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(2)

Conversion of placental alkaline phosphatase from a phosphatidylinositol-glycan-anchored protein to an integral transmembrane protein

(enzymes/chimeric cDNA/vesicular stomatis virus glycoprotein)

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ABSTRACT **Placental alkaline phosphatase (PLAP) is normally anchored to the plasma membrane of cells by a phosphatidylinositol-glycan anchor after removal of a carboxyl-terminal peptide from the nascent enzyme. To investigate the signals required for this processing we constructed a chimeric cDNA. The latter was designed to code for a truncated precursor form of PLAP, containing the phosphatidylinositol-glycan attachment site but incapable of any form of membrane attachment, fused to a carboxyl-terminal peptide of vesicular stomatis virus glycoprotein. Expression of the PLAP–vesicular stomatis virus glycoprotein chimeric cDNA in transfected COS cells produced an enzymatically active protein that was attached to the plasma membrane, with the PLAP domain on the outer surface. Assays for the presence of phosphatidylinositol-glycan attachment proved negative, whereas an antibody assay confirmed the presence of the vesicular stomatis virus glycoprotein carboxyl-terminal peptide, leading to the conclusion that the truncated PLAP is attached to the cells by the membrane-spanning domain of the vesicular stomatis virus glycoprotein. In light of previous findings on carboxyl-terminal requirements of PLAP these studies suggest that an essential signal for correct sorting between transmembrane insertion and phosphatidylinositol-glycan attachment resides in the cytoplasmic domain.**

Many proteins are known to be attached to the outer face of plasma membranes by means of a phosphatidylinositol-glycan (PI-G) anchor (1, 2). Studies on the structure and biosynthesis of such proteins have demonstrated, without exception, that the PI-G anchor is linked to the carboxyl terminus of the mature protein. During the processing of a nascent protein, a short carboxyl-terminal peptide is apparently cleaved and an ethanolamine-containing glycolipid is attached by an amide linkage to the α carbonyl of the newly exposed carboxyl-terminal residue (2, 3). This presumably takes place at the luminal side of the rough endoplasmic reticulum membrane (4, 5). The PI-G-tailed protein then proceeds through the remaining elements of the cellular translocation system to arrive at the plasma membrane. Mammalian alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1], a ubiquitous class of isozymes that cleave a broad range of phosphomonoester substrates, are PI-G-anchored membrane-bound glycoproteins (1, 2). We have utilized placental alkaline phosphatase (PLAP), the most stable of the isozymes, as a model to study the signaling mechanisms of the cellular PI-G-tailing machinery (6, 7). Our laboratory has demonstrated that during the processing of nascent PLAP, a 29-residue carboxyl-terminal peptide is removed and the PI-G anchor is attached to the newly available Asp-484 residue (3). In studying the structural requirements of the carboxyl-terminal signal peptide, we have demonstrated that a hydrophobic domain of a certain size is necessary for correct modification to occur (8). In this report, we have constructed and expressed a hybrid protein to determine whether the carboxyl terminus of a transmembrane protein can serve as a signal for the attachment of a PI-G moiety.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. Transformed African green monkey kidney cells (COS cells) (9) were maintained as previously described (10) and were transfected using DEAE-dextran and chloroquine (11).

Extraction of Alkaline Phosphatase. After transfection, the medium was removed and centrifuged for 2 min at 14,000 × *g* to remove cell debris, and the cells were washed and homogenized as described (6). Media and cell homogenates were assayed for alkaline phosphatase activity as has been described (12).

