Volume 326, number 1,2,3, 241–245 FEBS 12696 July 1993


# Anti-(p34 protein) antibodies inhibit ribosome binding to and protein translocation across the rough microsomal membrane

Tohru Ichimura[a], Yukiko Shindo[b], Yasuyo Uda[b], Tomoya Ohsumi[a], Saburo Omata[b] and Hiroshi Sugano[a,*]

[a]*Department of Biosystem Science, Graduate School of Science and Technology* and [b]*Department of Biochemistry, Faculty of Science, Niigata University, 2-Igarashi, Niigata 950-21, Japan*

Received 12 April 1993; revised version received 24 May 1993

The p34 protein is a non-glycosylated, integral membrane protein characteristic of rough microsomes and is believed to play a role in the ribosome-membrane association. Here, antibodies directed against p34 were examined as to their inhibitory effect on ribosome binding to and protein translocation across the microsomal membrane. Preincubation of the stripped (ribosome-depleted) membrane with anti-p34 immunoglobulins (IgGs) or their Fab fragments led to more than 80% inhibition of the binding of ribosomes and their large (60S) subunit to the membrane. The inhibition was dependent on the amount of antibodies used, but comparable amounts of IgGs and Fab fragments from nonimmune serum had less effect. The p34 antibodies were also inhibitory for cotranslational translocation of secretory proteins, i.e. placental lactogen and serum albumin, across the membrane. These results suggest that p34 is involved in the binding of ribosomes to the microsomal membrane and that it is in close proximity to the protein translocation site in the microsomal membrane.

Rough microsome; Ribosome-binding protein; Ribosome; Protein translocation; Rat; Liver

## 1. INTRODUCTION

The membranes of rough microsomes (RM) contain specific sites for binding with ribosomes synthesizing secretory or membrane proteins. These binding sites are postulated to be functionally associated with a putative protein translocation channel in the membrane which facilitates the import of the synthesized proteins into the lumen of RM [1,2]. In vitro studies have revealed that the binding sites of RM comprise a protein(s) which is highly sensitive to trypsin [3–6]. The binding of ribosomes to the trypsin-sensitive protein was shown to be salt-labile [3–6]. Recently, we isolated from rat liver RM a non-glycosylated, membrane protein with a relative molecular weight of 34 kDa (which we termed p34), and showed that it accounts for the majority of the ribosome-binding activity in the membrane protein fraction which is recovered with ribosomes after solubilization of RM membranes with certain non-ionic detergents [7,8]. The binding properties of this protein (e.g. trypsin- and salt-sensitivity) were also found to be similar to those of intact RM [8]. The p34 protein is localized specifically in ribosome-attached organelles (RM and nuclear envelope), and homologs of it are distributed widely among mammalian tissues [8]. However, although these data suggested the role of the p34 protein in the ribosome–membrane association, the physiological significance of this ribosome-binding protein has not yet been established.

In this paper, evidence is presented that p34 is closely associated with the ribosome-binding/protein translocation site in the RM membrane. It was found that incubation of the microsomal membrane with p34 antibodies abolished the capacity of the membrane for both ribosome binding and secretory protein translocation.

*Correspondence address:* T. Ichimura, Department of Biosystem Science, Graduate School of Science and Technology, Niigata University, 2-Igarashi, Niigata 950-21, Japan. Fax: (81) (25) 262-1175.

**Present address:* Keiwa College, Shibata-Tomizuka, Niigata 957, Japan.

*Abbreviations:* RM, rough microsomes; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; hPL, human placental lactogen; RSA, rat serum albumin.

## 2. EXPERIMENTAL

### 2.1. *Preparation and characterization of antibodies*

Rabbits (3 kg) were injected three times with ~100 μg of p34 protein (in polyacrylamide gel) in Freund's adjuvant as described [8]. An immunoglobulin fraction was obtained from the serum by precipitation with ammonium sulfate. Fab fragments were prepared from IgG using an Fab preparation kit from Pierce. The specificity of the antibodies was confirmed by immunoblot analysis as described [8].

