Guanosine Triphosphate Promotes the Post-translational Integration of Opsin into the Endoplasmic Reticulum Membrane*

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Membrane integration of a nascent opsin polypeptide was examined to determine whether insertion of proteins into the endoplasmic reticulum is dependent upon energy provided by ribonucleotide triphosphate hydrolysis. A discrete-sized nascent chain was obtained by in vitro translation of a mRNA which lacked a termination codon yet encoded the first 156 residues of bovine opsin. Ribosomes bearing the newly synthesized opsin chains were post-translationally incubated with canine pancreas microsomal membrane vesicles after addition of exogenous ribonucleotides or ribonucleotide analogues. Post-translational membrane integration and glycosylation of the 156-residue nascent polypeptide was found to require either the presence of guanosine triphosphate or a nonhydrolyzable GTP analogue. ATP did not promote post-translational integration of the nascent polypeptide. Although ribonucleotide hydrolysis was not obligatorily required for integration of opsin, we observed an increase in the proportion of glycosylated opsin chains in post-translational incubations that contained hydrolyzable ribonucleotide triphosphates. We conclude that a GTP-binding protein performs an essential role during integration of opsin into the endoplasmic reticulum.

The signal recognition particle (SRP) and the SRP receptor (or docking protein) were initially shown to function together to target polysomes bearing nascent secretory proteins to the endoplasmic reticulum for translation coupled translocation (1–5). Membrane insertion of structurally distinct classes of integral membrane proteins was subsequently shown to be mediated by SRP and the SRP receptor (6–10). Additional insight into the mechanisms of protein translocation and membrane protein integration has been provided by the development of translocation and integration assays that are not dependent upon the continued elongation of the polypeptide chain (8, 11–14). Post-translational translocation of nascent secretory proteins (11, 13) or integral membrane proteins (12) was shown to occur only in the presence of hydrolyzable ribonucleotide triphosphates. Perera et al. (11) recognized that the energy provided by ribonucleotide triphosphate hydrolysis could conceivably be directly coupled to transport of the nascent chain across the membrane bilayer or could instead serve in an auxiliary capacity to maintain the translocation competence of the polypeptide. In contrast, a similar experimental strategy revealed a requirement for GTP during attachment of secretory polysomes to the endoplasmic reticulum (14). The GTP requirement could be fulfilled by nonhydrolyzable GTP analogues but not by ATP or GDP (14).

As the above ribonucleotide requirements appear to be distinct, we investigated membrane integration of a nascent opsin polypeptide to determine whether either a requirement for ATP hydrolysis or alternatively a guanine ribonucleotide was an obligatory phenomenon during integration of membrane proteins with multiple transmembrane-spanning segments. The integral membrane protein opsin contains seven predominately hydrophobic transmembrane-spanning segments separated by short hydrophilic segments or loops which are exposed in an alternating fashion on the lumenal and cytoplasmic faces of the membrane (15). The first transmembrane-spanning segment can function both as a signal sequence to direct translocation of the amino-terminal luminal domain of opsin as a stop-transfer sequence to integrate the polypeptide in the membrane bilayer (9, 11). Additional functional signal sequences have been identified in all of the transmembrane-spanning segments of opsin with the exception of the seventh which is nonfunctional and the third which has not been tested (16).

Based upon the preceding observations concerning opsin biosynthesis, we conducted post-translational membrane integration experiments using a nascent opsin polypeptide under conditions of defined ribonucleotide composition. Translocation of the amino-terminal hydrophilic domain of opsin across the membrane bilayer was monitored by the acquisition of asparagine-linked oligosaccharide and resistance to proteolytic digestion. The nascent opsin polypeptide was post-translationally integrated into microsomal membranes provided that either GTP or a nonhydrolyzable GTP analogue was present. Glycosylation of the lumenally exposed domain of opsin did not require hydrolysis of GTP nor was it dependent upon the inclusion of ATP.

