

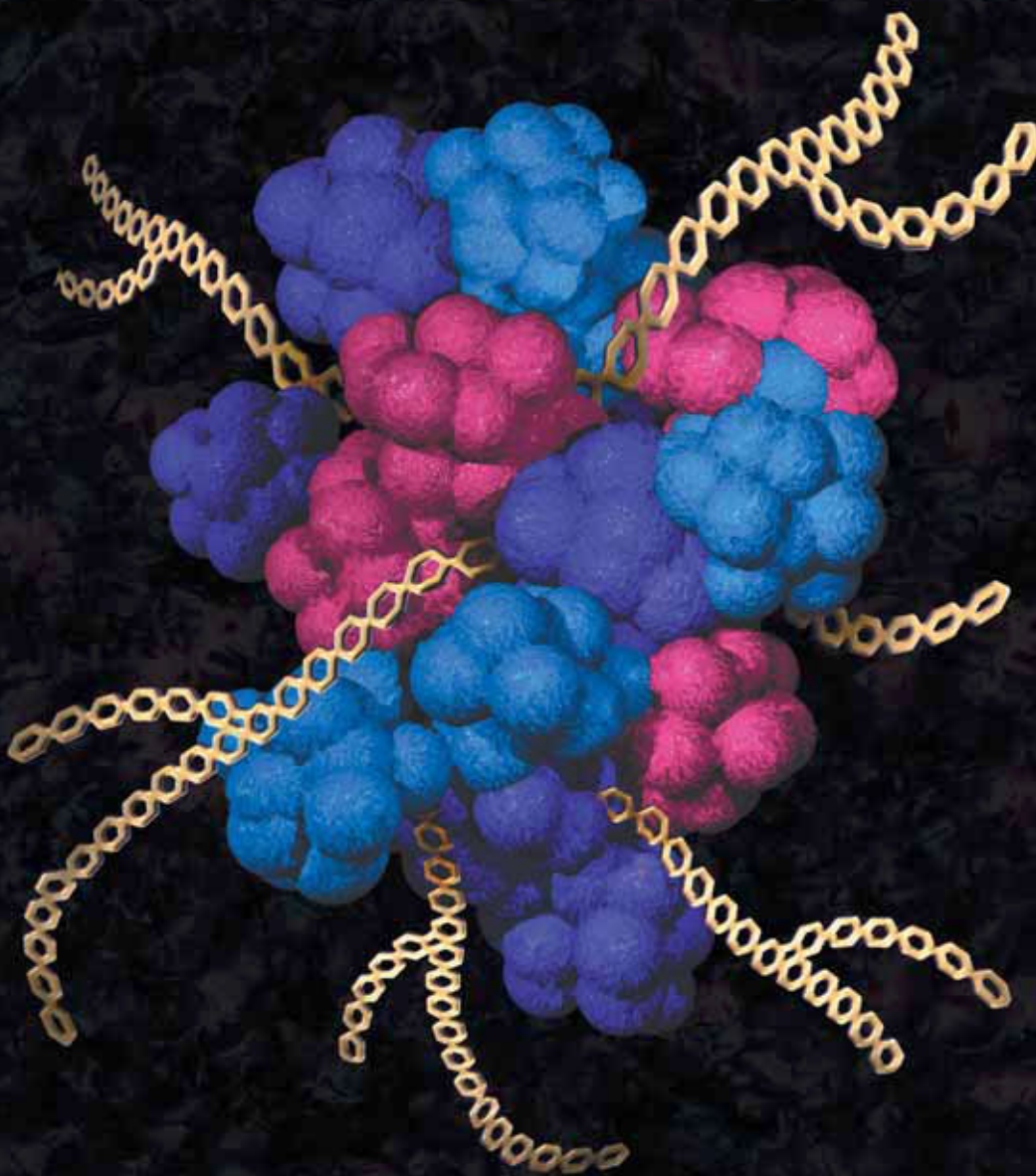
CHAPTER

8

About the Image:

Cell-free protein synthesis systems have been observed to result in a variety of co- and post-translational protein modifications, such as signal peptide cleavage or glycosylation (shown here), phosphorylation, myristoylation and protein folding.

