Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4982–4985, June 1992
Biochemistry

Biosynthesis of phosphatidylinositol-glycan (PI-G)-anchored membrane proteins in cell-free systems: PI-G is an obligatory cosubstrate for COOH-terminal processing of nascent proteins

(transamidase)

KRISHNA KODUKULA*, RODOLFO AMTHAUER*, DOUGLAS CINES†, EDWARD T. H. YEH‡, LARRY BRINK*, LAWRENCE J. THOMAS‡, AND SIDNEY UDENFRIEND*§

*Department of Neurosciences, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110; †Departments of Medicine and Pathology, University of Pennsylvania, Philadelphia, PA 19104; and ‡Department of Medicine, Arthritis Unit, Massachusetts General Hospital, Boston, MA 02114

Contributed by Sidney Udenfriend, February 27, 1992

ABSTRACT **It is generally recognized that nascent proteins destined to be processed to a phosphatidylinositol-glycan (PI-G)-anchored membrane form contain a hydrophobic signal peptide at both their NH_2 and COOH termini. In previous studies we showed that rough microsomal membranes (RM) prepared from CHO cells can carry out COOH-terminal processing. We have now investigated RM prepared from many additional cell types, including frog oocytes, B cells, and T cells, and found that all are competent with respect to COOH-terminal processing. Exceptions were certain mutant T cells that had been shown to be defective at various steps of PI-G anchor biosynthesis [Sugiyama, E., De Gasperi, R., Urakaze, M., Chang, H.-M., Thomas, L. J., Hyman, R., Warren, C. D. & Yeh, E. T. H. (1991) *J. Biol. Chem.* 266, 12119–12122]. In one such defective mutant, COOH-terminal processing activity of RM could be restored either by transfecting the intact cells with the gene for the deficient step in PI-G synthesis or by adding PI-G extracts to the RM *in vitro*. Cleavage of the COOH-terminal signal peptide in the RM is therefore dependent on the presence of intact PI-G incorporated into the mature protein.**

Nascent proteins destined to be processed to a phosphatidylinositol-glycan (PI-G)-anchored membrane form contain a hydrophobic signal peptide at both their NH_2 and COOH termini (1–4). During processing of such a nascent protein (preproprotein) there is first a cleavage of the NH_2-terminal signal peptide to yield the proprotein and a subsequent cleavage of the COOH-terminal signal peptide, accompanied by attachment of the PI-G moiety, to yield the mature protein. Initially we investigated the overall processing, in a cell-free system, of nascent human placental alkaline phosphatase (PLAP, 513 residues) (5, 6). To simplify analysis we introduced a shortened, engineered form of nascent PLAP (preprominiPLAP, 208 residues) (Fig. 1) to monitor processing to the mature protein (7). In studies relating to the COOH-terminal processing of the pro form to the mature form of miniPLAP, we were able to obtain indirect evidence for the incorporation of the PI-G moiety into the mature protein (8). What had not yet been obtained in our cell-free system was direct evidence that the COOH-terminally cleaved, mature product obtained from prominiPLAP contained the PI-G moiety. Several different mutant T-cell lines have been characterized that are unable to express PI-G-anchored membrane proteins such as Thy-1 (9, 10). These mutant cells were found to be defective at various steps of PI-G anchor biosynthesis. It was furthermore shown that a PI-G-deficient mutant could be rescued by transfection with cDNA from yeast (11). We have verified the PI-G deficiency in rough microsomal membranes (RM) prepared from the mutant cells. We have further shown that cleavage of the COOH-terminal signal peptide by RM from mutant cells is dependent on the addition of exogenous PI-G and that the latter is incorporated into the mature protein.