Immunofluorescence Microscopy. Indirect immunofluorescence microscopy was performed as described (13). After transfected cells were fixed to slides with ethanol/acetic acid (95:5), nonspecific binding sites were blocked by exposure to normal goat serum. For viewing surface antigen, cells were incubated with anti-PLAP antibodies (Dako, Santa Barbara, CA) diluted 1:500 in bovine serum albumin buffer (1% bovine serum albumin/0.3% gelatin/25 mM Na_2HPO_4, adjusted to pH 7.5/0.15 M NaCl). To view intracellular antigen, the cells were incubated with antibodies diluted 1:500 in Tween buffer (0.5 M NaCl/1% bovine serum albumin/5 mM NaH_2PO_4, adjusted to pH 6.5/0.5% Tween 20). Intact and permeabilized cells were washed to remove excess PLAP antibodies and incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin antibodies (Boehringer Mannheim) diluted 1:50 in Tween buffer. The slides were then processed for examination under ultraviolet light in a fluorescence microscope.

Biosynthetic Radiolabeling and Immunoprecipitation. Thirty-five millimeter dishes of COS cells (1×10^6 cells) were labeled 60 hr after transfection. For labeling with [^{35}S]methionine cells were preincubated for 1 hr in 1 ml of methionine-free Eagle's minimal essential medium (MEM)/5% dialyzed fetal calf serum. Fresh medium containing [^{35}S]methionine (1400 Ci/mmol, 50 μCi; Amersham; 1 Ci = 37 GBq) was then added, and incubation was continued for 4 hr or 12 hr as described in the figure legends. For labeling with [^{14}C]ethanolamine cells were preincubated for 1 hr with 1 ml of serum-free Iscove's modified Dulbecco's medium (GIBCO). Fresh medium con-

Abbreviations: PLAP, placental alkaline phosphatase; PI-G, phosphatidylinositol-glycan; VSV G protein, vesicular stomatis virus glycoprotein.

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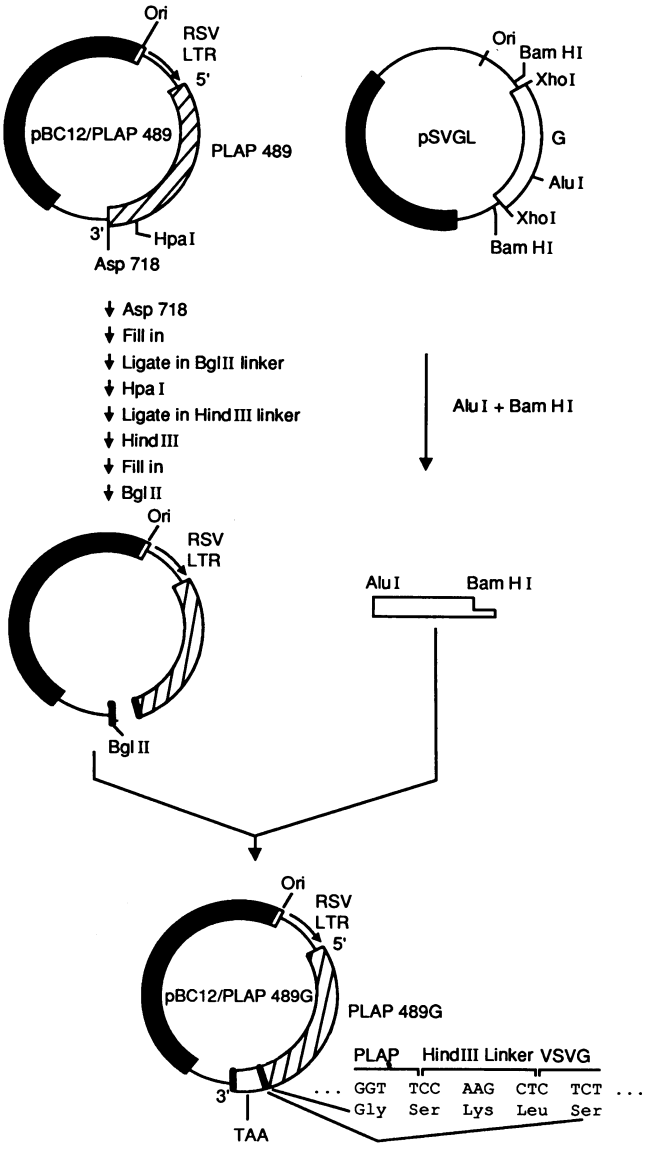

