Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells

(membrane glycoprotein/exocytosis/plasma membrane)

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ABSTRACT In this paper mechanically permeabilized cells have been used to dissect the transport of Semliki Forest virus glycoproteins from the trans-Golgi network to the plasma membrane. Transport from the Golgi complex was monitored by measuring the proteolytic cleavage of the Semliki Forest virus p62 glycoprotein into the E2 and E3 polypeptide chains. Cell surface appearance was measured by the exposure of the exoplasmic domain to antibodies directed against the viral glycoprotein. Both the cleavage of the p62 protein and the transport of the glycoprotein to the cell surface were reconstituted in permeabilized BHK cells when calcium and glucose were present in the medium. Detailed analysis showed that the cleavage of the p62 protein occurred before arrival to the plasma membrane.

Newly synthesized proteins destined for post-Golgi locations follow a common route from the endoplasmic reticulum to the Golgi complex (1). The available evidence suggests that further transport occurs after sorting in the last Golgi compartment, the trans-Golgi network (TGN), where the proteins destined for different locations are included into separate transport vesicles (2). The polypeptides directed to the plasma membrane are transported to the cell surface by carrier vesicles that bud from the TGN and fuse with the plasma membrane (3, 4). The precise sequence of events and the molecular machinery involved are not yet defined.

In this paper we have used permeabilized baby hamster kidney (BHK) cells to study the delivery of the Semliki Forest virus (SFV) glycoprotein from the TGN to the cell surface. The spike protein of SFV is composed of two polypeptide chains, p62 and E1, which are synthesized from the same mRNA (5). The p62 and E1 polypeptides associate in the endoplasmic reticulum to form a heterodimer. This complex is subsequently transported to the cell surface, and the p62 protein is proteolytically processed late in transport, giving rise to two smaller polypeptides (E2 and E3) that remain associated after the cleavage (6, 7). The cleavage takes place on the extracytoplasmic part of the protein, next to an arginine pair, followed by the removal of the carboxyl-terminal arginine (8). The pE2 glycoprotein of Sindbis virus (9) and the glycoproteins of several other envelope viruses [fowl plague virus (10), human influenza virus (11), retroviruses (12), paramyxoviruses (13), and human immunodeficiency virus (14)] undergo similar dibasic cleavages during transport and maturation. These cleavages are essential for infectivity (15). The viral glycoproteins are probably processed by host-cell enzymes, which cleave cellular plasma membrane glycoproteins with similar specificity (16). The exact cellular site of these proteolytic cleavages has not been determined (e.g., refs. 5, 17, and 18).

The spike glycoproteins of SFV and other enveloped RNA viruses accumulate in the membranes of the TGN after incubation of the infected cells at 20°C (19–21). The 20°C block has been shown to occur late in protein transport after completion of terminal glycosylation (19, 22, 23). Here we have used the 20°C block in combination with permeabilized cells (24) to dissect the sequence of events occurring during SFV spike protein transport from the TGN to the plasma membrane.

MATERIALS AND METHODS

Materials. Adenosine 5'-[γ -thio]triphosphate (ATP[γ -S]) was from Boehringer Mannheim, HA filters (0.45- μ m pore size) were from Millipore, and the monoclonal antibody 9AB4 (ascites fluid) was a gift from Brian Burke (Harvard Medical School, Boston). The rabbit polyclonal antibody against the SFV spike glycoproteins was raised against the 29S spike protein complex and was affinity-purified as described (25).

Cells, Virus, and Infection. BHK-21 cells were cultured for 2 days to about 90% confluency in 35-mm diameter dishes on poly(L-lysine)-coated 18 $\times$ 18 mm glass coverslips in Glasgow minimum essential medium containing 10% (vol/vol) tryptose phosphate broth, 5% (vol/vol) fetal calf serum, and 10 mM Hepes (pH 7.4).

A stock of SFV was prepared from subconfluent infected BHK cells, and the virus was purified as described (26).

For experiments the cell monolayers were infected for 1 hr at 37°C with 15 plaque-forming units of SFV per cell in 0.5 ml of infection medium [Earle's minimum essential medium containing 1% fetal calf serum and 10 mM Hepes (pH 7.4)]. The infection medium was then discarded, 2 ml of the same medium without virus was added, and the cells were incubated for 2 hr at 37°C in the presence of 5% CO₂/95% air.

