Rapid cellular removal of a membrane-inserted foreign polypeptide

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We have developed a system that makes it possible to study the fate of a foreign polypeptide that is inserted in the plasma membrane. Diphtheria toxin is a bacterial protein toxin that, upon acidification, has the ability to insert into the plasma membrane from the outside of eukaryotic cells. We present results that indicate endocytic uptake and degradation of the diphtheria toxin B-fragment after insertion into the membrane of Vero cells. The degradation rate of the fragment was found to be very high ($t_{1/2} = 6$ min) and dependent on cleavage of the extra-

cellular part of the polypeptide with protease. Degradation was strongly inhibited in ATP-depleted cells, as well as at temperatures below 18 °C, and it was partially inhibited when the cytosol was acidified to block endocytosis from clathrin-coated pits. Degradation was also reduced in the presence of NH_4Cl . The results indicate that the inserted and cleaved B-fragment is degraded by a process requiring endocytosis and transport to late endosomes or to lysosomes.

INTRODUCTION

Some proteins of bacterial and viral origin can be found in the plasma membrane of eukaryotic cells. Examples of such proteins are bacterial cytolysins and viral spike proteins [1,2]. Cytolysins insert into the membrane from the outside of cells, whereas most spike proteins are synthesized by the cells and enter the secretory pathway [2]. When diphtheria toxin bound to cells bearing toxin-specific receptors is exposed to low pH values, it inserts into the membrane to mediate translocation of an enzymically active polypeptide into the cytosol (for review, see [3]). The toxin is synthesized as a single polypeptide with a molecular mass of 58342 Da [4], and it must be proteolytically activated to be inserted into the membrane and to be toxic to cells [5]. When activated, the toxin consists of two polypeptides, A and B, that have distinct functions. At low pH values the B-fragment inserts into the plasma membrane whereas the enzymically active A-fragment is translocated to the cytosol [6,7]. A 25 kDa polypeptide derived from the C-terminal part of membrane-inserted B-fragment is protected against high concentrations of unspecific proteases added extracellularly, presumably because the polypeptide is partially embedded in the membrane and partially translocated to the cytosolic side of the membrane [8]. In contrast with the insertion of complement C9 and bacterial cytolysins, which results in rapid cell death [2], the insertion of diphtheria toxin B-fragment as such is not lethal and the cell survives for many hours until it succumbs to the effects of protein synthesis inhibition by the translocated A-fragment. This allows us to study how the cells handle a foreign protein inserted into the membrane. We have studied the degradation in Vero cells of the 25 kDa polypeptide derived from membrane-inserted diphtheria toxin B-fragment.

MATERIALS AND METHODS

Materials

Hepes, phenylmethanesulphonyl fluoride (PMSF), *N*-ethyl-

maleimide (NEM), Pronase E (from *Streptomyces griseus*), tosylphenylalanylchloromethane (Tos-Phe- CH_2Cl , 'TPCK')-treated trypsin and monensin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Na^{125}I was purchased from Amersham International, Amersham, Bucks., U.K. Diphtheria toxin (crude toxin) was purchased from Connaught Laboratories Ltd., Willowdale, Ontario, Canada.

Buffers

A number of buffers were used in this study details of which are as follows. Hepes medium: serum-free Dulbecco minimal essential medium (DMEM) buffered with 20 mM Hepes to the pH value indicated. Bicarbonate-buffered medium: serum-free DMEM buffered with bicarbonate. In buffers where the pH value was adjusted to pH 4.5, 10 mM sodium gluconate was added to increase the buffering capacity. Lysis buffer: this contained 0.1 M NaCl, 20 mM NaH_2PO_4 , 10 mM EDTA, 1% (v/v) Triton X-100, 10 mM NaF, 0.1 mM sodium vanadate, 1 mM PMSF, 1 mM NEM, and 200 units/ml aprotinin, at pH 7.4.

Cell culture

Vero cells were propagated as described previously [9]. Cells were seeded out into six- or 12-well plastic dishes 24 h prior to experiments at a density of 6×10^5 or 2.5×10^5 cells per well respectively.