MATERIALS AND METHODS

Cell Lines and Preparation of RM. Eight different T-cell lines were grown in RPMI 1640 medium, supplemented with L-glutamine and 10% fetal bovine serum. YH 16/33 (same as YH 16.33 in refs. 9 and 10), S1A, and EL4 are wild-type T cells that can synthesize intact PI-G. M31/25 is a mutant that is deficient in transferring *N*-acetylglucosamine to phosphatidylinositol. M38/20 is a mutant lacking dolichyl-phosphate-mannose synthase and thus cannot synthesize dolichyl phosphate mannose. Neither M31/25 nor M38/20, both of which are derived from YH 16/33, can synthesize the complete PI-G anchor. BL-25 is a M38/20 derived mutant that had been permanently transfected with the dolichyl-phosphate-mannose synthase gene from *Saccharomyces cerevisiae*. Thus it is a rescued mutant of M38/20 and capable of synthesizing PI-G (11). S1A/B is a class B mutant derived from S1A that is defective in its ability to add the third mannose residue to the PI-G core. Similarly EL4/F is a class F mutant derived from EL4 that is defective in adding the ethanolamine phosphate to the third mannose residue (9). Neither of the last two mutants can synthesize intact PI-G.

CHO, HeLa, COS, and WISH cells were grown in Iscove's modified Dulbecco's medium, and CACO cells were grown in Dulbecco's modified Eagle's medium; both were supplemented with 10% fetal bovine serum. All cultures were harvested at $\approx 1.0 \times 10^6$ cells per ml and RM were prepared as described previously (8). Membranes were washed with EDTA (12) where indicated and stored at −70°C until further use. Approximately 10 g of oocytes from *Xenopus laevis* was obtained by dissection, washed, and used to prepare RM in the usual manner.

Preparation of PI-G-Enriched Lipid Extracts from Cells and RM. The PI-G moiety was extracted either from CHO cells, HeLa cells, and wild-type T cells (YH 16/33) or from the RM of these cells and was partially purified by previously published methods (9, 13, 14) with minor modifications. Typically, $\approx 1.0 \times 10^9$ cells were washed twice with phosphate-buffered saline (PBS) and taken to complete dryness by lyophilization. The dried cells were extracted with chloroform/methanol (2:1, vol/vol) and the solvent phase was separated from the residue by centrifugation at 2000 × *g* for 10 min. The resulting pellet

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

Abbreviations: PI-G, phosphatidylinositol-glycan; PLAP, placental alkaline phosphatase; RM, rough microsomal membranes.

§To whom reprint requests should be addressed.

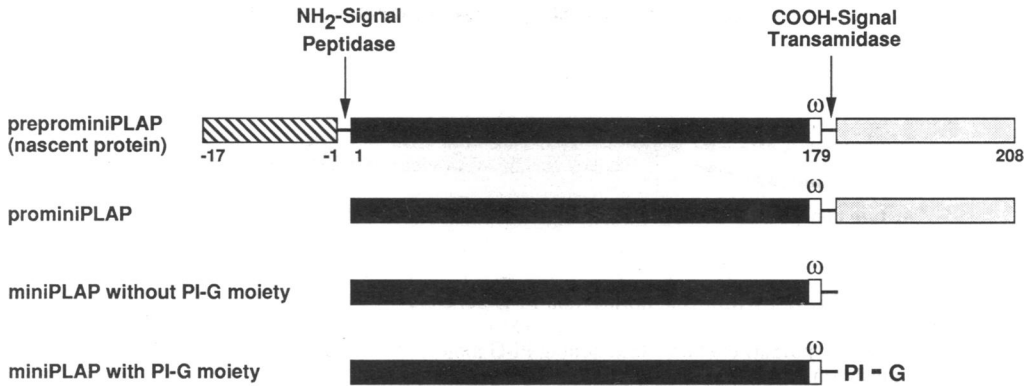

FIG. 1. Sequential processing of prepromini PLAP to mature miniPLAP. Both NH_2- and COOH-terminal signal peptides are present in the nascent protein (prepromini PLAP). The peptide at the NH_2 terminus (hatched bar) is removed cotranslationally by the signal peptidase, generating promini PLAP. The latter undergoes further processing, losing a COOH-terminal signal peptide (stippled bar) and concomitantly adding a PI-G moiety. The reaction is mediated by a putative COOH-terminal signal transamidase. ω represents the site of cleavage and PI-G addition onto nascent precursors of PI-G-tailed proteins. In miniPLAP the ω site is Asp-179. The major product of cell-free processing of promini PLAP is mature miniPLAP with the PI-G moiety attached to its COOH terminus (ω site). A minor product, without the PI-G moiety, is also observed.