Pulse-Chase. Three hours after infection, the BHK cells were washed once in medium without methionine containing low bicarbonate (0.35 g/liter), and 10 mM Hepes (pH 7.4). The cells were incubated for 30 min in 2 ml of the same medium in a 37°C waterbath, prior to labeling with 50 μ Ci (1 μ Ci = 37 kBq) of [³⁵S]methionine in 0.3 ml of the same medium for 5 min at 37°C. The medium was gently shaken at 1-min intervals to obtain a homogeneous labeling of the monolayers. Cells were cooled on ice, washed three times with the same medium containing 2.5 mM unlabeled methionine, and incubated for 1.5 hr in a waterbath at 19.5°C. Cells were then cooled on ice and washed twice with ice-cold washing buffer (75 mM KCl/50 mM Hepes-KOH, pH 7.4). When indicated, unlabeled methionine was added to the cells for 3 min at 37°C after the pulse and before the 19.5°C incubation.

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Abbreviations: SFV, Semliki Forest virus; TGN, trans-Golgi network; ATP[γ -S], adenosine 5'-[γ -thio]triphosphate.

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Preparation of Permeabilized Cells and Conditions for the Transport Assay. BHK cells grown on coverslips were infected with SFV, pulse-labeled, incubated at 19.5°C, and washed as described. For perforation, the coverslip was collected with tweezers, and the edge was blotted on filter paper to remove excess buffer. The coverslip was placed on a smooth plastic surface with the cells facing upwards and carefully covered with a nitrocellulose filter (Millipore, type HA, 0.45- μ m pore size, 2.5-cm diameter) that had been soaked in the washing buffer and briefly blotted between two layers of filter paper to remove any excess of buffer just before use. Care was taken to avoid any air-bubble formation between the filter and the cells. After 30 sec the filter was carefully removed starting from one edge of the coverslip, and the coverslip was transferred immediately to a new dish containing 0.4 ml of the assay buffer [buffer A: 115 mM KCl/2.5 mM $MgCl_2$ /2.5 mM methionine/25 mM Hepes-KOH, pH 7.4/12 mM glucose/10 μ M free Ca^{2+} ; the calcium concentration was regulated by using an EGTA/Ca buffer system (27); the final EGTA concentration in the buffer A was 2 mM]. The excess washing buffer was removed from cells to be left intact, and the coverslips were transferred directly to 0.4 ml of the same buffer A. Samples were incubated for 1 hr in a 37°C waterbath and cooled on ice to stop the transport. Modifications of buffer A are indicated in the figure legends. Scanning electron microscopic data have shown that the cells maintain their overall structure after permeabilization both before and after the incubation at 37°C (not shown). Each experiment was performed at least twice.

Immunoprecipitation and Quantitation of the Spike Glycoprotein Transport. Immunoprecipitation of the E2 protein exposed on the surface of the cells was performed by adding (i) 4 μ l of an affinity-purified polyclonal antibody (1.1 mg/ml) directed against the spike glycoproteins of SFV and (ii) 4 μ l of the monoclonal antibody 9AB4 (ascites fluid) directed against the exoplasmic portion of the p62 and E2 glycoproteins of SFV (28) to buffer A at the end of the experiment and incubating the cells on a rocking platform for 30 min on ice. The excess antibodies were removed, and the cells were solubilized with 0.3 ml of lysis buffer (0.15 M NaCl/50 mM Tris-HCl, pH 7.5/1 mM EDTA/1% Nonidet P-40) containing a cocktail of anti-proteases composed of 10 μ g (final concentration) of antipain, chymostatin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride per ml. Lysates were clarified by centrifugation for 5 min at 4°C in a Microfuge, and the pellets were discarded.

Only about 10% of the trichloroacetic acid-precipitable radioactivity present in untreated, nonpermeabilized coverslips was lost during perforation. The recovery of trichloroacetic acid-precipitable radioactivity from permeabilized cells after the manipulations preceding the surface immunoprecipitation was >60% (as compared to samples solubilized immediately after perforation). Samples containing equal amounts of trichloroacetic acid-precipitable radioactivity were subjected to immunoprecipitation.