These post-translational protein modifications have been accomplished in cell-free systems such as wheat germ extract and rabbit reticulocyte lysate, both in the presence and in the absence of canine pancreatic microsomal membranes.



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Chapter Eight: Post-Translational Modifications

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Introduction

Several cell-free protein synthesis systems have been used in recent years for the in vitro expression of proteins from numerous sources. A variety of co- and post-translational protein modifications have been observed in both rabbit reticulocyte lysate (RRL) and wheat germ extract (WGE), the most commonly used in vitro expression systems, in the presence and absence of canine pancreatic microsomal membranes. Such modifications have been observed in reactions programmed with template RNA, as well as coupled transcription/translation reactions programmed with DNA. Modifications that have been observed in RRL or WGE include signal peptide cleavage, glycosylation, acetylation, phosphorylation, iso-prenylation, myristoylation, protein folding and proteolytic processing.

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Signal Peptide Cleavage

The most commonly investigated co- and post-translational modifications to in vitro synthesized proteins are signal peptide cleavage and N-linked glycosylation. Proteins destined for secretion, membrane insertion or inclusion into the lumen of certain cellular organelles contain a characteristic sequence at their N-terminus, designated the signal sequence. The signal sequence interacts with the signal recognition particle (SRP), which targets the translating protein to the rough endoplasmic reticulum (RER) by interacting with the SRP receptor (docking protein). Upon insertion into the RER, the signal sequence is removed by a specific protease, termed signal peptidase (1).

Signal peptide cleavage may occur with the appropriate template in rabbit reticulocyte lysate (RRL) in the presence of canine pancreatic microsomal membranes (2), as the proteins are translocated into the microsomal membrane interior. The enzyme responsible for signal peptide cleavage (signal peptidase) has been purified from canine pancreatic microsomal membranes (3). Signal peptide processing, commonly observed as a shift to a lower molecular weight upon analysis with SDS-polyacrylamide gel electrophoresis, has been observed with a number of different protein substrates, including *E. coli* β -lactamase (4; Figure 1, Panel A), human interferon- γ (5), human prostatic acid phosphatase (6), human renin (7) and α -, β - and γ -preprotachykinins (8). Signal peptide cleavage has not been detected in RRL in the absence of microsomal membranes (4–8). This more rapidly migrating band arising from signal peptide processing would also be

predicted to be protected from exogenous protease degradation (see below).

Signal peptide cleavage has also been detected in WGE supplemented with canine microsomal membranes with in vitro synthesized proteins such as human placental SP₁ (9), prolactin and pregrowth hormone (3) and α -, β - and γ -preprotachykinins (8). Studies investigating the signal peptide processing of the preprotachykinins demonstrate that, at least for this particular protein substrate, RRL was much more efficient in processing compared to WGE (8). No signal peptide cleavage has been detected in WGE in the absence of microsomal membranes (3). Please note that Promega's Wheat Germ Extract has been processed for maximal translation. During this process the SRP is removed, thus Promega's WGE will not function with CMMs. Studies by a number of laboratories have demonstrated that WGE lacks the SRP found in RRL (10–12), and that addition of SRP to WGE relieves the translation block that can occur with certain templates in WGE in the presence of microsomal membranes (10–13). This translation block generally occurs with secretory or membrane proteins and is not observed with the in vitro expression of cytoplasmic proteins in this system (12). Interestingly, SRP and SRP receptor (docking protein) homologs have been identified in bacteria, and these homologs are functional in the RRL system (14).

Protein translocation into the interior of membranes is often confirmed using protease protection assays, in which the translation reaction is incubated with a protease such as proteinase K (5, 15–18) or trypsin (2, 8, 19–22).