FIG. 1. Construction of pBC12/PLAP 489G. Details of the construction are described elsewhere. pBC12 PLAP 489 contains the coding sequence of truncated prepro-PLAP 489 (hatched region) inserted by blunt-end ligation between the unique *Hin*dIII and *Bam*HI sites of the expression vector pBC12BI (8, 13). pBC12 PLAP 489G contains the sequence encoding prepro-PLAP 489 fused to the *Hin*dIII linker (thick black line) followed by the *Alu* I–*Bam*HI DNA fragment encoding the transmembrane and cytoplasmic regions of VSV G protein (open region) and the *Bgl* II linker (thick black line at 3′ terminus of chimera).

taining [^{14}C]ethanolamine (50 mCi/mmol, 80 μCi; Amersham) was then added, and incubation was continued for 4 hr.

Immunoprecipitation of labeled PLAP and analyses by NaDodSO$_4$/PAGE and fluorography were performed as described (7). Proteins were immunoprecipitated with a 500-fold dilution of antibodies to PLAP (Dako) or antiserum to a peptide containing the 15 carboxyl-terminal amino acids of vesicular stomatis virus glycoprotein (VSV G) (14).

Treatment of Cells with Phospholipase C. PLAP was released from transfected COS cells by phosphatidylinositol-specific phospholipase C prepared from *Bacillus thuringiensis* in this laboratory according to Taguchi *et al.* (15). The cells were washed twice in phosphate-buffered saline, twice in release buffer (25 mM Tris·HCl, pH 7.5/0.25 M sucrose/10 mM glucose/5 mM phenylmethylsulfonyl fluoride/5 mM iodoacetate/100 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone/1 mM leupeptin) and were then suspended in the same release buffer at a concentration of 5×10^6 cells per ml. One hundred-microliter aliquots of cell suspension were then incubated with 5 units of phosphatidylinositol-specific phospholipase C for 1 hr at 37°C. One unit releases 0.1 μmol of inositol in 2 hr at 25°C (16). After incubation the cells were pelleted by centrifugation at 14,000 $\times$ *g* for 2 min, and the supernatants were removed for analysis.

Triton X-114 Partitioning of Placental Alkaline Phosphatase. Homogenates of COS cells, cell culture media, or supernatants of phosphatidylinositol-specific phospholipase C incubations were subjected to partitioning with 0.5% Triton X-114 by the procedure of Bordier (17), as modified by Malik and Low (18). Aliquots of each phase were assayed for alkaline phosphatase activity.

Plasmid Construction. The vector pBC12/PLAP 489, a cDNA encoding a mutant form of prepro-PLAP 489 amino acids long, has been described (8). This vector was further modified (see Fig. 1) by insertion of a *Bgl* II linker (1052; New England Biolabs) into the unique Asp-718 site found in the 3′-noncoding region of the PLAP cDNA and by the insertion of a *Hin*dIII linker (1038; New England Biolabs) at the unique *Hpa* I site located at the 3′ terminus of the PLAP cDNA coding region. The *Alu* I–*Bam*HI fragment encoding the 48 amino acids comprising the carboxyl-terminal transmembrane and cytoplasmic domains of the VSV G protein was removed from the vector pSVGL (19) and inserted into the *Hin*dIII–*Bgl* II site of the PLAP vector to generate pBC12/PLAP 489G. This construct contains a chimeric gene that includes (*i*) the cDNA-encoding prepro-PLAP through amino acid Gly-489; (*ii*) *Hin*dIII linker-encoded amino acids Ser-Lys-Leu; and (*iii*) VSV G cDNA encoding 19 transmembrane and the 29 cytoplasmic residues (Fig. 2).