was extracted twice with chloroform/methanol/water (10:10:3, vol/vol) and the supernatants were evaporated to dryness under dry nitrogen. The dried extract was partitioned between water-saturated 1-butanol and water (1:1, vol/vol) and the upper (butanol) phase was removed and evaporated to dryness under nitrogen. Lyophilized RM were extracted in a similar manner but the chloroform/methanol/water extract was evaporated and used as the source of PI-G, omitting the butanol extraction step.

Reconstitution of Deficient RM with the PI-G-Enriched Lipid Extracts. EDTA-washed RM from M38/20 or S1A/B cells (at a prewash concentration of 100 A_{280} units/ml) were used. The dried lipid preparations from CHO, HeLa, or T cells (YH 16/33) were resuspended in buffer containing 250 mM sucrose, 50 mM triethanolamine at pH 7.5, 2 mM dithiothreitol, and 0.05% Triton X-100. Typically, 20 μl of lipid suspension in buffer and 20 μl of RM were mixed briefly in a hand-held microhomogenizer. Dilutions were maintained such that the final concentration of detergent was 0.005% in a translation and processing assay.

Cotranslational Processing of Prepromini PLAP in the Cell-Free System. Prepromini PLAP mRNA (7) was translated by using a rabbit reticulocyte lysate as described by Pelham and Jackson (15) with modifications (7, 8). RM from the various cell types and reconstituted RM were used at a final concentration of ≈8–10 A_{280} units/ml.

Immunoprecipitation, SDS/PAGE, and Fluorography. Antibodies that were used to detect all three forms of miniPLAP (shown in Fig. 1) were an affinity-purified rabbit polyclonal IgG (polyclonal antibody) against human PLAP obtained from Accurate Chemicals (Westbury, NY) and an antibody directed to a peptide sequence at the NH_2 terminus of mature miniPLAP (8). Another NH_2-terminal antibody has been described earlier that does not react with its epitope when the latter is present as an internal sequence in prepromini PLAP (7, 16). Interaction with the NH_2-terminus-specific antibody occurs when the epitope is exposed at the NH_2 terminus after cleavage of the NH_2-terminal signal peptide. Immunoprecipitations and PAGE were carried out as described (7). Gels were fixed, treated with Amplify (Amersham), dried, and exposed to preflashed X-Omat-AR (Kodak) film.

RESULTS AND DISCUSSION

All of our previous studies on cell-free COOH-terminal processing were carried out on CHO or HeLa cells. Since PI-G-tailed proteins have been found in almost all eukaryotic cells studied to date (3, 4) it appeared likely that RM from cells other than CHO and HeLa cells contain similar processing activity. As shown in Fig. 2, all the cells investigated,