**EXPERIMENTAL PROCEDURES**

Materials—SP6 RNA polymerase, the vector pSP65, placental RNase inhibitor (RNasin), and rabbit reticulocyte lysate were obtained from Promega Biotech. All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. [35S]Methionine was from Du Pont-New England Nuclear. GTP, ATP, puromycin dihydrochloride, endoglycosidase H, and proteinase K were from Boehringer Mannheim. Adenylyl-5'-imidodiphosphate (AMPPNP), guanylyl-5'-imidodiophosphate (GMPNP), and Sephacryl S-200 were from Pharmacia LKB Biotechnology Inc. Cycloheximide, emetine dihydrochloride, and phenylmethylsulfonyl fluoride were from Sigma. The nonionic detergent Nikkol (octaethylene glycol-mono-N-dodecyl ether) was obtained from Nikko Chemical Co., Ltd. Rough microso-

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1 The abbreviations used are: SRP, signal recognition particle; AMPPNP, adenylyl-5'-imidodiphosphate; GMPNP, guanylyl-5'-imidodiphosphate; SDS, sodium dodecyl sulfate.
nal membranes were isolated from canine pancreas as described previously (1).

**In Vitro Transcription and Translation**—The plasmid bd20 (17) was digested with SacI to obtain a 1382-base pair fragment containing the complete coding sequence of bovine opsin. The SacI fragment was inserted into the SacI site of pSP65 to obtain the plasmid pSP65-pSPOP. pSPOP was transcribed with SP6 RNA polymerase as described (14, 18) after linearization within codon 157 with AhaII. The mRNA transcript was isolated by extraction with phenol-chloroform and by successive precipitations with ethanol and with lithium chloride. The mRNA transcript (500 ng) was translated in a 100-μl system with microsomal nucleoside-digested rabbit reticulocyte lysate system (19) containing 80 μCi of [35S]methionine and further supplemented with placental RNase inhibitor.

**Post-translational Incubation of Polypeptides with Microsomal Membranes**—For post-translational assays, protein synthesis was terminated after 15 min of translation at 30 °C by adjustment to 1 mM emetine dihydrochloride (or 250 μM cycloheximide). In *vitro* assembled polysomes were separated from ribonucleotides at 4 °C using a 1-ml Sephacryl S-200 gel filtration column equilibrated in 50 mM triethanolamine-OAc (pH 7.5), 150 mM KOAc, 2.5 mM Mg(OAc)2, 3 mM dithiothreitol, 0.002% Nikkol as described (14). The void volume fraction (150 μl) containing the polysomes was further supplemented with tRNA (11, 12, 14) and precleared with microsomal membranes (1 ml/10 μl, as defined previously (1)) and exogenous ribonucleotides as described in individual experiments.

**GTP Is Required for Membrane Integration of Polypeptides**—A nascent opsin polypeptide was assayed by alkaline extraction of membranes (7, 20). Post-translational membrane integration assays were chilled on ice and diluted with 9 volumes of 0.1 M Na2CO3 (pH 11.5). After 10 min at 0 °C, the samples were layered over a 50-μl cushion of 0.2 M sucrose, 0.1 M Na2CO3 (pH 11.5), 100 mM KOAc, 2.5 mM Mg(OAc)2, and centrifuged for 8 min in a Beckman airfuge A-100/30 rotor at 30 psi. The supernatant (S) fraction including the sucrose cushion was removed from the airfuge tube and the protein was recovered by precipitation with one-fifth volume of 100% trichloroacetic acid. The ribonucleotide-depleted polysome fraction was supplemented with microsomal membranes (1 μl/10 μl, as defined previously (1)) and electrophoresis was accomplished as described (24).

**Miscellaneous Analytic Procedures**—Post-translational digestion of translation products with endoglycosidase H was conducted as described (21). Peptidyl-tRNA was selectively precipitated from *in vitro* translation reactions with cetyltrimethylammonium bromide as described (22). For precipitation of proteins protected by microsomal membranes, translation products were digested for 1 h at 0 °C with proteinase K (50 μg/ml) in a total volume of 50 μl as described (14). Protease digestions were terminated by adjustment of the sample to 1 mM phenylmethylsulfonyl fluoride and the protease digestion products were precipitated by precipitation of the sample with an equal volume of 20% trichloroacetic acid. Translation products were prepared for gel electrophoresis and subsequent fluorography with diphenylazoxazole as described previously (14). The alkaline Triton X buffer used during preparation of samples for electrophoresis hydrolyzes the acyl linkage of the tRNA to the nascent polypeptide. The quantitation of radioactivity in specific polypeptides by scintillation counting after gel electrophoresis was accomplished as described previously (24).