RESULTS

Expression and Distribution of PLAP 489G. COS cells were transfected with plasmids encoding cDNAs for the nascent forms of PLAP 513, 489, and 489G. Transient (48-hr) expression of PLAP in COS cells was monitored by labeling cell protein with [^{35}S]methionine. PLAP was immunoprecipitated from the cells and media with antibodies to PLAP. As shown in Fig. 3, PLAP 489G was found entirely associated with the cell layer; both the quantity and distribution of PLAP 489G were similar to wild-type PLAP 513. In contrast, PLAP 489 was expressed at approximately 5-fold higher levels than

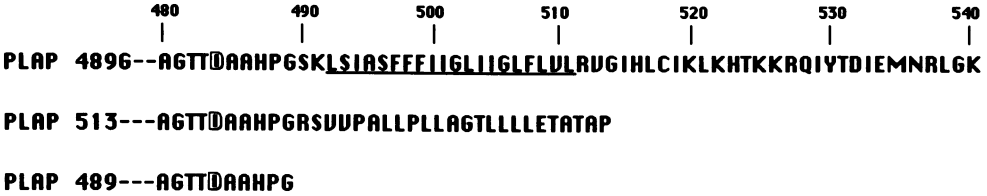

FIG. 2. Carboxyl-terminal amino acid sequences of nascent PLAP 489G, PLAP 513, and PLAP 489. The amino acid sequences are presented in one-letter code. Asp-484 (D), the attachment site of the PI-G tail in PLAP 513, is outlined. The membrane-spanning domain of PLAP 489G is underlined.

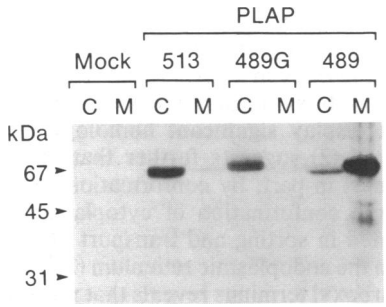

FIG. 3. Distributions of various forms of PLAP in cell cultures. Transfected COS cells were incubated with [^{35}S]methionine for 12 hr. Labeled PLAP was immunoprecipitated from cell homogenates (lanes C) and medium (lanes M) with anti-PLAP antibodies and analyzed by NaDodSO$_4$/PAGE followed by fluorography.

PLAP 513 or 489G. Furthermore, PLAP 489 was found almost entirely in the medium. It should be noted that all transfections were performed with equal, excess amounts of vector DNA. Assays of PLAP activity showed that the mutant and wild-type forms of the protein were enzymatically active and that the above immunoprecipitations reflect the distribution of the active proteins (data not shown).

PLAP 489G Is Not PI-G Tailed. PLAP 513 (wild type) is attached by a PI-G tail to the plasma membrane from which it can be released by treatment with phosphatidylinositol-specific phospholipase C (6). In the process of release the protein is converted from a hydrophobic to a hydrophilic form. To determine the nature of cell-associated PLAP, we transfected COS cells with vectors encoding prepro-PLAP 513 and 489G and then incubated the cells in isotonic buffer in the presence or absence of phosphatidylinositol-specific phospholipase C. Although almost 50% of PLAP 513 was readily removed from the cells by a 1-hr incubation with lipase, no PLAP 489G was released by similar treatment. Furthermore, upon partitioning with Triton X-114, the membrane-bound form of PLAP 513 was found to be extremely hydrophobic, whereas PLAP 489G partitioned primarily as a hydrophilic protein (data not shown). To examine PI-G tailing more directly biosynthetic radiolabeling was performed using [^{35}S]methionine and [^{14}C]ethanolamine. It has been shown that PI-G-containing proteins such as PLAP 513 can be biosynthetically labeled by radioactive precursors of the PI-G moiety (7, 8, 19). As shown in Fig. 4, both PLAP 513 and PLAP 489G incorporated [^{35}S]methionine; however, PLAP 489G was not labeled by the PI-G precursor [^{14}C]ethanolamine.

Immunocytochemical Localization of PLAP 489G. We demonstrated previously that mature, wild-type PLAP is located on the outer surface of the plasma membrane. In contrast, mutant PLAP 489 is secreted rather than being membrane-bound (6, 8). To localize PLAP 489G the transfected COS cells were examined by immunofluorescent microscopy. Fig. 5 shows that PLAP 513 appeared on the cell surface. However, while truncated PLAP 489 was secreted into the medium, PLAP 489G appeared on the exoplasmic surface of intact cells despite the lack of a PI-G anchor.