### 2.2. *Inhibition of ribosome binding by antibodies*

Rat liver RM were treated with 0.5 mM puromycin/0.5 M KCl as described [4,9]. The stripped microsomes possessed a number of binding sites, 65 nmol/g membrane proteins, as determined by the method of Yoshida et al. [9]. The stripped microsomes (20 μg protein) were

incubated with the indicated amounts of antibodies (see the legends to Figs. 1 and 2) for 5 h at 4°C in 100 μl of TKM buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$) containing a mixture of protease inhibitors (50 U/ml trasylol, and 0.5 mg/ml each of pepstatin, chymostatin, antipain and leupeptin). The process of antibody binding to the microsomal membrane was saturable under these conditions (not shown). $^3$H-Labeled ribosomes (1.4 μg RNA) or their 60S subunit (1 μg RNA) were then added, and incubation was continued for 10 min at 4°C. The samples were each layered on a sucrose gradient (a linear 0.5 ~1 M sucrose gradient in TKM buffer (4.1 ml) on top of 0.8 ml of 2 M sucrose in the same buffer), followed by centrifugation for 50 min at 100,000 × g. The gradients were collected as 15 equal fractions and a pellet fraction. Radioactivity was determined with an Aloka LSC-903 scintillation counter (Aloka Co. Ltd., Tokyo).

### 2.3. *Inhibition of translocation by antibodies*

For each assay, IgG fractions and monovalent Fab fragments were all prepared in PBS (10 mM phosphate, 145 mM NaCl, pH 7.2). The antibody preparation was mixed with a mixture of protease inhibitors (see above) in a total volume of 100 μl and then incubated at 22°C for 15 min. Rat liver rough microsomes (20 μg protein), which had previously been stripped with sodium pyrophosphate [10], were then added, and incubation was continued with gentle shaking for 30 min at 22°C, followed by 30 min at 4°C. The microsomes were recovered by centrifugation for 30 min at 4°C in a microfuge, rinsed once with PBS, and then resuspended in 20 μl of 50 mM HEPES (pH 7.5) and 250 mM sucrose. In vitro translation/translocation reactions contained, in 25 μl: 10 μl membrane suspension, 10 μl rabbit reticulocyte lysate (Amersham Corp., code N.90Y), 0.25 μg messenger RNA, 5 μCi [$^{35}$S]methionine (Amersham Corp.), 60 mM KCl, 1.2 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 600 U/ml placental RNase inhibitor and the above protease inhibitors. After 60 min at 30°C, the reactions were transferred to ice, and aliquots (12.5 μl) were treated with 50 μg/ml each of trypsin and chymotrypsin for 60 min at 0°C. To stop proteolysis, 1,500 U/ml Trasylol was added for 15 min at 0°C. Proteins were precipitated with cold acetone (−20°C), and analyzed by SDS/ 12% polyacrylamide gel electrophoresis and subsequent fluorography [11].

### 2.4. *Others*

$^3$H-Labeled ribosomes and their 60S subunit were prepared as described [7,9]. The specific activities of the preparations were 1902 and 1659 dpm/μg RNA, respectively. Messenger RNAs for hPL and RSA were isolated from human term placenta and rat liver, respectively [11]. Protein concentrations were determined by the method of Lowry et al. [12], with bovine serum albumin as a standard. The concentrations of RNA were determined using $A_{1\,cm}^{1\%} = 265$ at 260 nm [13]. The concentrations of ribosomes and the 60S subunit were calculated by assuming that they contain 52% RNA, and that $M_r$ is $4.5 \times 10^6$ for ribosomes and $3.0 \times 10^6$ for the 60S subunit [14].