**RESULTS**

**Post-translational Membrane Integration of a Nascent Opin Chain**—Previous research from several laboratories has demonstrated that in *vitro* translation of a mRNA lacking a termination codon results in the synthesis of a discrete-sized peptidyl-tRNA (11, 12, 14). We chose to prepare a nascent opsin polypeptide using this strategy based upon the observation that other nascent polypeptides produced by this method can be post-translationally translocated across microsomal membranes provided they remain bound to the ribosome in tRNA linkage (11, 12). A plasmid (pSPOP) containing a bovine opsin cDNA insert was linearized with the restriction enzyme AhaII prior to ribonucleotide transcription with bacteriophage SP6 RNA polymerase to prepare a truncated mRNA encoding the first 156 codons of bovine opsin. The resulting polypeptide contains the entire first three transmembrane-spanning segments of bovine opsin plus the 36-residue amino-terminal luminal exposed domain (Fig. 1). We chose to investigate post-translational membrane integration of this relatively short polypeptide to minimize any potential perturbations which could be induced by folding of the nascent chain.

Translation of the mRNA transcript in the reticulocyte lysate system yielded a single major translation product referred to as op-156 (Fig. 2, lane a). Specific precipitation of the op-156 peptidyl-tRNA with cetyltrimethylammonium bromide demonstrated that the majority of the op-156 chains were in tRNA linkage before but not after incubation of the translation reaction with puromycin (data not shown). Thus, the nascent polypeptide remains both physically and functionally bound to the ribosome as peptidyl-tRNA. Cotranslational membrane integration of op-156 (Fig. 2, lane b) was accompanied by the addition of high mannose oligosaccharide to asparagine residues 2 and 15 (15, 25, and Fig. 1) to produce a more slowly migrating form of the polypeptide (g-op-156). Based upon the luminal location of the oligosaccharyl transferase (26, 27), we can conclude that glycosylation of the polypeptide is indicative of translocation of the amino-terminal domain of the protein across the membrane bilayer.

![Fig. 1. Schematic diagram of the polypeptide encoded by the truncated op-156 mRNA. The transmembrane-spanning sequences (solid bars) and the sites for asparagine-linked glycosylation (Y) are shown.](image1)

![Fig. 2. Cotranslational and post-translational integration of a 156-residue opsin nascent chain. A mRNA transcript encoding the first 156-amino acid residues of bovine opsin was translated in a reticulocyte lysate translation system either in the absence (a) or presence (b) of canine pancreas rough microsomal membranes. A 100-μl translation was adjusted to 1 mM emetine after 15 min of translation in the absence of membranes (c-h). The op-156 polysomes were separated from ribonucleotides by chromatography on a 1-ml Sephacryl S-200 column as described under 'Experimental Procedures.' Ribonucleotide-depleted op-156 polysomes were post-translationally incubated with microsomal membranes for 15 min at 25 °C either in the absence (c) or presence (d-h) of 100 μM GTP plus 1 mM ATP. Translation products were either prepared directly for electrophoresis (a-e), immunoprecipitated with preimmune serum (f), immunoprecipitated with antibody raised against bovine opsin (g) or incubated with endoglycosidase H prior to electrophoresis (h). The polypeptides corresponding to the unglycosylated (op-156), monoglycosylated (Y) and fully glycosylated (g-op-156) forms of the opsin nascent chain were resolved on 15% gradient polyacrylamide gels in SDS and visualized by fluorography of the diphenylazoxazole-impregnated gel. The difference in efficiency of post-translational glycosylation observed between lanes d and e can be ascribed to a difference in glycosylation capacity of the two membrane preparations used in this experiment.](image2)
Post-translational membrane integration experiments were performed by allowing translation to occur for 15 min prior to the addition of a protein synthesis elongation inhibitor (cycloheximide or emetine). The op-156 polysomes were separated from ribonucleotides by gel filtration chromatography prior to incubation with microsomal membranes. The glycosylated form of op-156 was produced during the post-translational incubation in the presence (Fig. 2, lane d) but not in the absence (Fig. 2, lane c) of exogenous ribonucleotides. The polypeptide profile after immunoprecipitation with antibody to bovine opsin (Fig. 2, lane g), but not nonimmune control serum (Fig. 2, lane f) was identical to the total products of the post-translational incubation (Fig. 2, lane e). Endoglycosidase H digestion eliminated both the fully glycosylated form of op-156 and a more rapidly migrating polypeptide marked by an asterisk (Fig. 2, lane h), thereby demonstrating that both polypeptides contain asparagine-linked oligosaccharide. Control incubations of endoglycosidase H with the unglycosylated form of op-156 did not induce an alteration in polypeptide mobility (data not shown). As opsin contains two glycosylation sites (25), the polypeptide of intermediate mobility presumably corresponds to a monoglycosylated form of op-156. The post-translational integration experiments described below were analyzed without immunoprecipitation of the opsin-specific polypeptides as we did not detect any other translation products in this region of the polyacrylamide gel.