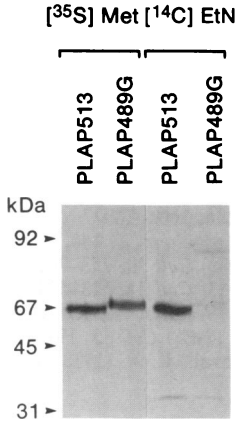

FIG. 4. Biosynthetic radiolabeling of PLAP 513 and PLAP 489G. Transfected cells were incubated with [^{35}S]methionine or [^{14}C]ethanolamine for 4 hr as described. Labeled PLAP was immunoprecipitated from the cell homogenate by anti-PLAP antibodies and analyzed by NaDodSO$_4$/PAGE followed by fluorography.

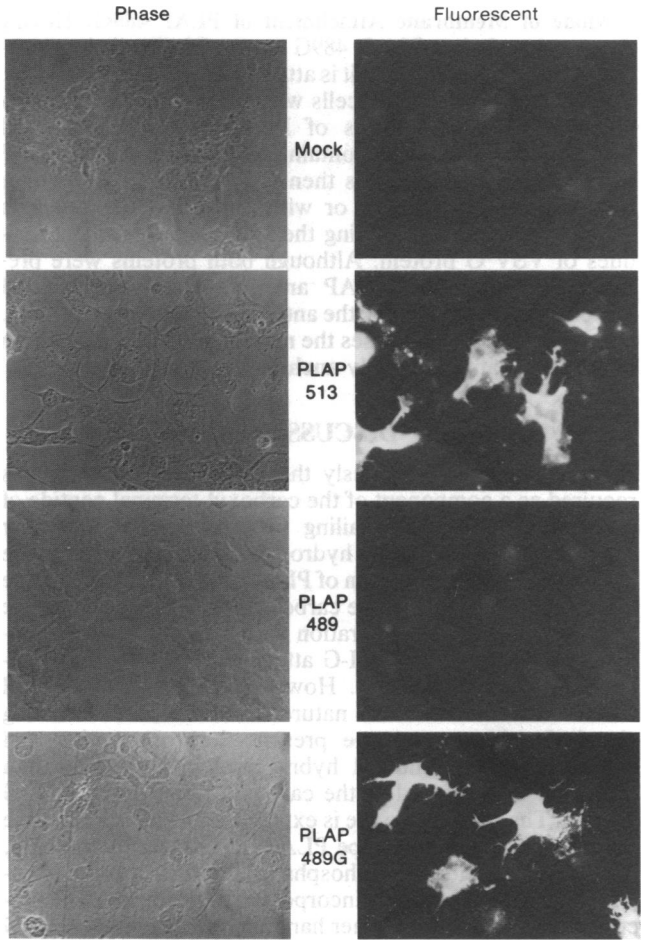

FIG. 5. Detection of PLAP 489G and PLAP 513 by immunofluorescent staining of transfected COS cells. Transfected cells were fixed, and nonspecific binding sites were blocked with normal goat serum. Intact and permeabilized cells were treated with anti-PLAP antibodies, and bound antibody was visualized by staining with rhodamine-conjugated rabbit anti-rabbit immunoglobulin antibodies.

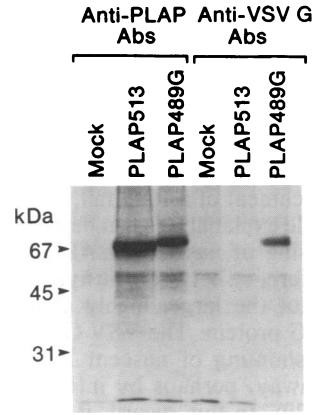

FIG. 6. Immunoprecipitation of PLAP 513 and PLAP 489G with anti-PLAP antibodies and anti-VSV G antisera. Transfected cells were incubated with [^{35}S]methionine for 12 hr as described. Labeled PLAP was immunoprecipitated from the cell homogenate by anti-PLAP or anti-VSV G antibodies and analyzed by NaDodSO$_4$/PAGE followed by fluorography.