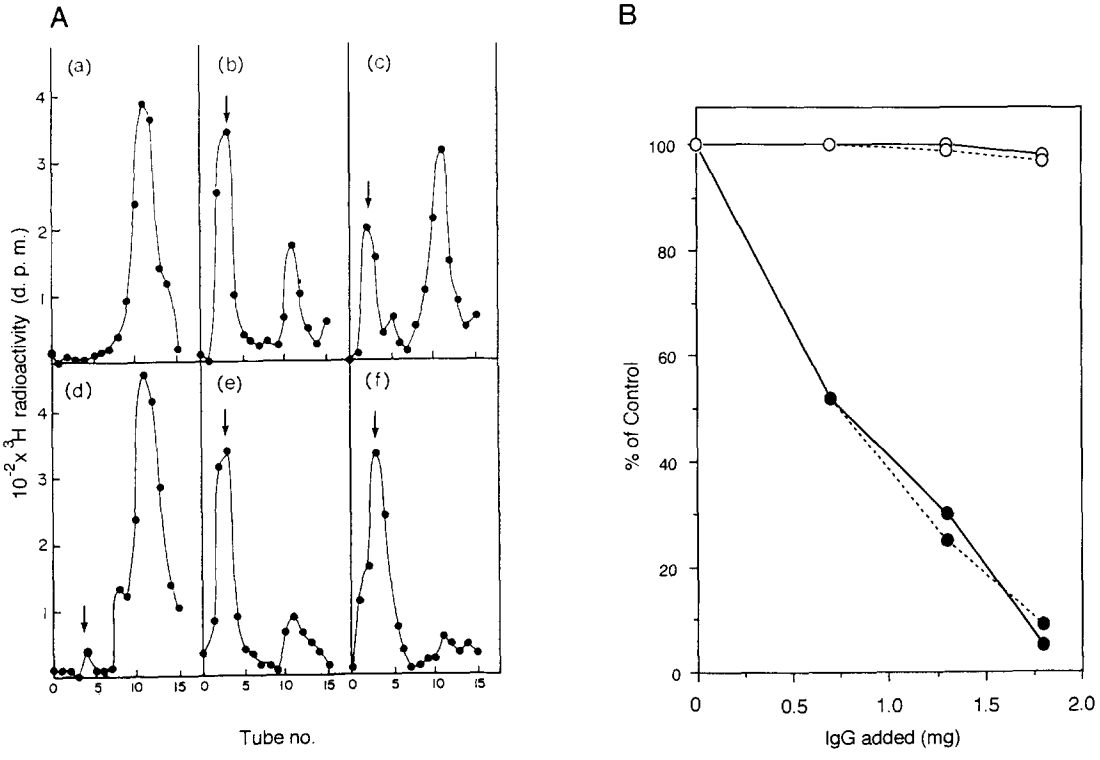


Fig. 1. Antibodies against the p34 protein inhibit ribosome binding to the stripped RM membrane. (A) Each binding mixture, comprising stripped RM (20 μg protein) that had been incubated in TKM buffer with p34-IgG (0.7 (c) and 1.8 (d) mg) or n-IgG (0.7 (e) and 1.8 (f) mg), and 1.4 μg of RNA of $^3$H-labeled ribosomes in 100 μl of TKM buffer, was centrifuged through a sucrose gradient (100,000 × g, 50 min), and then the resulting distribution of ribosomes was determined. As controls, the sedimentation of ribosomes in the absence (a) and presence (b) of stripped membranes is also shown. The direction of sedimentation was from right to left. Arrows indicate the position of the complex between the membrane and ribosomes. The recovery of total radioactivity was 68–83%. (B) Stripped RM (20 μg protein) were preincubated with various amounts of p34-IgG or n-IgG (0.7, 1.3 and 1.8 mg), and then analyzed as to the binding activity toward ribosomes or the 60S subunit as in (A). The binding activity was calculated from the ratio of the radioactivity of ribosomes (solid line) or the 60S subunit (dotted line) bound to the membrane against the total radioactivity recovered, and expressed as a percentage of the binding activity observed without antibody treatment closed circle, p34-IgG; open circle, n-IgG.