Membrane Integration of Opsin Requires GTP—Having characterized the polypeptides that accumulate during post-translational integration assays, we decided to determine whether ATP or GTP alone could support membrane integration of op-156. Control experiments demonstrated that glycosylated forms of op-156 were not observed when either membranes (Fig. 3, lane a) or ribonucleotides (Fig. 3, lane b) were deleted during the post-translational incubation. A post-translational incubation of op-156 polysomes with microsomal membranes plus GTP (Fig. 3, lane c) led to the appearance of glycosylated op-156. The nonhydrolyzable GTP analogue, GMPPNP was tested and found to promote the glycosylation of the nascent opsin chain (Fig. 3, lane e). Glycosylation of op-156 was not observed in the presence of microsomal membranes plus 750 μM ATP (Fig. 3, lane d) or 750 μM AMPPNP (data not shown). Additional post-translational membrane integration experiments were supplemented with 10 mM MgATP either in the presence (Fig. 3, lane g) or absence (Fig. 3, lane h) of 100 μM GTP. MgATP alone, even when present at 10 mM did not lead to the appearance of glycosylated opsin nascent chains. The extent of op-156 glycosylation in reactions containing GTP (Fig. 3, lanes c and i) was similar to that observed in reactions containing both GTP plus ATP (Fig. 3, lanes f and g).

The assays described above detected membrane integration indirectly by monitoring the appearance of glycosylated forms of the protein. The observed requirement for GTP could conceivably be ascribed to the initial synthesis of the lipid-linked oligosaccharide or to the subsequent transfer of the oligosaccharide to the nascent chain rather than to the membrane insertion of the nascent polypeptide. To address such a possibility directly, we analyzed post-translational integration reactions by extraction of nonintegrated polypeptides with sodium carbonate (pH 11.5). The alkaline sodium carbonate extraction converts the membrane vesicles into sheets and removes peripheral proteins from both faces of the bilayer (20). Integral membrane proteins are recovered in the membrane or pellet (P) fraction irrespective of carbohydrate content, while soluble and peripheral proteins are recovered in the supernatant (S) fraction. When both GTP and ATP were included in the post-translational assay, the glycosylated forms of op-156 sedimented exclusively with the alkaline extracted membrane fraction (Fig. 4A, P). In contrast, op-156 was predominately recovered in the supernatant fraction (Fig. 4C) when ribonucleotides were deleted. Approximately 50% of the unglycosylated op-156 nascent chains were recovered in the pellet fraction when GTP was included during the incubation with membranes (Fig. 4 B and D). The efficiency of op-156 glycosylation was somewhat variable and can presumably be ascribed to the quantity of endogenous lipid-linked oligosaccharide present in individual microsomal membrane preparations. The nonhydrolyzable GTP analogue, GMPPNP, could substitute for GTP in the post-translational assay, albeit with detectably lower efficiency, to promote both glycosylation and membrane integration of op-156 (Fig. 4, compare D and E). No detectable membrane integration of the opsin nascent chain was observed in post-translational membrane integration of opsin nascent chains. Ribonucleotide-depleted op-156 polysomes were adjusted to 1 mM emetine and incubated with microsomal membranes and ribonucleotides: A, 100 μM GTP plus 1 mM ATP; B, 100 μM GTP; C, no ribonucleotides; D, 100 μM GTP; E, 100 μM GMPPNP, and F, 1 mM ATP. After a 15-min incubation at 25 °C, the 10-μl reactions were chilled on ice and diluted with 9 volumes of 0.1 M Na2CO3 (pH 11.5). The samples were fractionated on alkaline sucrose gradients into supernatant (S) and pellet (P) fractions as described under “Experimental Procedures.” Samples A–C and samples D–F are from two separate experiments which have been aligned here to show a common migration of the opsin specific polypeptides.