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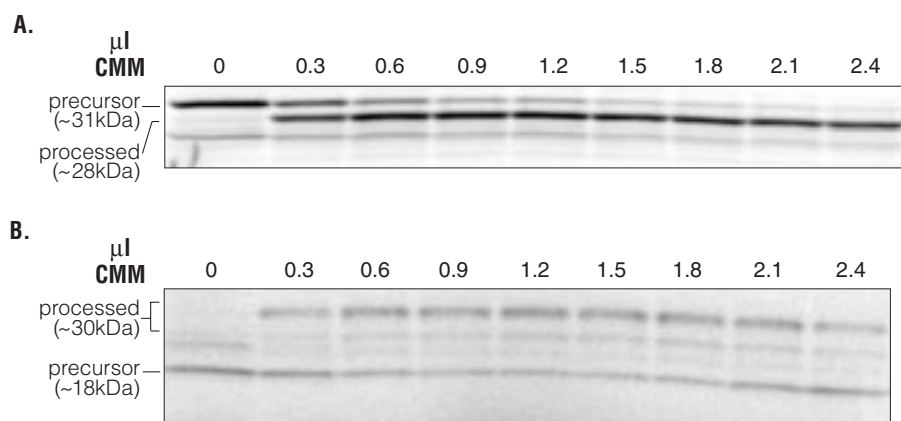


Figure 1. Processing and glycosylation activity of Canine Pancreatic Microsomal Membranes. The positive control mRNAs (**Panel A**, 0.1 μ g of *E. coli* β -lactamase; **Panel B**, 0.1 μ g of *S. cerevisiae* α -factor) were translated using Promega's Rabbit Reticulocyte Lysate in a 25 μ l reaction for 60 minutes in the presence of the indicated amounts of Canine Microsomal Membranes^(c) (CMM) using [³⁵S]met. Aliquots (1 μ l) were analyzed by SDS-PAGE on a 4–20% Novex[®] gel, transferred to a sheet of PVDF (Bio-Rad[®], Sequi-Blot) and exposed to a phosphorimaging cassette plate for 12 hours.

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The protease reactions are performed in the presence or absence of a nonionic detergent, such as Triton® X-100 (2,8,17,18,22) or Nonidet® P-40 (21), because protection due to microsomal incorporation only occurs in the absence of detergent. Translocation may also be detected by separation of the intact membranes from the translation reaction using ultracentrifugation, followed by the subsequent detection of the translocated or intramembrane proteins in the membrane pellet (19). Protease protection of human placental lactogen after post-translational insertion into microsomal membranes was observed by Caulfield *et al.* (23). In addition, studies by Miao *et al.* (22) suggested that cotranslational insertion into microsomal membranes can occur in the absence of signal peptide cleavage.

Glycosylation

Glycoproteins are generated by the addition of oligosaccharides to the NH₂ group of asparagine (N-linked) or to the OH group of serine, threonine, or hydroxylysine (O-linked glycosylation). N-linked glycosylation initiates in the lumen of the RER and further processing occurs in the Golgi apparatus. O-linked glycosylation occurs in the Golgi apparatus (1).

N-linked glycosylation has been detected with a number of different templates when expressed in RRL in the presence of canine microsomal membranes. These include *S. cerevisiae* α -maturing factor (4; Figure 1, Panel B), human interferon- γ (5), human prostatic acid phosphatase (6), human renin (7), rabies virus glycoprotein (16), pro-sucrase-isomaltase (17), asialoglycoprotein receptor H1 (21), human insulin proreceptor (24), P-glycoprotein (25), tissue-type plasminogen activator (TPA; 26), mannose 6-phosphate receptor (MPR; 27) and influenza hemagglutinin (28). Translation reactions for TPA (26), MPR (27) and influenza hemagglutinin (28) were performed in the presence of reduced glutathione (GSSG) to allow for correct glycosylation and protein folding, and thus native enzymatic activity. In vitro expression of the cystic fibrosis transmembrane conductance regulator (CFTR) in RRL in the presence of microsomal membranes showed the CFTR to be glycosylated in the absence of signal peptide cleavage and not glycosylated when translated in WGE in the presence of microsomal membranes (29). The lack of glycosylation of the CFTR protein in WGE was postulated to be due to the lack of SRP in WGE, and expression of CFTR in RRL in the absence of membranes required the presence of Triton® X-100 to release the SRP block (29).