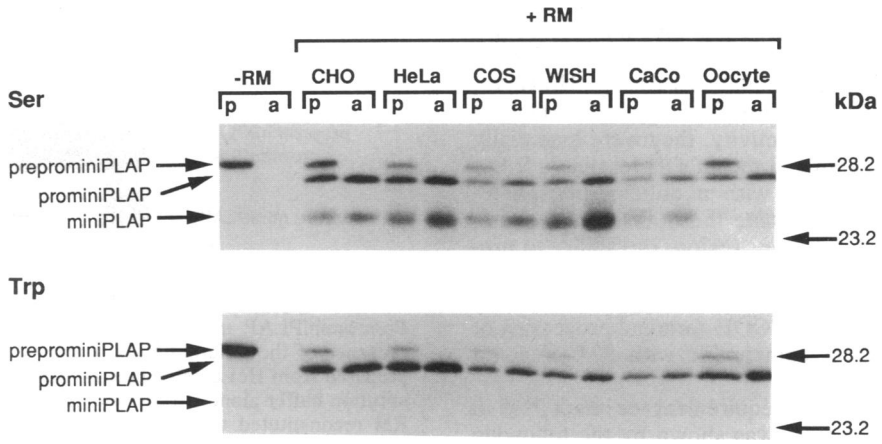

FIG. 2. COOH-terminal processing by RM preparations from various cells. Translation and processing were performed in the presence of RM prepared from the various cells shown. MiniPLAP-Ser mRNA was used to generate the substrate for overall processing of prepromini PLAP-Ser-179. MiniPLAP-Trp mRNA was used to generate prepromini PLAP-Trp-179, which cannot serve as a substrate for COOH-terminal processing. After incubation, reaction mixtures were immunoprecipitated with either polyclonal antibody (p) or NH_2-terminal-specific antibody (a).

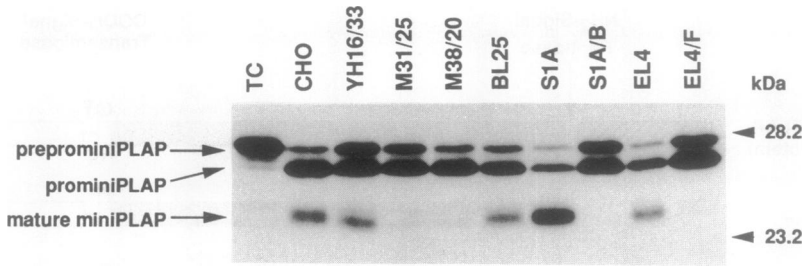

FIG. 3. Processing of preprominiPLAP by RM from normal and PI-G-deficient T cells. PreprominiPLAP mRNA was translated by using a rabbit reticulocyte lysate system in the presence of EDTA washed RM from different cell types. RM from CHO, YH 16/33, S1A, and EL4 (wild-type cells) and BL-25 (a rescued mutant) contain a functioning PI-G moiety. M31/25, M38/20, S1A/B, and EL4/F are cells with defects in generating PI-G. TC is the translation control, where no RM was added to the translation mixture. All samples were immunoprecipitated with a polyclonal antibody and then subjected to SDS/PAGE followed by fluorography.

including frog oocytes, can carry out both NH_2- and COOH-terminal processing. In all cases the mature products migrated on SDS gels with the same apparent molecular mass as was obtained with RM from CHO cells. Additional evidence of the specific nature of the COOH-terminal processing in all these cells was obtained by showing that substitution at the ω site (Fig. 1) by an amino acid other than one of the six that are allowable (17), in this case tryptophan instead of aspartic acid, led to a total loss of COOH-terminal processing activity but did not affect NH_2-terminal processing (7) (Fig. 2). PI-G transamidase, which we have demonstrated in T cells (see below) and also in B cells from patients with paroxysmal nocturnal hemoglobinuria (18, 19) (data not shown), is thus a ubiquitous enzyme.

In previous studies with wild-type PLAP and miniPLAP we had attempted to demonstrate incorporation of the elements of PI-G into the mature product in cell-free preparations. It should be noted that incorporation of labeled PI-G into variable surface glycoprotein in trypanosomal lysates had already been demonstrated (20). However, in our experiments transfer of labeled precursors (ethanolamine, mannose, inositol, fatty acids, and inorganic phosphate) to PLAP and miniPLAP were unsuccessful (ref. 5 and K.K. and S.U., unpublished results). The failure to detect incorporation of label into the femtomole quantities of mature protein that were formed in the cell-free system was due to the limiting specific activities of the labeled compounds available combined with a dilution of any newly labeled PI-G with endogenous PI-G present in the RM. The availability of mutant cells that cannot produce intact PI-G made it possible to reinvestigate the incorporation of PI-G into the COOH-terminal processed product. Accordingly, RM were utilized from a variety of Thy-1-deficient T cells known to be defective in PI-G biosynthesis. As shown in Fig. 3, RM prepared from CHO cells and from wild-type T cells carried out both NH_2- and COOH-terminal processing. However, although RM from all the Thy-1-deficient mutants retained NH_2-terminal signal peptidase activity, they were essentially unable to carry out processing at the COOH terminus. It has been shown that transfection with a dolichyl-phosphate-mannose synthase gene can rescue the dolichyl-phosphate-mannose-deficient mutant(s)—i.e., restore the ability to produce PI-G-tailed Thy-1 on the cell surface and yield intact PI-G (11). As shown in Fig. 3, RM prepared from such rescued cells (BL-25) were able to carry out COOH-terminal processing of prominiPLAP to an extent comparable with RM prepared from wild-type cells.