The total amounts of p62 and E2 proteins were quantitated by immunoprecipitation from solubilized lysates by adding equal amounts of the two antibodies (diluted 1:100). Immunoprecipitation was performed overnight at 4°C.

The immunocomplexes were precipitated by adding 1 μ l of rabbit anti-mouse IgG antiserum and 100 μ l of a 50% suspension of protein A-Sepharose to the lysates. After a 2-hr incubation at 4°C with rocking, the immunoprecipitates were washed and processed for electrophoresis on 10% acrylamide gels as described (19). To allow separation of E2 from E1 during NaDodSO₄/PAGE, dithiothreitol and iodoacetamide were omitted, and samples were heated 3 min at 70°C before electrophoresis (7).

The gels were fixed, treated with Entensify (DuPont/New England Nuclear Research Products), and dried. Preflashed

x-ray film (Kodak XAR-5) was used for fluorography (4–20 hr exposure).

The p62 and E2 protein bands were quantitated by densitometric scanning of the fluorographs on a Quick Scan R & D densitometer (Helena Laboratories, Beaumont, TX).

The amount of cleaved p62 was calculated from densitometric values of total immunoprecipitates by correcting the E2 values with a factor that takes into consideration the loss of methionines present in the E3 glycoprotein—the second product of the cleavage, which migrates close to the dye front on 10% acrylamide gels. The amount of cleaved p62 in each lane of the gel was calculated by considering the sum of cleaved p62 and of uncleaved p62 to be 100%.

The relative amounts of E2 transported to the cell surface in different samples of the same experiment were calculated as the amount of E2 immunoprecipitated from the cell surface divided by the amount of E2 immunoprecipitated from total lysates.

Hoechst Staining. Permeabilized and intact cells grown on coverslips were incubated before or after the transport assay for 5 min at room temperature in phosphate-buffered saline containing 2.5 μ g of Hoechst dye (Hoechst 33258; Serva, Heidelberg; M_r = 534) per ml. The cells were washed once in phosphate-buffered saline, fixed 4 min at –20°C in methanol, washed twice in phosphate-buffered saline, mounted, and observed with a fluorescent microscope.

For the quantitation, the number of dye-positive cells was compared to the number of cells visible by phase-contrast microscopy.

RESULTS

Arrest of p62 Cleavage in Infected Cells Incubated at 19.5°C. BHK cells were infected for 3.5 hr at 37°C with SFV, pulse-labeled for 5 min with [³⁵S]methionine, and incubated for 1.5 hr at 19.5°C to determine whether the p62 glycoprotein is cleaved when the protein accumulates in the TGN. For the analysis, the p62 protein was immunoprecipitated with anti-spike protein antibodies. About 30% of the p62 glycoprotein was processed to the E2 glycoprotein at 19.5°C (Fig. 1, lane b). However, when the cells were subsequently incubated for 1 hr at 37°C, nearly all of the p62 protein was found to be

FIG. 1. The cleavage of the p62 protein is arrested in cells incubated at 19.5°C. Infected BHK cells were pulse-labeled for 5 min with [³⁵S]methionine as described. Cells were immediately cooled on ice and solubilized (lane a) or incubated for 1.5 hr at 19.5°C in minimal essential medium containing 2.5 mM unlabeled methionine. At the end of this incubation period, the cells were immediately solubilized (lane b) or incubated for an additional hour at 37°C in buffer A and then solubilized (lane c). Aliquots of the lysates were immunoprecipitated with a polyclonal antibody directed against the spike proteins of SFV, and the immunoprecipitates were processed for NaDodSO₄/PAGE and fluorography as described.

converted to the mature E2 protein (lane c). These results demonstrate that the low temperature slows down the processing of the p62 protein, suggesting that the cleavage takes place in the TGN or after the exit of the glycoprotein from the TGN.

Ca²⁺ and Glucose Are Required for the Cleavage of p62 in Permeabilized Cells. We used permeabilized cells to find out whether the cleavage of p62 occurs intracellularly or at the cell surface. This approach has been widely used in studies of regulated secretion (29). Infected BHK cells were permeabilized by using the nitrocellulose filter stripping method (24). The cells were grown on polylysine-coated coverslips to prevent detachment of the cells during the perforation procedure. Perforation conditions were optimized to allow permeation of small molecules into the cells. The nuclear stain Hoechst 33258 was used to monitor the permeabilization efficiency. The nuclei of intact infected cells treated with Hoechst 33258 showed little or no staining (Fig. 2*a*), while 100% of the cells attached to the coverslip after perforation had strongly stained nuclei (Fig. 2*b*).