N-linked glycosylation is generally detected by the shift to a higher molecular weight form upon analysis by SDS-polyacrylamide gel electrophoresis. The presence of N-linked glycosylation is then confirmed by digestion of the expressed protein with either endoglycosidase H (6,9,15,21,22), N-glycanase (5), glycopeptidase A (7), or α -mannosidase (9) prior to gel electrophoresis. After disruption of the lipid bilayer of the membranes with 0.1% of the nonionic detergent Nikkol, core glycosylation does not occur, although signal peptide cleavage remains intact, allowing for the discrimination between these two processing events (2,28). Glycosylation can also be inhibited by the addition of N-benzoyl-Asn-Leu-Thr-N-methylamide to the translation reaction (27).

N-linked glycosylation has also been detected in WGE supplemented with canine microsomal membranes with various in vitro expressed substrate proteins (18,25,30). Studies by Spiess *et al.* (21) indicated that different stop-transfer signals for translocation into microsomal membranes may be utilized with different efficiencies in RRL compared to WGE. In addition, studies by Zhang and Ling (25) suggested that the membrane topology of expressed proteins in the presence of microsomal membranes is different depending on the in vitro expression system utilized (RRL versus WGE). Studies by Lopez *et al.* (31) suggested that RRL contains a cytoplasmic component necessary for the generation of secretory forms for certain proteins, which is not present in WGE.

O-linked glycosylation has been observed in the absence of microsomal membranes in RRL, while this same modification was not observed with the same template in WGE (32). The addition of O-linked oligosaccharides probably occurs post-translationally, as opposed to the co-translational addition of N-linked oligosaccharides (32). The lack of glycosylation in WGE in the absence of microsomal membranes was also observed by Kottler *et al.* (33).

Acetylation and Phosphorylation

In addition to signal peptide cleavage and N-linked glycosylation, other co- and post-translational protein modifications have been observed in these in vitro expression systems. The removal of the N-terminal methionine residue has been observed in RRL for various protein substrates including ovalbumin and others (34-37), and N-Met removal is sometimes followed by N-terminal acetylation (34). N-terminal acetylation is usually produced early in translation when the nascent chain is still attached to the ribosome, but little is known

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about its significance (1). N-terminal acetylation can also occur in the absence of the removal of the N-terminal methionine residue (35,38–40). Acetylation can be inhibited by the addition of S-acetyl CoA to the translation reaction (35).

The addition of phosphate groups to serine, threonine or tyrosine residues in cellular proteins is a common post-translational modification, and for many proteins this modification alters their biological activity or function. Phosphorylation is mediated by the action of a wide variety of protein kinases present in the cellular milieu (1).

Phosphorylation of in vitro expressed proteins has also been observed. Examples of proteins phosphorylated in RRL include human type I keratins (40), rat *c-fos* (41), max proteins (42), human immunodeficiency virus type I vpu protein (43) and eIF-4E (44). Phosphorylation of the type I keratins appeared to occur post-translationally, following release from ribosomes (40). Phosphorylation events are likely due to cAMP-dependent protein kinase (PKA; 41), protein kinase C (PKC; 44), casein kinase II (42,43,45) and potentially other kinases as well. The serine/threonine phosphorylation of the max proteins could be inhibited by staurosporine (42). Phosphorylation in WGE and RRL has been demonstrated for in vitro expressed chicken *myoD1* (46). The addition of 3mM EDTA to the reaction blocked the phosphorylation of *myoD1* (46).

Isoprenylation and Myristoylation

The linkage of isoprenoid (lipid) groups to cysteine residues represents an important type of post-translational protein modification in eukaryotic cells and may play a role in membrane localization (47). Isoprenylation of in vitro expressed proteins translated in the RRL system have also been demonstrated. Isoprenylation was observed for human p11 protein (48), the G-protein γ subunit (47,49), Rap2a and Rap2b (50), the Hepatitis delta virus large antigen (51) and the human homolog of bacterial DnaJ (52). The lipid moieties attached include both farnesyl (49,50,52) and geranylgeranyl groups (49–51). Farnesylation may be inhibited by the addition of α -hydroxy-farnesylphosphonic acid to the translation reaction (52). In the presence of microsomal membranes, isoprenylation, as well as methylation and AAX endopeptidase cleavage, have also been demonstrated in RRL for the *ras* superfamily of GTPases (53). In addition, the glypiation of protein substrates has been demonstrated for mouse Thy-1 antigen and human decay-accelerating factor when expressed in RRL supplemented with microsomal membranes (54). Glypiation involves the

addition of glycosyl-inositolphospholipid structures to the C-terminus of proteins (54).