Purification and radiolabelling of diphtheria toxin

Diphtheria toxin was purified as described [9], dialysed against distilled water and stored frozen (-20 °C). The toxin was labelled with ^{125}I according to Fraker and Speck [10] to a specific radioactivity of 15000–45000 c.p.m. per ng of toxin. Before use the toxin was nicked with trypsin [5] to yield nicked diphtheria toxin (nDT).

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; nDT, nicked diphtheria toxin; FCS, fetal-calf serum; DMEM, Dulbecco minimum essential medium; VSV G-protein, vesicular stomatitis virus G-protein; Tos-Phe- CH_2Cl , tosylphenylalanylchloromethane.

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PAGE in the presence of SDS and subsequent densitometry

After solubilization or fractionation of the cells, SDS/PAGE (12.5% (w/v) acrylamide, 0.27% *N,N'*-methylenebisacrylamide) was carried out as described previously [11]. When not otherwise indicated, the samples were not treated with reducing agents before electrophoresis (non-reducing SDS/PAGE). Densitometry was carried out using a Computer Densitometer model 300A from Molecular Dynamics and ImageQuant for analysis of the data.

RESULTS

Degradation of protected fragments derived from diphtheria toxin

Exposure of cells with surface-bound diphtheria toxin to low pH values induces translocation of a fraction of the toxin molecules. Subsequent treatment of the cells with Pronase removes non-inserted toxin, whereas translocated A-fragment and the C-

Figure 1 Stability of Pronase-protected diphtheria toxin polypeptides

^{125}I -NDT was bound to cells for 15 min at 25 °C. The toxin-containing medium was removed and replaced with Hepes medium, pH 4.5, containing 10 mM gluconic acid. After 2 min, the low-pH medium was removed and the cells were treated with 3 mg/ml Pronase for 5 min at 37 °C. The cells were washed in Hepes medium and subsequently incubated for the indicated periods of time at 37 °C in Hepes medium containing 5% (v/v) FCS. The cells were then washed and lysed in lysis buffer. Acid-precipitable material was analysed by SDS/PAGE and autoradiography.

Figure 2 Time-curve for degradation of the membrane-inserted 25 kDa polypeptide

Membrane-inserted 25 kDa polypeptide from ^{125}I -NDT was obtained as described in Figure 1, and the cells were incubated for 0, 5, 10 or 15 min in bicarbonate-buffered medium containing 5% (v/v) FCS. The cells were then washed and lysed and trichloroacetic acid-precipitable material was analysed by SDS/PAGE and autoradiography. Bands corresponding to the 25 kDa fragment were analysed by computerized densitometry of the autoradiogram and the absorbance value (A_{632}) plotted as a percentage of the control (0 min). The error bars represent the S.D. from the mean of three experiments. Inset shows the autoradiogram of a typical experiment.

terminal part (25 kDa) of the B-fragment are protected [7,8]. To study the stability of the pronase-protected fragments, cells with bound ^{125}I -NDT were exposed to low pH values, treated with Pronase, and then incubated for increasing periods of time. Then the cells were lysed and radioactive polypeptides were analysed by SDS/PAGE. It can be seen from Figure 1 that there was a striking difference in the stability of the two protected fragments. The 25 kDa polypeptide disappeared rapidly, whereas the A-fragment was stable for the 2 h duration of the experiment. In other experiments the A-fragment was found to be intact 6 h after translocation (results not shown). This is in accordance with the findings of Yamaizumi et al. [12] that A-fragment introduced into cells by the erythrocyte ghost fusion method was very stable in the cytosol. The polypeptide migrating slightly slower than the 25 kDa polypeptide is presumably produced by cleavage closer to the N-terminal end of the B-fragment [8]. This polypeptide disappeared with the same kinetics as the 25 kDa polypeptide.

In the next set of experiments we measured degradation of the membrane-inserted 25 kDa polypeptide after shorter time intervals and analysed the SDS/PAGE results by autoradiography and computerized densitometry. Figure 2 shows the densitometric quantification of the bands corresponding to the 25 kDa polypeptide, whereas the inset shows a typical autoradiogram. The half-life of the 25 kDa polypeptide was calculated, from the results in Figure 2, to be approx. 6 min and only 10% (0–20% in different experiments) of the polypeptide was intact after a 15 min incubation of the cells. The rate of degradation was found to be the same whether the cells were incubated in bicarbonate-buffered DMEM with or without 5% fetal-calf serum (FCS) in Hepes-buffered DMEM or in PBS (results not shown).