That this was indeed due to a requirement for intact PI-G in the cell-free processing system was shown by the following experiments. When PI-G-enriched lipid extracts from CHO, HeLa, or wild-type T cells were reconstituted with RM from the PI-G-deficient mutant M38/20, COOH-terminal processing activity was restored (Fig. 4). The putative mature miniPLAP formed by RM reconstituted with the lipid extracts comigrated with mature miniPLAP formed by RM reconstituted with the lipid extracts comigrated with mature miniPLAP from CHO cells (≈24.7 kDa). In addition specific site-directed antibodies (7) reacted with this protein in exactly the same manner as with mature miniPLAP formed by HeLa or CHO cells (data not shown). COOH-terminal processing did not take place when PI-G-deficient RM was fortified with phosphatidylethanolamine instead of the lipid extracts (data not shown). Results similar to those with the mutant M38/20 were also obtained with the mutant S1A/B (data not shown).

It is evident that there was great variability in the activity obtained with different batches of lipid extracts. In fact, less than half of the many extracts we prepared were active. However, at this stage of our knowledge of PI-G chemistry, such variations are not surprising. First of all, there was no way to quantify the PI-G content in each extract. In addition, large and variable amounts of extraneous lipids were present in the extracts. These nonspecific lipids frequently interfered during translation and processing.

The present experiments provide valuable information relating to many aspects of the biosynthesis of PI-G-tailed proteins. First of all, they corroborate conclusions based on studies in intact cells that the defect in Thy-1-deficient mutants is their inability to synthesize PI-G (9, 10, 21) and that mutants "rescued" by transfection with an exogenous DNA for the putative defective gene(s) do indeed produce PI-G (11).

With respect to our studies on COOH-terminal processing (4–8, 17) these findings lend final assurance that what we have called mature miniPLAP is indeed PI-G-tailed. We had already shown that mature miniPLAP behaves like a PI-G-tailed protein in its distribution between Triton X-114 and water and in its migration on SDS gels (8). Had COOH-terminal cleavage occurred without PI-G addition the product

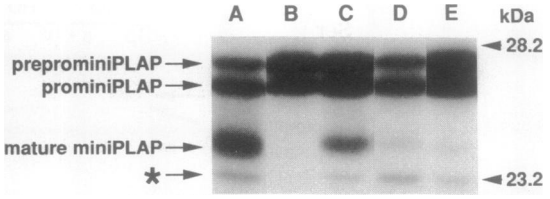

FIG. 4. Reconstitution of PI-G-deficient RM with lipid extracts. PreprominiPLAP mRNA was cotranslationally processed in the presence of the different RMs. Lane A, normal control using RM prepared from HeLa cells; lane B, M38/20 RM treated with reconstitution buffer alone without any lipid extract; lanes C–E, M38/20 RM reconstituted with lipid extracts from CHO, HeLa, and YH 16/33 cells, respectively. The asterisk indicates the minor product formed when translation and processing are carried out with normal RM or with reconstituted PI-G-deficient RM. We believe that this represents cleaved, but not PI-G-tailed, miniPLAP. Samples were immunoprecipitated with antibody and subjected to SDS/PAGE and fluorography.