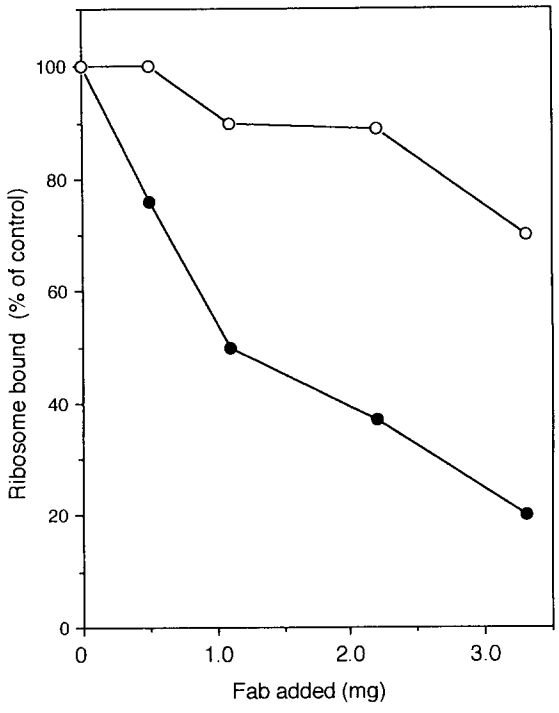


Fig. 2. Monovalent Fab fragments of p34-IgG inhibit ribosome re-binding. Stripped RM (20 $\mu$g protein) were preincubated with various amounts of Fab fragments of p34-IgG (closed circle) or n-IgG (open circle) (0.5, 1.1, 2.2 and 3.3 mg), and then analyzed as to their ribosome-binding activity as in Fig. 1.

## 3. RESULTS

Previous studies revealed that the stripped microsomal membrane possesses the capacity of re-binding an equivalent amount of ribosomes to that on the intact RM membrane [4,15,16]. Therefore, we first examined the effect of p34 antibodies on this process of rebinding of ribosomes to the stripped membrane. The stripped membrane was preincubated with IgGs produced from either antiserum or non-immune serum (termed p34-IgGs and n-IgGs, respectively), and then their ribosome-binding capacity was determined using $^3$H-labeled ribosomes. As shown in Fig. 1A(c and d), incubation with p34-IgGs decreased the capacity of stripped membrane to rebind ribosomes. On the other hand, incubation with n-IgGs had no effect (Fig. 1A(e and f)). The inhibitory effect observed was dependent on the amount of antibodies used, and more than 90% of the capacity to bind ribosomes was lost (Fig. 1B, solid line). Preincubation with p34-IgG was also inhibitory toward the binding of the large (60S) ribosomal subunit, which is an essential subunit for ribosome attachment to the membrane. The observed inhibition was very similar to in the case of ribosomes (Fig. 1B, dotted line).

We also examined the effect of monovalent Fab fragments to determine whether the observed inhibition was caused by cross-linking to p34 proteins on the stripped membrane. The Fab fragments produced from the p34-IgG fraction caused, like in the case of p34-IgGs, marked inhibition of ribosome re-binding (Fig. 2). Thus, the inhibition does not seem to be the consequence of cross-linking of p34 proteins through divalent antibodies. The Fab fragments from the n-IgG fraction were also inhibitory (up to 30%) with the use of 3.3 mg protein, but the effect was much lower than in the case of anti-p34 Fab fragments (Fig. 2).

The ribosome-binding site is postulated to be functionally associated with the site of translocation. We assumed, therefore, that, if the effect of p34 antibodies is physiologically important, the antibodies might inhibit the capacity of RM membranes to translocate secretory proteins. To test this assumption, a translocation assay was performed (Fig. 3). As expected, preincubation of the microsomal membrane with p34-IgGs caused inhibition of the translocation of human placental lactogen (hPL) (Fig. 3A, lanes 3 and 4, and 3′ and 4′). On the other hand, under the same conditions comparable amounts of n-IgG had less effect (lanes 5 and 6, and 5′ and 6′). The p34-IgGs also inhibited the translocation of another secretory protein, rat serum albumin (Fig. 3B, lanes 2 and 2′). The translocation capacity of the microsomal membrane was unimpaired, however, when a translocation assay was performed using microsomes preincubated with p34-IgG fractions from which antibodies had been removed by adsorption to protein A-Sepharose. This suggests that the inhibition was indeed caused by the antibodies and not by a contaminating agent in the immunoglobulin fraction (data not shown). Fig. 3C (lanes 3 and 4, and 3′ and 4′) demonstrates that the Fab fragments from p34-IgGs also inhibited the translocation of hPL. Thus, the inhibition is essentially due to the binding of the antibodies to the p34 protein.