We have previously presented evidence that the Pronase-protected 25 kDa fragment is derived from the C-terminal part of diphtheria toxin B-fragment and that it probably is inserted as an integral membrane polypeptide. Diphtheria toxin is endocytosed slowly ($t_{1/2} = 25$ min at 37 °C, see [13]). When Vero cells with bound ^{125}I -NDT were incubated for 15 min after exposure to low pH values and then treated with Pronase (Figure 3), the same amount of 25 kDa polypeptide was obtained as if the cells had been treated with Pronase immediately after the low-pH treatment (compare lane 1 and lane 2). On the other hand, when cells were treated with Pronase immediately after the exposure to low pH and then washed and incubated for 15 min, we observed only traces of radioactive material corresponding to 25 kDa (Figure 3, lane 3, in agreement with the results in Figure 2). This indicates that the inserted B-fragment is not efficiently degraded until an extracellular part is removed by Pronase.

Some of the 25 kDa polypeptide seen in lane 1 could have been produced by proteolytic activity in a compartment reached after endocytosis, but this is less likely, since inhibition of endosomal and lysosomal proteolysis with NH_4Cl did not reduce the amount of 25 kDa polypeptide obtained after 15 min incubation before the Pronase treatment (Figure 3, lane 4). Interestingly, it appears that degradation of the 25 kDa polypeptide was less efficient as long as Pronase was present in the external medium (Figure 3, lane 5). Control experiments showed that Pronase treatment did not induce a general increase in internalization of a fluid-phase marker such as horseradish peroxidase (results not shown). Rather, the results suggest that the Pronase treatment alters the membrane-inserted B-fragment in such a way that it can be internalized and degraded.

No distinct degradation products could be detected under the conditions used, which were such that polypeptides with a molecular mass less than 5000 Da would migrate at the front of

Figure 3 Effect of Pronase treatment on internalization of membrane-inserted 25 kDa polypeptide

¹²⁵I-NDT was bound to cells for 15 min at 25 °C. The toxin-containing medium was then removed and substituted with Hepes medium, pH 4.5, containing 10 mM gluconic acid. After 2 min the low-pH medium was removed and the cells were either treated immediately with 3 mg/ml Pronase for 5 min (lanes 2 and 3), or first incubated for 15 min at 37 °C with medium containing 5% (v/v) FCS and then treated with Pronase (lanes 1 and 4). In another experiment the Pronase treatment lasted for 15 min rather than 5 min (lane 5). The cells were then lysed and the trichloroacetic acid-precipitable material was analysed by SDS/PAGE and autoradiography. Lane 1, after exposure to pH 4.5 the cells were incubated for 15 min in Hepes medium at 37 °C, then they were treated with Pronase and lysed; lane 2, after exposure to pH 4.5 the cells were immediately treated with Pronase followed by lysis; lane 3, after exposure to pH 4.5 the cells were treated with Pronase and then incubated for 15 min in Hepes medium at 37 °C and lysed; lane 4, after exposure to pH 4.5 the cells were incubated for 15 min in Hepes medium at 37 °C containing 30 mM NH₄Cl, then the cells were treated with Pronase and lysed; lane 5, after exposure to pH 4.5 the cells were treated with Pronase for 15 min at 37 °C and lysed.

the gel and not be distinguished. It can be concluded that the 25 kDa polypeptide is broken down to fragments with low molecular mass.

It could be that the 25 kDa polypeptide was expelled from the cell membrane into the culture medium rather than being degraded by the cells. To test this possibility the trichloroacetic acid-precipitable material in the medium was analysed by SDS/PAGE. No 25 kDa polypeptide was detected in the medium after 15 min of incubation (results not shown). Furthermore, the 25 kDa polypeptide could not be extracted from the membranes of intact cells with carbonate and at pH 11.5, indicating that it is inserted as an integral membrane protein. The possibility that treatment of the cells with 3 mg/ml Pronase permeabilized the cell membrane and that Pronase thereby could attack the 25 kDa polypeptide from the inside is not likely, since the cells could be cultured after the enzyme treatment at a level of close to 100% recovery.