The permeabilized cells were used to establish conditions that supported cleavage of the p62 protein after release of the temperature block. When the permeabilized cells were incubated in the presence of an ATP regenerating system sufficient to support intracellular transport in other cell-free assays (30, 31), no significant cleavage of p62 was observed (not shown). We found that glucose and Ca²⁺ were essential for the cleavage reaction to occur. When permeabilized cells were incubated in buffer A, the efficiency of p62 protein cleavage was 60–75% of that observed in intact cells incubated in the same buffer (Fig. 3*A*, compare lanes a and c with lanes d and f, respectively). The cleavage of p62 observed in the permeabilized cells was 80% dependent on the addition of Ca²⁺ and glucose (Fig. 3*A*, lanes e and f). In intact cells the cleavage proceeded efficiently in the absence of glucose and Ca²⁺ (Fig. 3*A*, lanes b and c). The omission of either one of these two components reduced the efficiency of p62 cleavage in permeabilized cells to background levels (Fig. 3*A*, compare lanes e, g, and h). The stimulatory effect of Ca²⁺ on the cleavage of p62 in permeabilized cells was significant even at concentrations of 0.1 μ M Ca²⁺. When ATP[γ -S] (2.5 mM) was added to the incubation medium after permeabilization, practically no cleavage of p62 could be observed (Fig. 3*B*).

FIG. 3. Cleavage of p62 protein in permeabilized BHK cells requires Ca²⁺ and glucose and is inhibited by ATP[γ -S]. Infected BHK cells were pulse-labeled for 5 min with [³⁵S]methionine, and the label was chased for 1.5 hr at 19.5°C. The cells were left intact (A, lanes a–c) or were permeabilized (A, lanes d–h; B, lanes a–c). The samples were then incubated at 37°C for 1 hr, where indicated, in complete buffer A (A, lanes c and f; B, lane b), in buffer A lacking Ca²⁺ (A, lane g), lacking glucose (A, lane h), lacking both Ca²⁺ and glucose (A, lanes b and e), or in complete buffer with 2.5 mM ATP[γ -S] (B, lane c). After immunoprecipitation of the viral spike proteins from lysates, NaDodSO₄/PAGE and fluorography were performed as described.

Quantitation from the fluorogram showed that 85% of the cleavage was inhibited in presence of the ATP analog.

The permeabilized cells remained permeable to Hoechst dye after 1 hr of incubation at 37°C, while intact cells incubated under the same conditions were still impermeable to the dye (Fig. 2*d* and *c*).

Transport of E2 to the Cell Surface of Permeabilized Cells. Having demonstrated that the p62 protein had been cleaved in permeabilized cells, we wanted to test whether the cleavage product E2 was transported to the cell surface of these cells. Pulse-labeled BHK cells were subjected to a 1.5-hr incubation at 19.5°C, and antibodies directed against the spike proteins were used to immunoprecipitate [³⁵S]methionine-labeled spike proteins exposed on the surface of permeabilized and intact cells. Immediately after the 19.5°C incubation, viral glycoprotein was not detected at the cell surface of permeabilized (Fig. 4, lane a) or intact (not shown) cells. After incubation for 1 hr at 37°C, similar amounts of E2

FIG. 2. Accessibility of permeabilized cells to Hoechst staining. BHK cells were infected with SFV for 3.5 hr and incubated for 1.5 hr at 19.5°C. Cells were then permeabilized (b and d) or left intact (a and c). Cells in c and d were subsequently incubated for 1 hr at 37°C in the buffer A. All four samples were stained with Hoechst dye as described. (Bar = 10 μ m.)