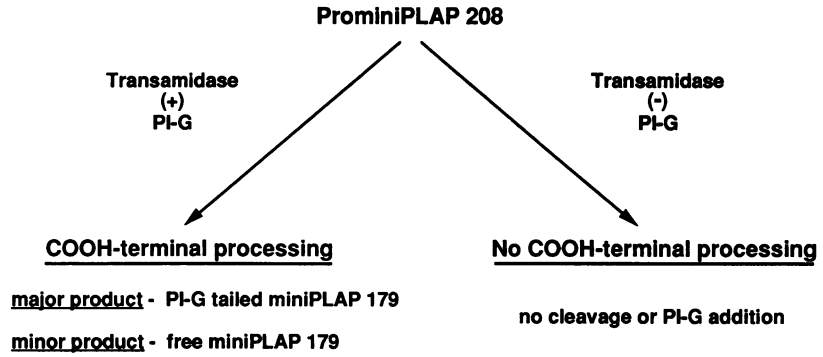

FIG. 5. Processing of prominiPLAP (208 residues) at its COOH terminus is mediated by a transamidase in the presence of a PI-G moiety. COOH-terminal processing results in the formation of two products (both 179 residues); a major one in which mature miniPLAP is PI-G-tailed and a minor product that is also cleaved at the ω site but is not PI-G-tailed. In the absence of PI-G, cleavage does not occur even in the presence of the "transamidase."

would have had an observed molecular mass of 23.2 kDa instead of 24.7 kDa (Fig. 1). The difference between the two values ($\approx$1.5 kDa) can be accounted for by the presence of a PI-G moiety. The non-PI-G-tailed form of miniPLAP used as a standard reference was generated from the appropriately truncated cDNA that codes for preprominiPLAP Δ179 (8).

Finally, the PI-G-deficient mutants raise some interesting questions about the PI-G-tailing mechanism. The mutant cells contain the putative transamidase, as shown by the ability to restore PI-G-tailing activity in both intact cells and cell-free extracts by overcoming the PI-G deficiency. However, even in the absence of added PI-G one might expect formation of an "activated transamidase-prominiPLAP complex" (Fig. 5). By analogy with other transamidases and transpeptidases (22, 23) such a complex, in the absence of the normal acceptor, would be expected to react slowly with water to cleave the COOH-terminal signal peptide and yield non-PI-G-tailed miniPLAP 179. However, in our studies no COOH-terminal processing of any kind occurred in the absence of added intact PI-G. Small amounts of a product consistent with non-PI-G-tailed PLAP-179 have been observed frequently (Fig. 4) but only in the presence of PI-G. This generally represents a minor protein band (23.2 kDa) following the major PI-G-tailed form (24.7 kDa). Thus, the transamidase responsible for COOH-terminal cleavage and PI-G addition may form a very stable complex with prominiPLAP, one that reacts far better with PI-G than with water. An alternative explanation is that the transamidase may exist in combination with PI-G, the latter perhaps maintaining the enzyme in an appropriate conformational state to react with the substrate, in this case, prominiPLAP. The absolute requirement of PI-G for cleavage, with or without PI-G addition to the protein, rules out the remote possibility of a three-step reaction involving proteolysis, activation of the newly exposed COOH terminus, and condensation with PI-G. In accord with our findings, an absolute requirement for PI-G in intact cells for the biosynthesis of PI-G-anchored surface proteins (24, 25) as well as for cleavage at the correct processing site (25) has recently been reported. In those studies PI-G biosynthesis was specifically inhibited by either mannosamine (24) or fluoroglucose (25). The fact that traces of non-PI-G-tailed cleaved product are generated indicates that the stoichiometry between PI-G-tailed cleaved product are generated indicates that the stoichiometry between PI-G and prominiPLAP is not absolute and that some reaction of the prominiPLAP-transamidase with water does occur. However, as stated above, even this requires the presence of PI-G.