Mode of Membrane Attachment of PLAP 489G. Having demonstrated that PLAP 489G is not PI-G tailed, we attempted to determine how it is attached to the cell membrane. To accomplish this, COS cells were transfected with cDNA encoding precursor forms of PLAP 513 and 489G and incubated with medium containing [^{35}S]methionine. PLAP from each of the cells was then immunoprecipitated either with antibodies to PLAP or with antiserum directed to a synthetic peptide comprising the 15 carboxyl-terminal residues of VSV G protein. Although both proteins were precipitated by the anti-PLAP antibodies, only the hybrid protein was recognized by the anti-VSV G antiserum (Fig. 6). Thus PLAP 489G possesses the membrane-spanning domain of VSV G that presumably anchors the hybrid PLAP.

DISCUSSION

We demonstrated previously that a hydrophobic region is required as a component of the carboxyl-terminal peptide of prepro-PLAP for PI-G tailing to occur (8). Removal or excessive truncation of the hydrophobic domain results in the synthesis of a secreted form of PLAP. We also found that the amino acid sequence of the carboxyl-terminal signal peptide could tolerate limited alteration without significant diminution in the efficiency of PI-G attachment as long as hydrophobicity was maintained. However, many questions still remain as to the structural nature of the critical PI-G-tailing signal. In this report, we present the construction and expression of an unusual hybrid protein consisting of a truncated PLAP fused to the carboxyl terminus of VSV G protein. The hybrid enzyme is expressed and functions at the same efficiency as wild-type PLAP but is more hydrophilic, is resistant to release by phosphatidylinositol-specific phospholipase C, and fails to incorporate radioactive PI-G precursors. It does, on the other hand, contain an intact VSV G carboxyl terminus. This hybrid PLAP 489G is apparently attached to the outer surface of cells by the transmembrane domain of VSV G protein, not by a PI-G anchor. Hybrid 489G was designed to resemble the carboxyl-terminal structural organization of the wild-type PLAP 513, which has a 17-amino acid hydrophobic domain beginning seven residues after Asp-484 and ending at the acidic Glu-508, which, in turn, is followed by five more amino acid residues (Fig. 2). The hybrid protein also contains the normal PI-G attachment site, Asp-484, with the transmembrane region of VSV G, beginning eight amino acids to the carboxyl-terminal side of that residue. The combined linker–VSV G membrane-spanning region consists of 20 residues that are mostly hydrophobic. This group is followed by a highly charged cytoplasmic domain of 29 amino acids. Thus, even though the hybrid contains the normal attachment site in proper register as well as a hydrophobic domain comparable in size to that found in wild-type PLAP 513, PI-G anchoring did not occur. In light of the present findings, our data indicate that the carboxyl terminus of wild-type PLAP does contain the signal governing the post-translational attachment of a PI-G tail.