## 4. DISCUSSION

The present study demonstrates the antibody-mediated inhibition of ribosome binding to and protein translocation across the microsomal membrane. Since p34 constitutes 1 ± 0.2% of the total protein in the RM membrane, as determined by quantitative immunoblotting [17], and immunoglobulins as well as their Fab fragments should only cover a very small fraction of the membrane surface, the observed inhibition by antibodies (Figs. 1 and 2) appears to be due to masking of a specific site in the membrane that could facilitate in vivo association with ribosomes. Previous reconstitution studies [7,8] showed that the p34 protein accounts for the major ribosome-binding activity in an detergent extract of the membrane, and that its ribosome-binding properties, including protease- and salt-sensitivity, are very similar to those of ribosome binding to stripped RM. Considering these facts, the present results suggest the possibility that the p34 protein directly participates

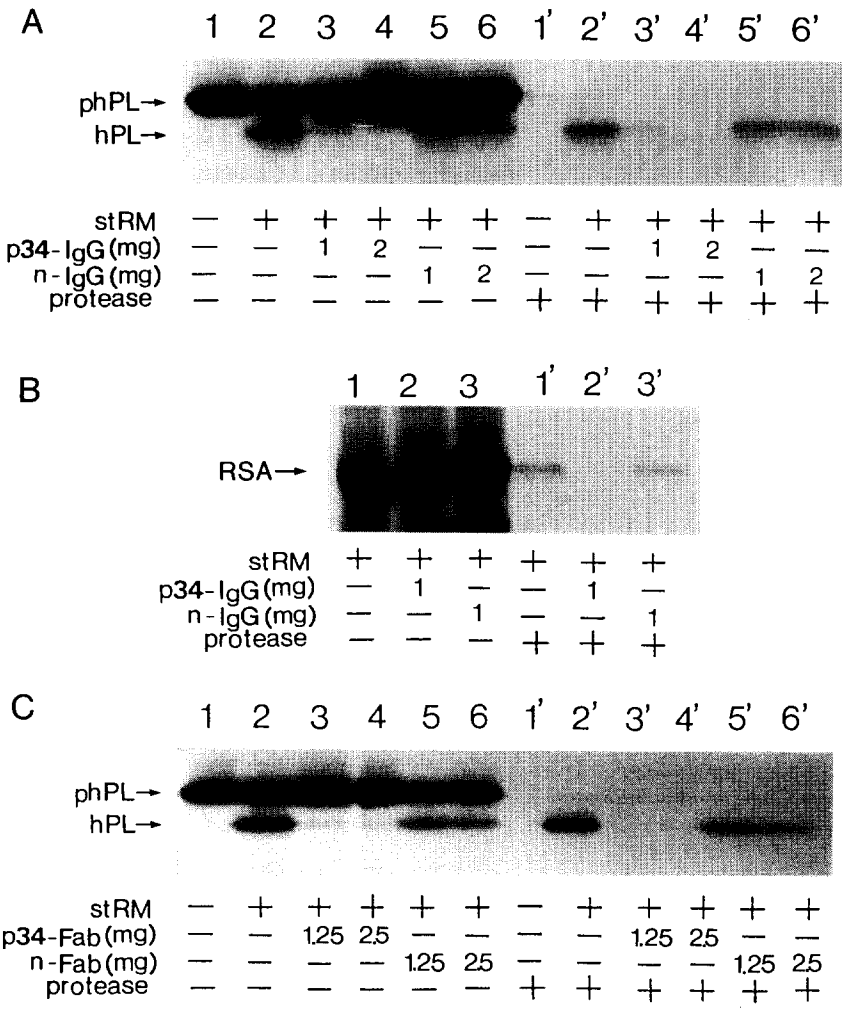


Fig. 3. Antibodies against p34 inhibit translocation. (A,B) Stripped microsomes (20 μg protein) were incubated with the indicated amounts of p34-IgGs or n-IgGs, and subsequently sedimented and resuspended. These microsomes were examined for the translocation of hPL (A) or BSA (B) in a cell-free system (see section 2). To determine translocation, half of each sample was treated with 50 μg/ml each of trypsin and chymotrypsin for 60 min at 0°C. (C) The translocation assay, using hPL, was carried out as in (A) except that Fab fragments were used instead of IgGs. phPL, pre-human placental lactogen.

in the observed ribosome–membrane association [3–6,15,16]. However, one cannot exclude another possibility that other ribosome receptors exist in close proximity to p34 in the membrane and thus that the inhibition by p34 antibodies toward other receptors could be indirectly caused by steric hindrance due to antibodies bound to p34. In any case, the inhibition of protein translocation by p34 antibodies observed here suggests that the p34 protein could be located in the proximity of the translocation site in the microsomal membrane.