Internalization of the 25 kDa polypeptide

To study whether endocytic uptake is required for degradation of the 25 kDa polypeptide, we inhibited endocytosis in two different ways. Total endocytosis is strongly reduced when the intracellular ATP level is reduced to < 10% of the normal level [14]. When cellular ATP was reduced to this extent, by treatment with 2-deoxyglucose and oligomycin before insertion of the 25 kDa polypeptide, degradation was completely blocked (results not shown). The protection could be due to reduced endocytosis, but may also be due to reduced activity of the ATP-dependent proton pumps responsible for acidification of the endosomes or to other energy-requiring mechanisms.

Figure 4 Effect of temperature on 25 kDa polypeptide degradation

After insertion of diphtheria toxin B-fragment and Pronase treatment as described in Figure 1, the cells were incubated for 45 min at the indicated temperature in bicarbonate-buffered medium with 5% (v/v) FCS. The cells were then washed and lysed in lysis buffer. Trichloroacetic acid was added to the post-nuclear supernatant to a final concentration of 5% and precipitated material was analysed by SDS/PAGE and autoradiography. Bands corresponding to the 25 kDa fragment were analysed by computerized densitometry of the autoradiogram. The error bars represent the S.D. from the mean of three experiments. Inset shows an autoradiogram from a typical experiment.

Sandvig et al. have previously found that endocytosis from coated pits is strongly inhibited by acidification of the cytosol [15,16]. We therefore studied whether degradation of the membrane-inserted 25 kDa polypeptide was inhibited under these conditions. For this purpose, cells with membrane-inserted 25 kDa polypeptide were incubated in the presence of acetic acid, pH 5.0, to acidify the cytosol. Densitometric analysis of autoradiograms showed that approx. 50% of the 25 kDa polypeptide was degraded when the cytosol was acidified, whereas more than 90% was degraded in control cells. Control experiments showed that endocytosis of ¹²⁵I-labelled transferrin was reduced from 40% of the totally bound transferrin per 15 min to 7% (results not shown), indicating that coated-pit endocytosis was indeed inhibited by acidification of the cytosol [17]. The results indicate that endocytosis from clathrin-coated pits is only partly involved in degradation of the 25 kDa polypeptide. It is possible that the rest of the material is taken up by non-clathrin-coated endocytosis, which is less sensitive to acidification of the cytosol [16], but it cannot be excluded that some of the material is degraded while still remaining in the plasma membrane.

Temperature requirement for 25 kDa polypeptide degradation

If endocytosis is required for degradation of the 25 kDa polypeptide, it is most likely that degradation takes place in late endosomes or in lysosomes. Transport from peripheral early endosomes to late endosomes and lysosomes is inhibited below 20 °C [18,19]. We therefore investigated the effect of temperature on the degradation of the 25 kDa polypeptide (Figure 4). The temperature curve was found to have a break-point at approx. 18 °C. This finding is consistent with the possibility that degradation starts in a compartment beyond the early endosomes, most likely in late endosomes or in lysosomes.

Effects of NH₄Cl, protease inhibitors, and 3-methyladenine on 25 kDa polypeptide degradation

Endosomes are rapidly acidified by proton pumps in the vesicle membrane [20–22]. This acidification can be counteracted by weak bases [23]. When NH₄Cl is added to the cells, membrane permeant NH₃ diffuses into the vesicular compartments and

Figure 5 Effect of NH_4Cl on the degradation rate

Cells with membrane-inserted ^{125}I -labelled 25 kDa polypeptide (see Figure 1) were Pronase treated, washed and incubated in bicarbonate-buffered medium in the absence (—) or presence (+) of 20 mM NH_4Cl for increasing periods of time. The cells were then washed and lysed in lysis buffer. Trichloroacetic acid was added to the post-nuclear supernatant to a final concentration of 5%, and precipitated material was analysed by SDS/PAGE and autoradiography.

binds protons to form NH_4^+ . Vero cells with membrane-inserted 25 kDa polypeptide were incubated in the presence of NH_4Cl and then the degradation rate of the 25 kDa polypeptide was measured. Figure 5 shows a typical autoradiogram. In the presence of NH_4Cl the degradation rate of the 25 kDa polypeptide was clearly reduced. The ionophore monensin, which disrupts the proton gradient across vesicular membranes, had a similar inhibitory effect to NH_4Cl (results not shown). The results indicate that the polypeptide is degraded in a low-pH compartment.