Several possible explanations can be offered to explain the different processing of nascent PLAP 513 and 489G. The most obvious difference between the hybrid and the wild type is the presence of the large, highly charged, cytoplasmic domain of VSV G protein. The VSV G cytoplasmic domain may block the shunting of nascent PLAP 489G into the PI-G-tailing pathway, perhaps by interaction with another sorting system (20). In this regard, it should be noted that none of the deduced amino acid sequences from PI-G-tailed protein cDNAs have revealed a sizeable, charged cytoplasmic domain (2). Alternatively, there may be significant structural and conformational differences between the hydrophobic domains of the two carboxyl termini. Although previous alterations in the carboxyl terminus of prepro-PLAP permitted PI-G tailing (8), those alterations were not as extensive as the ones in PLAP 489G. The fact that known sequences of carboxyl-terminal peptides of PI-G-tailed proteins do not display significant homology at the primary structural level (2) suggests further that the signal is one defined, at least in part, by conformation. Here it is worth noting that the conformation of cytoplasmic domains has been implicated in sorting and transport of transmembrane proteins from the endoplasmic reticulum (21). Lastly, inspection of the carboxyl terminus reveals that prepro-PLAP 489G has a positively charged amino acid at the carboxyl-terminal border of its hydrophobic region; this contrasts with wild-type prepro-PLAP and other known PI-G-tailed proteins that have been cloned and sequenced thus far (2). The positive charge might interact strongly with the negatively charged polar heads on the cytoplasmic side of the endoplasmic reticulum, thereby limiting the nascent protein's mobility and subsequent interaction with the PI-G-tailing apparatus. It was shown recently that substitution of an asparagine for a valine at position 295 of the deduced transmembrane sequence of the protein Qa-2 converted it from a PI-G-tailed form to an integral membrane form (22).

With the conversion of a PI-G-tailed protein to an integral transmembrane protein, further experiments can be performed to delineate more specifically the structural elements that permit cells to sort proteins into their appropriate pathways and to trace the different pathways in the cell.

We thank Dr. John K. Rose of the Yale University School of Medicine for providing us with vector pSVGL and antiserum to the peptide comprising the 15 carboxyl-terminal amino acids of VSV G protein.

1. Cross, G. A. M. (1987) *Cell* **48**, 179–181.
2. Low, M. G. (1987) *Biochem. J.* **244**, 1–13.
3. Micanovic, R., Bailey, C. A., Brink, L., Gerber, L., Pan, Y.-C. E., Hulmes, J. D. & Udenfriend, S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1398–1402.
4. Bangs, J. D., Hereld, D., Krakow, J. L., Hart, G. W. & Englund, P. T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3207–3211.
5. Ferguson, M. A. J., Duszenko, M., Lamont, G. S., Overath, P. & Cross, G. A. M. (1986) *J. Biol. Chem.* **261**, 356–362.
6. Berger, J., Howard, A. D., Gerber, L., Cullen, B. R. & Udenfriend, S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4885–4889.
7. Howard, A. D., Berger, J., Gerber, L., Familetti, P. & Udenfriend, S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6055–6059.
8. Berger, J., Howard, A. D., Brink, L., Gerber, L., Hauber, J., Cullen, B. R. & Udenfriend, S. (1988) *J. Biol. Chem.* **263**, 10016–10021.
9. Gluzman, Y. (1981) *Cell* **23**, 175–182.
10. Cullen, B. R. (1986) *Cell* **46**, 973–982.
11. Cullen, B. R., Lomedico, P. T. & Ju, G. (1984) *Nature (London)* **307**, 241–245.
12. McComb, R. B. & Bowers, G. N., Jr. (1972) *Clin. Chem.* **18**, 97–104.
13. Cullen, B. R. (1987) *Methods Enzymol.* **152**, 684–703.
14. Guan, J. L., Cao, H. & Rose, J. K. (1988) *J. Biol. Chem.* **263**, 5306–5313.
15. Taguchi, R., Asahi, Y. & Ikezawa, H. (1980) *Biochim. Biophys. Acta* **619**, 48–57.
16. Sundler, R., Alberts, A. W. & Vagelos, P. R. (1978) *J. Biol. Chem.* **253**, 4175–4179.
17. Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607.
18. Malik, A. S. & Low, M. G. (1986) *Biochem. J.* **240**, 519–527.
19. Rose, J. K. & Bergmann, J. E. (1982) *Cell* **30**, 753–762.
20. Rose, J. K. & Bergmann, J. E. (1983) *Cell* **34**, 513–524.
21. Copeland, C. S., Zimmer, K. P., Wagner, K. R., Healey, G. A., Mellman, I. & Helenius, A. (1988) *Cell* **53**, 197–209.
22. Waneck, G. L., Stein, M. E. & Flavell, R. A. (1988) *Science* **241**, 697–699.