FIG. 3. Ribonucleotide requirements for post-translational glycosylation of opsin. Ribonucleotide-depleted op-156 polysomes were adjusted to 1 mM emetine and incubated either in the presence (b–i) or absence (a) of microsomal membranes after supplementation with ribonucleotides: (a) 100 μM GTP, 750 μM ATP; (b) no ribonucleotides; (c) 100 μM GTP; (d) 750 μM ATP; (e) 100 μM GMPPNP; (f), 100 μM GTP plus 750 μM ATP; (g) 10 mM MgATP plus 100 μM GTP; (h) 10 mM MgATP; (i) 100 μM GTP. After a 15-min incubation at 25 °C, the samples were prepared for SDS-polyacrylamide gel electrophoresis. Lanes g–i were taken from a separate experiment and are aligned to show a common migration of the op-156-specific polypeptides.

FIG. 4. Ribonucleotide requirements for post-translational membrane integration of opsin nascent chains. Ribonucleotide-depleted op-156 polysomes were adjusted to 1 mM emetine and incubated with microsomal membranes and ribonucleotides: A, 100 μM GTP plus 1 mM ATP; B, 100 μM GTP; C, no ribonucleotides; D, 100 μM GTP; E, 100 μM GMPPNP, and F, 1 mM ATP. After a 15-min incubation at 25 °C, the 10-μl reactions were chilled on ice and diluted with 9 volumes of 0.1 M Na2CO3 (pH 11.5). The samples were fractionated on alkaline sucrose gradients into supernatant (S) and pellet (P) fractions as described under "Experimental Procedures." Samples A–C and samples D–F are from two separate experiments which have been aligned here to show a common migration of the opsin specific polypeptides.
incubations supplemented with ATP (Fig. 3F).

**Ribonucleotide Hydrolysis Stimulates Membrane Integration of Opin**—We were unable to detect an obligate requirement for ATP or ribonucleotide hydrolysis by either the criterion of resistance to alkaline extraction or addition of asparagine-linked oligosaccharide. Protease digestion experiments were conducted to determine whether the glycosylated form of op-156 produced during incubation with GMPPNP differed in accessibility to proteases from that produced in the presence of GTP plus ATP. The *in vitro* assembled polymers were incubated with microsomal membranes either in the presence of ATP plus GTP, or with AMPPNP plus GMPPNP. The nonhydrolyzable ATP analogue AMPPNP was included in the latter incubation as a competitive inhibitor to insure that trace quantities of ATP could not account for the observed membrane integration of op-156. Aliquots from the post-translational incubations were removed at several time points from the alkaline sucrose gradient gel. For ATP or ribonucleotide hydrolysis by either the criterion of resistance to alkali extraction or addition of asparagine-linked oligosaccharide. Protease digestion experiments were conducted to determine whether the glycosylated form of op-156 was substantially greater after a 20-min post-translational incubation (Fig. 5, lanes e and i) and the glycosylated polypeptide remained resistant to protease digestion (Fig. 5, lanes f and j). Protection of the opsin nascent chain from proteolytic digestion was strictly limited to those polypeptides that contained oligosaccharide. A prominent protease digestion product was observed which is apparently derived from the membrane integrated, nonglycosylated form of op-156 (Fig. 5, lanes d, f, h, and j, but not lane b, denoted by a downward pointing arrowhead). As both the first and second transmembrane spanning segments of opsin contain functional signal sequences (16), an inverted, hence, nonglycosylated form of the polypeptide could potentially arise during a post-translational incubation by membrane insertion of the first two hydrophobic segments of opsin as a hairpin.