It has been reported that macrophages may degrade endocytosed proteins while they are in endosomes, and cathepsin D was found to be involved in the degradation [24]. Cathepsin D is inhibited by pepstatin A, but we found no effect of this protease inhibitor on degradation of the 25 kDa polypeptide. Also, degradation was not inhibited either by the unspecific protease inhibitor PMSF or by leupeptin that inhibits several lysosomal proteases. On the other hand, degradation could be inhibited to some extent by addition of Tos-Phe- CH_2Cl to the medium during internalization of the membrane-inserted 25 kDa polypeptide. Also, calpeptin, an inhibitor of calpains, inhibited the degradation to some extent (results not shown).

Cellular proteins have been shown to be delivered to lysosomes by the autophagic pathway under certain conditions [25]. Autophagy in hepatocytes is effectively inhibited by 3-methyladenine [26]. Degradation of the 25 kDa polypeptide was not inhibited by this compound, indicating that autophagy is not involved (results not shown).

DISCUSSION

We have previously presented evidence that the diphtheria toxin B-fragment inserts into the plasma membrane of Vero cells at low pH values and apparently behaves like an integral membrane protein [8]. This allows studies of how cells handle a foreign membrane protein. In this work we present evidence that after Pronase cleavage, the inserted polypeptide is rapidly endocytosed and degraded. The 25 kDa polypeptide appears to be taken up both by non-clathrin-coated and clathrin-coated endocytosis [27]. Degradation of the 25 kDa polypeptide appears to depend on acidification of the compartment where degradation takes place and on an intracellular transport step that can be inhibited at temperatures below 20 °C.

The half-life of the 25 kDa polypeptide in Vero cells was estimated to be 6 min, which is comparable with the half-lives of

receptor-bound ligands such as glucagon [28] and insulin [29]. However, endocytosed ^{125}I -labelled intact diphtheria toxin has been found to be broken down after more than 1 h in Vero cells, this was measured as release of free radioactivity [13,30,31].

The degradation of the 25 kDa polypeptide is much faster than that which has been described for naturally occurring membrane proteins and other membrane-inserted foreign polypeptides. The half-life of Fc receptors with bound IgG was estimated to be 5 h [32] and that of high-affinity interleukin 2 receptors to be 1 h [33]. G-protein from vesicular stomatitis virus (VSV G-protein) inserted into the plasma membrane by low pH is rapidly internalized (60 % within 5 min) in BHK-21 cells. This protein is to some extent recycled back to the cell surface from an early endocytic compartment, while the rest is routed along the endocytic pathway and degraded in lysosomes [34]. Degradation of VSV G-protein was first detected after 15 min incubation at 37 °C and the protein had a half-life of > 30 min [34]. The VSV G-protein was not proteolytically cleaved after insertion and the kinetics of degradation resembles that of the uncleaved B-fragment which is much more stable than the 25 kDa polypeptide. Alterations in the cleaved protein could generate a signal that results in internalization and degradation. One possibility is that cleavage induces aggregation of the 25 kDa polypeptide, as aggregation was found to increase the degradation rate of other proteins [32,34].

Transport from peripheral early endosomes to late endosomes has been shown to be blocked below 20 °C [18,19]. The finding that the degradation of the 25 kDa polypeptide is inhibited at 18 °C indicates that it depends on a process that involves budding and fusion of membranes.

In conclusion, we have found that the membrane-inserted foreign 25 kDa polypeptide is removed by the cells by endocytosis and subsequent degradation. This process was found to be much more rapid than that which has been described for other membrane proteins; to our knowledge the diphtheria toxin-derived 25 kDa polypeptide has the shortest half-life described so far. The reason for this may be that the cell recognizes the 25 kDa polypeptide as foreign and that the cell has mechanisms by which it can destroy foreign membrane-inserted proteins.

