique to factor VIII but may be characteristic of GRP78-associated proteins. Previous *in vitro* studies have shown that dissociation of proteins and peptides from GRP78 requires ATP (9, 20). Since CCCP acts to uncouple oxidative phosphorylation resulting in depletion of ATP levels (11), our results suggest that dissociation from GRP78 *in vivo* may also require ATP. However, effects of CCCP treatment other than ATP depletion cannot be ruled out. ATP-dependent release from GRP78 association may be a necessary step in transport out of the ER for associated proteins.

Since secretion of factor VIII and unglycosylated tPA was blocked at a lower CCCP concentration than was secretion of vWF and wild-type tPA, we suggest that secretion of GRP78-associated proteins requires higher ATP levels in the ER compared to unassociated proteins. However, the presence of ATP in the ER has yet to be confirmed. Dissociation from GRP78 may be more sensitive to ATP levels than other steps in the secretory process. Alternatively, GRP78-associated proteins may have increased ATP requirements for correct folding. The use of CCCP to stabilize GRP78 complexes with proteins transiting the ER should facilitate studies on the role GRP78 plays in protein folding and transport. The inhibition of secretion of GRP78-unassociated proteins observed at higher concentrations of CCCP likely occurs at a later step in the secretory pathway such as formation or targeting of transport vesicles (21). Our results do not exclude the possibility that all proteins associate with GRP78 to some degree and require ATP for release. Some proteins such as vWF may only very transiently associate with GRP78 and are not detected at this step.

Consistent with our observations of differential sensitivity to CCCP concentration, transport of a temperature-sensitive vesicular stomatitis virus G protein from the ER requires higher intracellular ATP levels than wild-type G protein (22, 23). At nonpermissive temperature, temperature-sensitive G protein is found as a disulfide-bonded aggregate that is associated with GRP78 (24). ATP is required for disaggregation after shift to permissive temperature (22). This ATP requirement may be related to release from GRP78. The findings with the temperature-sensitive G protein and those presented here suggest that increased intracellular ATP levels may facilitate secretion of GRP78-associated proteins.

We thank R. Wise, D. Pittman, and D. Israel for critically reading the manuscript. We acknowledge D. Bole for providing anti-BiP antibody and M. Green for providing anti-ERp72 and anti-ERp99 antiserum.

1. Bole, D. G., Hendershot, L. M. & Kearney, J. F. (1986) *J. Cell Biol.* **67**, 852–862.
2. Gething, M. J., McCammon, K. & Sambrook, J. (1986) *Cell* **46**, 939–950.
3. Dorner, A. J., Bole, D. G. & Kaufman, R. J. (1987) *J. Cell Biol.* **105**, 2665–2674.
4. Hurtley, S. M., Bole, D. G., Hoover-Litty, H., Helenius, A. & Copeland, C. S. (1989) *J. Cell Biol.* **108**, 2117–2126.
5. Hendershot, L., Bole, D., Kohler, G. & Kearney, J. F. (1987) *J. Cell Biol.* **104**, 761–767.
6. Dorner, A. J., Krane, M. G. & Kaufman, R. J. (1988) *Mol. Cell. Biol.* **8**, 4063–4070.
7. Kaufman, R. J., Wasley, L. C., Davies, M. V., Wise, R. J., Israel, D. I. & Dorner, A. J. (1989) *Mol. Cell. Biol.* **9**, 1233–1242.
8. Ng, D. T. W., Randall, R. E. & Lamb, R. A. (1989) *J. Cell Biol.* **109**, 3273–3289.
9. Munro, S. & Pelham, H. R. B. (1986) *Cell* **46**, 291–300.
10. Jamieson, J. D. & Palade, G. E. (1968) *J. Cell Biol.* **39**, 589–603.
11. Heytler, P. G. (1963) *Biochemistry* **2**, 357–361.
12. Argon, Y., Burkhardt, J. K., Leeds, J. M. & Milstein, C. (1989) *J. Immunol.* **142**, 554–561.
13. Kaufman, R. J., Wasley, L. C., Spiliotes, A. J., Gossels, S. D., Latt, S. A., Larsen, G. R. & Kay, R. M. (1985) *Mol. Cell. Biol.* **5**, 1750–1759.
14. Hansen, L., Blue, Y., Barone, K., Collen, D. & Larsen, G. R. (1988) *J. Biol. Chem.* **263**, 15713–15719.
15. Dorner, A. J., Wasley, L. C. & Kaufman, R. J. (1989) *J. Biol. Chem.* **264**, 20602–20607.
16. Dorner, A. J. & Kaufman, R. J. (1990) *Methods Enzymol.* **185**, 577–596.
17. Lewis, M. J., Mazzarella, R. A. & Green, M. (1985) *J. Biol. Chem.* **260**, 3050–3057.
18. Mazzarella, R. A. & Green, M. (1987) *J. Biol. Chem.* **262**, 8875–8883.
19. Mazzarella, R. A., Srinivasan, M., Haugejorden, S. M. & Green, M. (1990) *J. Biol. Chem.* **265**, 1094–1101.
20. Flynn, G. C., Chappell, T. G. & Rothman, J. E. (1989) *Science* **245**, 385–390.
21. Balch, W. E. & Keller, D. S. (1986) *J. Biol. Chem.* **261**, 14690–14696.
22. Balch, W. E., Elliott, M. M. & Keller, D. S. (1986) *J. Biol. Chem.* **261**, 14681–14689.
23. Doms, R. W., Keller, D. S. & Balch, W. E. (1987) *J. Cell Biol.* **105**, 1957–1969.
24. Hurtley, S. M. & Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5**, 277–307.