A significantly higher proportion of the opsin chains were integrated and glycosylated in the post-translational assays that were supplemented with the hydrolyzable ribonucleotide triphosphates (Table I). If binding of GMPPNP by an essential translocation component would allow a given translocation site to be used only a single time, then the above result could be readily explained by the presence of an insufficient quantity of translocation sites. However, as the above incubations contained a vast excess of microsomal membranes relative to precursor proteins this trivial explanation can presumably be ruled out.

**DISCUSSION**

The development of elongation-independent assays for membrane protein integration has allowed investigators to probe aspects of the integration process that were formerly inaccessible to analysis. Nonetheless, the majority of currently available evidence indicates that both protein translation and membrane protein integration occur primarily in a cotranslational fashion in mammalian cells (as reviewed in Ref. 28). Therefore, the low molecular weight opsin chain selected for this investigation should adequately represent the size class of polypeptides that are targeted to the endoplasmic reticulum *in vivo*. Post-translational membrane integration of the nascent opsin polypeptide was found to be strictly dependent upon the presence of either GTP or a nonhydrolyzable GTP analogue. Neither GTP hydrolysis nor an ATP source were essential for transport of the amino-terminal domain of opsin across the membrane bilayer. The preceding observations support the participation of a GTP-binding protein as an essential component for the integration of membrane proteins with multiple transmembrane-spanning segments as well as for the translocation of secretory proteins (14). The identity and precise function of the GTP-binding protein remains to be defined. However, based upon previous work (14), as well as that described here, we propose that the GTP-binding protein functions subsequent to SRP and SRP receptor-mediated targeting of the ribosome to the membrane. Nascent chain transport and integration can then proceed without elongation of the polypeptide or hydrolysis of ribonucleotide triphosphates. As noted previously (14), elongation factors eEF-1α and eEF-2 are GTP-binding proteins of well-characterized function in protein synthesis. The use of truncated mRNAs in conjunction with several different protein synthesis elongation inhibitors in our experimental design should exclude the possible participation of either elongation

![FIG. 5. Protease resistance of post-translationally integrated opsin nascent chains.](image_url)

**TABLE I**

Quantitation of glycosylation and integration of opsin nascent chains

Additional aliquots from the post-translational incubation described in Fig. 5 were removed for centrifugation through alkaline sucrose gradients and analyzed by SDS-polyacrylamide gel electrophoresis. The radioactive bands corresponding to op-156 and op-156 were excised from the polyacrylamide gel and quantitated by scintillation counting. The % integration represents the fraction of opsin specific polypeptides recovered in the membrane pellet fraction.

| Time of Incubation with ribonucleotides | Integration | Glycosylation* |
|----------------------------------------|-------------|----------------|
| No nucleotides                         | %           | %              |
| 2 min                                  | 0           | 0              |
| GMPPNP plus AMPPNP                     | 42          | 11             |
| 90 s                                   | 54          | 36             |
| 20 min                                 | 58          | 26             |
| 20 min                                 | 69          | 54             |

*The % glycosylation is the average of the % glycosylation determined from gel lanes a, c, e, g, and i from Fig. 5 and from the identical time points from the alkaline sucrose gradient gel.
factor as a mediator of the GTP-dependent event in membrane protein integration.

Although the hydrolysis-resistant GTP analogue clearly supported membrane integration and glycosylation of op-156, we observed a stimulation of both integration and glycosylation in the presence of ATP plus GTP. The ribonucleotide hydrolysis-dependent stimulation of integration may represent an increase in the proportion of op-156 that is competent for integration. A more extreme example of this phenomenon may account for the ATP hydrolysis requirement for the integration of the 340-residue human glucose transporter nascent chain (12). Nascent polypeptides of this size class may require substantial energy-dependent unfolding prior to integration. The GTP requirement would then be obscured by ribonucleotide diphosphate kinase in conjunction with the ribonucleotide factor as a mediator of the GTP-dependent event in membrane protein integration.