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REFERENCES

- Rodriguez-Boulant, E. and Pendergast, M. (1980) *Cell* **20**, 45–54
- Bhakdi, S. and Tranum-Jensen, J. (1987) *Rev. Physiol. Biochem. Pharmacol.* **107**, 147–223
- Olsnes, S., Moskaug, J. Ø., Stenmark, H. and Sandvig, K. (1988) *Trends. Biochem. Sci.* **13**, 348–351
- Greenfield, L., Bjorn, M. J., Horn, G., Fong, D., Buck, G. A., Collier, R. J. and Kaplan, D. A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6853–6857
- Collier, R. J. and Kandel, J. (1971) *J. Biol. Chem.* **246**, 1496–1503
- Sandvig, K., Tønnessen, T. I., Sand, O. and Olsnes, S. (1986) *Cancer Res.* **261**, 11639–11644
- Moskaug, J. Ø., Sandvig, K. and Olsnes, S. (1988) *J. Biol. Chem.* **263**, 2518–2525
- Moskaug, J. Ø., Stenmark, H. and Olsnes, S. (1991) *J. Biol. Chem.* **266**, 2652–2659
- Sandvig, K. and Olsnes, S. (1981) *J. Biol. Chem.* **256**, 9068–9076
- Fraker, P. J. and Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
- Olsnes, S. and Eiklid, K. (1980) *J. Biol. Chem.* **255**, 284–289
- Yamaizumi, M., Uchida, T., Takamatsu, K. and Okada, Y. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 461–465
- Dorland, R. B., Middlebrook, J. L. and Leppla, S. H. (1979) *J. Biol. Chem.* **254**, 11337–11342
- Steinman, R. M., Silver, J. M. and Cohn, Z. A. (1974) *J. Cell Biol.* **63**, 949–969

- 15 Sandvig, K., Olsnes, S., Peterson, O. W. and van Deurs, B. (1988) *J. Cell. Biochem.* **36**, 73–81
- 16 Sandvig, K., Olsnes, S., Petersen, O. W. and van Deurs, B. (1987) *J. Cell Biol.* **105**, 679–689
- 17 Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, R. W. and Schneider, W. J. (1985) *Annu. Rev. Cell Biol.* **1**, 1–39
- 18 Dunn, W. A., Hubbard, A. L. and Aronsen, N. N., Jr. (1980) *J. Biol. Chem.* **255**, 5971–5978
- 19 Griffiths, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. (1988) *Cell* **52**, 329–341
- 20 Tycko, B. and Maxfield, F. R. (1982) *Cell* **28**, 643–651
- 21 van Renswoude, J., Bridges, K. R., Harford, J. B. and Klausner, R. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6186–6190
- 22 Geisow, M. J. and Evans, W. H. (1984) *Exp. Cell Res.* **150**, 36–46
- 23 Ohkuma, S. and Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3327–3331
- 24 Diment, S. and Stahl, P. D. (1985) *J. Biol. Chem.* **260**, 15311–15317
- 25 Seglen, P. O. (1987) in *Lysosomes: Their Role in Protein Breakdown* (Glaumann, H. and Ballard, F. J., eds.), pp. 365–382, Academic Press, London
- 26 Seglen, P. O. and Gordon, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1889–1892
- 27 Sandvig, K., Olsnes, S., Peterson, O. W. and van Deurs, B. (1989) in *Methods in Cell Biology* (Tartakoff, A. M., ed.), pp. 365–382, Academic Press, San Diego
- 28 Authier, F., Janicot, M., Lederer, F. and Desbuquois, B. (1990) *Biochem. J.* **272**, 703–712
- 29 Backer, J. M., Kahn, C. R. and White, M. F. (1990) *J. Biol. Chem.* **265**, 14828–14835
- 30 Leppla, S. H., Dorland, R. B. and Middlebrook, J. L. (1980) *J. Biol. Chem.* **255**, 2247–2250
- 31 Chignola, R., Colombatti, M., Dell'Arciprete, L., Candiani, C. and Tridente, G. (1990) *Int. J. Cancer* **46**, 1117–1123
- 32 Mellman, I. and Plutner, H. (1984) *J. Cell Biol.* **98**, 1170–1177
- 33 Duprez, V. and Dautry-Varsat, A. (1986) *J. Biol. Chem.* **261**, 15450–15454
- 34 Gruenberg, J. E. and Howell, K. E. (1987) *Proc. Natl. Sci. U.S.A.* **84**, 5758–5762