FIG. 4. Transport of E2 protein to the cell surface. Infected, pulse-labeled BHK cells were incubated for 1.5 hr at 19.5°C and prepared for perforation as described. Lanes: a–c, permeabilized cells; d and e, intact cells. Cells were incubated for 1 hr at 37°C in buffer A containing 10 μ M free Ca^{2+} and glucose (lane b) or lacking 10 μ M free Ca^{2+} and glucose (lane d) or containing 2 mM EGTA only (lanes c and e). The cells of lane a were left on ice. After surface immunoprecipitation of the viral spike proteins, lysates were treated for NaDodSO₄/PAGE and fluorography as described.

protein, but no p62 protein, were detected at the cell surface of intact (lane d) and permeabilized cells (Fig. 4, lane b). The substitution of the Ca^{2+} /EGTA buffer with 2 mM EGTA alone in buffer A to remove all free Ca^{2+} from the permeabilized cells during the 1 hr at 37°C inhibited the delivery of E2 to the cell surface (Fig. 4, lane c). In addition, the cleavage of p62 in permeabilized cells was also inhibited by EGTA. EGTA had no effect on the processing of p62 or on the surface appearance of E2 in intact cells (Fig. 4, lane e). Quantitation from fluorograms showed that 90% of E2 protein transport to the cell surface was inhibited by incubation without glucose and Ca^{2+} , whereas no significant inhibition was detected when the same conditions were applied to intact cells. The amount of E2 protein detected at the cell surface of permeabilized cells was also drastically reduced when either glucose or Ca^{2+} were deleted from buffer A (not shown).

Cleavage of p62 Glycoprotein Occurs Intracellularly Before Cell Surface Appearance. The previous experiments suggested that the amount of E2 protein produced during the 19.5°C incubation remained in an intracellular compartment and was therefore inaccessible to added antibodies. One way to prove that the cleavage of the p62 protein indeed occurs intracellularly would be to define conditions that allow transport of the E2 protein (cleaved at 19.5°C) to the cell surface but do not support further cleavage of the p62 protein. We found that a 3-min chase at 37°C between the pulse and the chase at 19.5°C increased the amount of the p62 protein cleaved during subsequent incubation at 19.5°C to about 55% of the total pulse-labeled p62 (Fig. 5A, lane a). This E2 protein could still not be immunoprecipitated at the cell surface (Fig. 5B, lane a). However, when further cleavage of p62 after perforation was inhibited with ATP[γ -S] (Fig. 5A, compare lane b with lane c), we found that already cleaved E2 protein appeared at the cell surface at 37°C (Fig. 5B, compare lane a with lane c). Quantitations from fluorograms showed that, after incubation with ATP[γ -S], >80% of the cleavage was inhibited, whereas the efficiency of the transport of the cleaved E2 protein to the cell surface was not significantly affected (10% inhibition). These findings indicate that our assay for measuring surface appearance is a valid one. The nucleotide analog blocked the cleavage of p62 but did not hinder the surface appearance of the E2 protein formed before inhibitor addition. We know that cleavage by itself is not essential for the transport to the cell surface. In fact, when intact BHK cells were incubated with chloroquine, we found a strong inhibition of the cleavage, but transport to the cell surface of the uncleaved p62 glycoprotein still occurred (not shown). Our results show that the p62 cleavage occurs

FIG. 5. ATP[γ -S] does not inhibit the appearance of the cleaved E2 protein at the surface of the cells. Infected, pulse-labeled BHK cells were incubated with unlabeled methionine for 3 min at 37°C, followed by 1.5 hr at 19.5°C. Permeabilized (lanes a–c) or intact (lanes d–e) cells were then incubated for 1 hr at 37°C, where indicated; 2.5 mM ATP[γ -S] (lanes c and e) was present during the incubation. At the end of the incubation, all samples were incubated with anti-spike antibodies. (A) Immunoprecipitation from total lysates. (B) Surface immunoprecipitation.

intracellularly and precedes the surface appearance of the SFV spike glycoprotein.

DISCUSSION

Different methods of cell permeabilization have been described that allow direct access to the cytosol (29). The ability to modify the intracellular environment has made it possible to analyze the molecular mechanisms involved in the stimulation of regulated secretion (32–35). In this paper we have used mechanically permeabilized BHK cells to study the constitutive transport of a newly synthesized glycoprotein from the TGN to the plasma membrane.