Beside the p34 protein, two RM membrane proteins with a relative molecular weights of 180 kDa [18] and 40 kDa [19] have so far been shown to be ribosome receptors. Antibodies to one of these, the 180 kDa protein, were recently demonstrated, like in the case of p34 antibodies, to inhibit both ribosome binding and secretory protein translocation [20]. Immunodepletion of the p34 protein from the stripped membrane, followed by quantitation of the ribosome-binding activity and of protein translocation after reconstitution of this preparation into liposomes will help clarify whether p34 is a component of the translocation apparatus that plays an essential role in ribosome binding and protein translocation in the microsomal membrane. Studies along these lines are currently in progress.

Recent studies have indicated that the turning on/off of ribosomes on the microsomal membrane (ribosomal cycle) regulates protein translocation across the membrane. Simon and Blobel [2] demonstrated, by means of an electrophysiological technique, that the protein-conducting channel opens when ribosomes are attached to the membrane and closes when ribosomes become detached from the membrane. From this viewpoint, our results concerning the p34 protein may comprise further evidence that the ribosome–membrane association is essential for protein import into the lumen of RM.

However, whether or not the ribosome-binding activity of the p34 protein is really involved in the regulation of the activity of the protein-conducting channel remains unknown.

*Acknowledgements:* This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

[1] Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 852–862.
[2] Simon, S.M. and Blobel, G. (1991) Cell 65, 371–380.
[3] Shires, T.K., Narurkar, L. and Pitot, H.C. (1971) Biochem. J. 125, 67–79.
[4] Borgese, N., Mok, W., Kreibich, G. and Sabatini, D.D. (1974) J. Mol. Biol. 88, 559–580.
[5] Jothy, S., Bilodeau, J.-L. and Simpkins, H. (1975) Can. J. Biochem. 53, 1039–1045.
[6] Hortsch, M., Avossa, D. and Meyer, D.I. (1986) J. Cell Biol. 103, 241–253.
[7] Tazawa, S., Unuma, M., Tondokoro, N., Asano, Y., Ohsumi, T., Ichimura, T. and Sugano, H. (1991) J. Biochem. 109, 89–98.
[8] Ichimura, T., Ohsumi, T., Shindo, Y., Ohwada, T., Yagame, H., Momose, Y., Omata, S. and Sugano, H. (1992) FEBS Lett. 296, 7–10.
[9] Yoshida, H., Tondokoro, N., Asano, Y., Mizusawa, K., Yamagishi, R., Horigome, T. and Sugano, H. (1987) Biochem. J. 245, 811–819.
[10] Amar-Costesec, A., Wibo, M., Thines-Sempoux, D., Beaufay, H. and Berthet, J. (1974) J. Cell Biol. 62, 717–745.
[11] Nanba, S., Hikawa, A., Kitoo, N., Horigome, T., Omata, S. and Sugano, H. (1984) J. Biochem. 96, 1133–1142.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Wilson, S.H. and Hoagland, M.B. (1965) Proc. Natl. Acad. Sci. USA 54, 600–607.
[14] Hamilton, H.G., Pavlovec, A. and Peterman, H.L. (1971) Biochemistry 10, 3424–3427.
[15] Suss, R., Blobel, G. and Pitot, H.C. (1966) Biochem. Biophys. Res. Commun. 23, 299–304.
[16] Kreibich, G., Marcantonio, E.E. and Sabatini, D.D. (1983) Methods Enzymol. 96, 520–530.
[17] Tajima, S., Lauffer, L., Rath, V.L. and Walter, P. (1986) J. Cell Biol. 103, 1167–1178.
[18] Savitz, A.J. and Meyer, D.I. (1990) Nature 346, 540–544.
[19] Gorlich, D., Prehn, S., Hartmann, E., Kalies, K.-U. and Rapoport, T.A. (1992) Cell 71, 489–503.
[20] Savitz, A.J. and Meyer, D.I. (1993) J. Cell Biol. 120, 853–863.