We thank Dr. Anant K. Menon for his helpful suggestions on PI-G extraction and Louise Gerber for valuable technical help. We acknowledge Enid Alston for her secretarial assistance. D.C. and E.T.H.Y. were partially supported by National Institutes of Health Grants HL 40387 and HL 45851, respectively.

1. Ferguson, M. A. J. & Williams, A. F. (1988) *Annu. Rev. Biochem.* **57**, 285–320.
2. Low, M. G. (1989) *Biochim. Biophys. Acta* **988**, 427–454.
3. Cross, G. A. M. (1990) *Annu. Rev. Cell Biol.* **6**, 1–39.
4. Udenfriend, S., Micanovic, R. & Kodukula, K. (1991) *Cell Biol. Int. Rep.* **15**, 739–759.
5. Bailey, C. A., Gerber, L., Howard, A. D. & Udenfriend, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 22–26.
6. Micanovic, R., Kodukula, K., Gerber, L. & Udenfriend, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7939–7943.
7. Kodukula, K., Micanovic, R., Gerber, L., Tamburrini, M., Brink, L. & Udenfriend, S. (1991) *J. Biol. Chem.* **266**, 4464–4470.
8. Kodukula, K., Cines, D., Amthauer, R., Gerber, L. & Udenfriend, S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1350–1353.
9. Sugiyama, E., De Gasperi, R., Urakaze, M., Chang, H.-M., Thomas, L. J., Hyman, R., Warren, C. D. & Yeh, E. T. H. (1991) *J. Biol. Chem.* **266**, 12119–12122.
10. Thomas, L. J., DeGasperi, R., Sugiyama, E., Chang, H.-M., Beck, P. J., Orleans, P., Urakaze, M., Kamitani, T., Sambrook, J. F., Warren, C. D. & Yeh, E. T. H. (1991) *J. Biol. Chem.* **266**, 23175–23184.
11. DeGasperi, R., Thomas, L. J., Sugiyama, E., Chang, H. M., Beck, P. J., Orlean, P., Albright, C., Waneck, G., Sambrook, J. F., Warren, C. D. & Yeh, E. T. H. (1990) *Science* **250**, 988–989.
12. Walter, P. & Blobel, G. (1983) *Methods Enzymol.* **96**, 84–93.
13. Mato, J. M., Kelly, K. L., Abler, A. & Jarett, L. (1987) *J. Biol. Chem.* **262**, 2131–2137.
14. Menon, A. K., Mayor, S., Ferguson, M. A. J., Duszenko, M. & Cross, G. A. M. (1988) *J. Biol. Chem.* **263**, 1970–1977.
15. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
16. Garattini, E., Margolis, J., Heimer, E., Felix, A. & Udenfriend, S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6080–6084.
17. Micanovic, R., Gerber, L. D., Berger, J., Kodukula, K. & Udenfriend, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 157–161.
18. Rosse, W. F. (1990) *Blood* **75**, 1595–1601.
19. Schubert, J., Uceichowski, P., Delany, P., Tischler, H.-J., Kolanus, W. & Schmidt, R. E. (1990) *Blood* **76**, 1181–1187.
20. Mayor, S., Menon, A. K. & Cross, G. A. M. (1991) *J. Cell Biol.* **114**, 61–71.
21. Conzelmann, A., Spiazzi, A., Bron, C. & Hyman, R. (1988) *Mol. Cell. Biol.* **8**, 674–678.
22. Tipper, D. J. & Strominger, J. L. (1965) *Proc. Natl. Acad. Sci. USA* **54**, 1133–1141.
23. Tate, S. S. & Meister, A. (1981) *Mol. Cell. Biochem.* **39**, 357–368.
24. Lisanti, M. P., Field, M. C., Caras, I. W., Menon, A. K. & Rodriguez-Boulan, E. (1991) *EMBO J.* **10**, 1969–1977.
25. Takami, N., Oda, K. & Ikehara, Y. (1992) *J. Biol. Chem.* **267**, 1042–1047.