Integration of the 340-residue human glucose transporter requires substantial energy-dependent unfolding prior to integration of the 340-residue human glucose transporter. Nonetheless, it remains to be determined whether the hydrolysis-resistant GTP analogue clearly supports membrane integration and glycosylation of op-156, or is instead an artificially induced consequence of the in vitro post-translational translocation assay procedure.

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REFERENCES
1. Walter, P., and Blobel, G. (1981) J. Cell Biol. 91, 551-556
2. Walter, P., and Blobel, G. (1981) J. Cell Biol. 91, 557-561
3. Meyer, D. I., Krause, E., and Dobberstein, B. (1982) Nature 297, 647-650
4. Gilmore, R., Walter, P., and Blobel, G. (1982) J. Cell Biol. 95, 470-477
5. Gilmore, R., and Blobel, G. (1983) Cell 35, 677-685
6. Anderson, D. J., Walter, P., and Blobel, G. (1982) J. Cell Biol. 93, 501-506
7. Anderson, D. J., Mostov, K. E., and Blobel, G. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 7249-7253
8. Mueckler, M., and Lodish, H. F. (1986) Cell 44, 629-637
9. Friedlander, M., and Blobel, G. (1985) Nature 318, 338-343
10. Holland, E. C., and Dickamer, K. (1985) J. Biol. Chem. 261, 1296-1292
11. Perera, E., Rothman, R. E., and Lingappa, V. R. (1986) Science 232, 345-352
12. Mueckler, M., and Lodish, H. F. (1986) Nature 322, 549-552
13. Caultfield, M. P., Duong, L. T., and Rosenblatt, M. (1986) J. Biol. Chem. 261, 10953-10956
14. Connolly, T., and Gilmore, R. (1986) J. Cell Biol. 103, 2253-2261
15. Raghaire, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fang, S.-L., Mohana Rao, J. K., and Argos, P. (1983) Biophys. Struct. Mech. 9, 235-244
16. Audigier, Y., Friedlander, M., and Blobel, G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5783-5787
17. Nathans, J., and Hogness, D. (1988) Cell 34, 907-914
18. Krieg, P. A., and Melton, D. A. (1984) Nucleic Acids Res. 12, 7057-7070
19. Pelham, H. R. B., and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247-256
20. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazzarow, P. B. (1982) J. Cell Biol. 93, 97-102
21. Erickson, A. H., Conner, G. E., and Blobel, G. (1981) J. Biol. Chem. 256, 11224-11231
22. Hobden, A. H., and Cundliffe, E. (1978) Biochem. J. 170, 57-61
23. Gilmore, R., and Blobel, G. (1985) Cell 42, 497-505
24. Walter, P., Jackson, R. C., Marcus, M. M., Lingappa, V. R., and Blobel, G. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1795-1799
25. Hargrave, P. A. (1977) Biochim. Biophys. Acta 492, 83-94
26. Snider, M. D., and Robbins, P. W. (1982) J. Biol. Chem. 257, 6796-6801
27. Hanover, J. A., and Lennarz, W. J. (1980) J. Biol. Chem. 255, 3800-3804
28. Sabatini, D. D., Kreibich, G., Morimoto, T., and Adesnik, M. (1982) J. Cell Biol. 92, 1-22
29. Waters, M. G., Chirico, W. J., and Blobel, G. (1986) J. Cell Biol. 103, 2625-2636
30. Wiech, H., Sagstetter, M., Muller, G., and Zimmermann, R. (1987) EMBO J. 6, 1011-1016
31. Schlenstedt, G., and Zimmermann, R. (1987) EMBO J. 6, 699-703
32. Hansen, W., Garcia, P. D., and Walter, P. (1986) Cell 45, 397-406
33. Waters, M. G., and Blobel, G. (1986) J. Cell Biol. 102, 1548-1550
34. Rothblatt, J. A., and Meyer, D. I. (1986) EMBO J. 5, 1031-1036