The cleavage of the p62 protein was used to assay the exit of the viral membrane protein from the TGN, and the surface appearance of E2 was used to monitor delivery to the plasma membrane. We found that glucose had to be added to the permeabilized cells to allow cleavage and transport of the p62 protein. When NaF was present, both processes were blocked (I.d.C., unpublished observation). Previous studies have shown that there is an energy requirement during exit of proteins from the TGN (36–38). The block of the p62 cleavage in the absence of an energy source could be explained by the fact that energy is required to maintain the correct environment in the lumen of the compartment in which the cleavage occurs or by the energy requirement for vesicle formation from the TGN, if the cleavage occurs only once the transport vesicles have formed. The fact that ATP[γ -S] blocks the cleavage but not the transport to the cell surface of the already cleaved E2 protein could be due to differences in intracellular levels of ATP required for these two events. In the permeabilized BHK cells, energy is probably supplied from glycolysis, suggesting that the holes in the plasma membrane are not large enough to allow the glycolytic enzymes to leak out of the cells. We also could demonstrate that Ca^{2+} is required for cleavage and transport to the cell surface. No requirement for Ca^{2+} has been found for the reconstitution of other steps of biosynthetic plasma membrane protein transport, although it has long been known that an increase of the cytoplasmic Ca^{2+} concentration is one of

the events preceding regulated secretion (39). Whether there is a specific demand for Ca^{2+} in any of the events involved in constitutive protein exocytosis between TGN and plasma membrane or whether this reflects the requirement for a basal cytoplasmic Ca^{2+} concentration in the intact cells to preserve cytoplasmic organization remains to be established.

Using permeabilized cells, we were able to demonstrate unequivocally that the cleavage of the p62 protein and the appearance of the E2 protein on the cell surface are temporally distinct events. The fact that the cleavage of the p62 protein along the constitutive exocytic pathway occurs during or after the exit of the viral proteins from the TGN is reminiscent of what is known about the maturation of prohormones along the regulated secretory pathway. Hormone maturation has been shown to involve cleavages of the prohormones adjacent to pairs of basic amino acid residues (40). Proinsulin has been shown to be cleaved only after the maturing secretory granule has budded from the TGN (41). Also cleavage of proopiomelanocortin in AtT20 cells occurs in secretory granules (42). The enzymes responsible for the dibasic cleavages in animal cells have not been identified yet. It also remains to be established whether different cleavage enzymes are involved in the constitutive and regulated pathways (43) or whether the same proteases are activated independently along the two pathways. However, it is clear that dibasic cleavages in the constitutive and in the regulated pathways have to occur at different sites.

The intracellular cleavage of the p62 viral glycoprotein can now be used as a marker for vesicle formation from the TGN. Therefore, it is possible to use the cleavage to assay vesicle formation in a cell-free system by using an isolated TGN fraction as the donor membrane (44). This has recently been confirmed by preliminary experiments (unpublished observations). Isolation of the budded transport vesicles will facilitate the identification of molecules involved in the transport of proteins along the constitutive exocytic pathway.

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- Pfeffer, R. S. & Rothman, J. E. (1987) *Annu. Rev. Biochem.* **56**, 829–852.
- Griffiths, G. & Simons, K. (1986) *Science* **234**, 438–443.
- Palade, G. (1975) *Science* **189**, 347–358.
- Kelly, R. B. (1985) *Science* **230**, 25–32.
- Simons, K. & Warren, G. (1984) *Adv. Prot. Chem.* **36**, 79–132.
- Simons, K., Keränen, S. & Kääriäinen, L. (1973) *FEBS Lett.* **29**, 87–91.
- Ziemiacki, A., Garoff, H. & Simons, K. (1980) *J. Gen. Virol.* **50**, 111–123.
- Garoff, H., Frischauf, A.-M., Simons, K., Lehrach, H. & Delius, H. (1980) *Nature (London)* **288**, 236–241.
- Rice, C. M. & Strauss, J. H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2062–2066.
- Porter, A. G., Barber, C., Carey, N. H., Hallewell, R. A., Threlfall, G. & Emtage, J. S. (1979) *Nature (London)* **282**, 471–477.
- Min Jou, W., Verhoeven, M., Devos, R., Saman, E., Fang, R., Huylebroeck, D., Fiers, W., Threlfall, G., Barber, C., Carey, M. & Emtage, S. (1980) *Cell* **19**, 683–696.
- Shinnick, T. M., Lerner, R. A. & Sutcliffe, J. G. (1981) *Nature (London)* **293**, 543–548.
- Paterson, R. G., Harris, T. J. R. & Lamb, R. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6706–6710.
- McCune, J. M., Rabin, L. B., Feinberg, M. B., Lieberman, M., Kosek, J. C., Reyes, G. R. & Weissman, I. L. (1988) *Cell* **53**, 55–67.
- White, J., Kielian, M. & Helenius, A. (1983) *Q. Rev. Biophys.* **16**, 151–195.
- Hynes, R. O. (1987) *Cell* **48**, 549–554.
- Weiss, R., Teich, N., Varmus, H. & Coffin, J., eds. (1984) *The Molecular Biology of Tumor Viruses: RNA Tumor Viruses* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed., Vol. 1.
- Morrison, T., Ward, L. J. & Semerjian, A. (1985) *J. Virol.* **53**, 851–857.
- Matlin, K. S. & Simons, K. (1983) *Cell* **34**, 233–243.
- Saraste, J. & Kuismanen, E. (1984) *Cell* **38**, 535–549.
- Griffiths, G., Pfeiffer, S., Simons, K. & Matlin, K. (1985) *J. Cell Biol.* **101**, 949–964.
- Fuller, S. D., Bravo, R. & Simons, K. (1985) *EMBO J.* **4**, 297–308.
- Green, J., Griffiths, G., Louvard, D., Quinn, P. & Warren, G. (1981) *J. Mol. Biol.* **152**, 663–698.
- Simons, K. & Virta, H. (1987) *EMBO J.* **6**, 2241–2247.
- Balcarova, J., Helenius, A. & Simons, K. (1981) *J. Gen. Virol.* **53**, 85–92.
- Kääriäinen, L., Simons, K. & von Bonsdorff, C. H. (1969) *Ann. Med. Exp. Biol. Fenn.* **47**, 235–248.
- Amos, W. B., Routledge, L. M., Weis-Fogh, T. & Yew, F. F. (1976) *Symp. Soc. Exp. Biol.* **30**, 273–301.
- Burke, B., Walter, C., Griffiths, G. & Warren, G. (1983) *Eur. J. Cell Biol.* **31**, 315–324.
- Gomperts, B. D. & Fernandez, J. M. (1985) *Trends Biochem. Sci.* **10**, 414–417.
- Balch, W. E., Dunphy, W. G., Braell, W. A. & Rothman, J. E. (1984) *Cell* **39**, 405–416.
- Balch, W. E., Wagner, K. R. & Keller, D. S. (1987) *J. Cell Biol.* **104**, 749–760.
- Baker, P. F. & Knight, D. E. (1978) *Nature (London)* **276**, 620–622.
- Knight, D. E., Tonge, D. A. & Baker, P. F. (1985) *Nature (London)* **317**, 719–721.
- Howell, T. W., Cockcroft, S. & Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 191–197.
- Perrin, D., Langley, O. K. & Aunis, D. (1987) *Nature (London)* **326**, 498–501.
- Jamieson, J. D. & Palade, G. (1968) *J. Cell Biol.* **39**, 589–603.
- Balch, W. E., Glick, B. S. & Rothman, J. E. (1984) *Cell* **39**, 525–536.
- Balch, W. E. & Keller, D. S. (1986) *J. Biol. Chem.* **261**, 14690–14696.
- Douglas, W. W. (1968) *Br. J. Pharmacol.* **34**, 451–474.
- Docherty, K. Y. & Steiner, D. F. (1982) *Annu. Rev. Physiol.* **44**, 625–638.
- Orci, L., Ravazzola, M., Storch, M.-J., Anderson, R. G. W., Vassalli, J.-D. & Perrelet, A. (1987) *Cell* **49**, 865–868.
- Tooze, J., Hollinshead, M., Frank, R. & Burke, B. (1987) *J. Cell Biol.* **105**, 155–162.
- Gumbiner, B. & Kelly, R. B. (1982) *Cell* **28**, 51–59.
- de Curtis, I., Howell, K. E. & Simons, K. (1988) *Exp. Cell Res.* **175**, 248–265.