Proteins expressed in vitro using the RRL system may also be modified by myristoylation (49,55–58). The linkage of myristoylate groups to proteins may play a role in membrane localization, similar to isoprenylation (47). Using synthetic octapeptide substrates, N-myristoyltransferase (NMT) activity was detected in both RRL and WGE (58). Myristoylation was also detected when the vaccinia virus gene encoding the major late myristoylated virion protein L1R was expressed in WGE (59). Myristoylation of the L1R protein in the WGE also involved removal of the N-terminal methionine residue (59).

Protein Folding and Chaperones

Protein folding and renaturation in vivo are often mediated by cellular chaperones, present both in the cytoplasm and endoplasmic reticulum of cells (see reference 60 for a review). Chaperones generally interact with hydrophobic regions of newly synthesized or denatured proteins to prevent aggregation or spurious interactions with other proteins. Molecular chaperones and chaperone activity have been detected in RRL and canine pancreatic microsomal membranes and include Hsp90 (61,63–67), Hsp70 (61–63,65,66), BiP (67), protein disulfide isomerase (67), p60 (61), p48 (61), p23 (61,63), calnexin (67), Hop (63), Hip (63), Hsp 56 immunophilin (65), Hsp40 (66) and TriC (66). Frydman *et al.* (66) quantitated the levels of Hsp70, Hsp90 and TriC in RRL to be 2 μ M, 2 μ M and 0.7 μ M, respectively. Functional Hsp90 has not been detected in WGE (68,69), and differential folding has been observed with various protein substrates when expressed in RRL and WGE, resulting in active protein only in the RRL (68–70). The correct folding for in vitro expressed mutant p53 was demonstrated to require functional Hsp90 (68). Similarly, functional Hsp90 was required for the in vitro expression of functional glucocorticoid receptor (69). The in vitro synthesis of an active protein in some instances requires the addition of oxidized glutathione to RRL (26–28,71).

Proteolytic Processing and Ubiquitin

An additional type of co- or post-translational modification, which may occur during in vitro expression, is proteolytic processing. Rabbit reticulocyte lysate has been shown by a number of laboratories to contain an active ubiquitin conjugation and degradation pathway, including active 26S and 20S proteasome systems (72–80). In general, small proteins (less than 15kDa) expressed in RRL may be particularly

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susceptible to degradation by the ubiquitin pathway, and WGE may be a more suitable system for the in vitro expression of small proteins.

The preferential degradation of in vitro expressed ornithine decarboxylase (74) and coronavirus infectious bronchitis virus 1a polyprotein (75) have been demonstrated in RRL. Studies by Wajnberg and Fagan (73) and Sato *et al.* (80) have shown that hemin and polyamines can inhibit the ubiquitin proteasome degradation pathway, and in general, commercial preparations of RRL contain exogenously added hemin. The addition of ATP γ S to the translation reaction can inhibit degradation without interfering with ubiquitination (81). Protein degradation pathways independent of ubiquitin and ATP have been identified in RRL as well and appear to target oxidatively damaged proteins for proteolysis (82). In addition, the differential degradation of Sos1 versus mSos2 has been observed in RRL but not in WGE (81). Both RRL and WGE appear to contain aminopeptidase-, chymotrypsin- and elastase-like

activities, while in addition, WGE also possesses trypsin-like and post-proline cleavage activities (83,84). Thus the stability of an in vitro expressed protein can vary depending on the type of expression system used and specific characteristics of the expressed protein.

Rabbit reticulocyte lysate, wheat germ extract and canine pancreatic microsomal membranes are complex biological systems, and thus the various co- and post-translational modifications observed in in vitro expression reactions utilizing these systems may vary among different preparations of lysate, extract, or microsomes. The protein product of an in vitro expression reaction may deviate from the predicted molecular weight due to one or more of the many possible modifications discussed here, as well as other uncharacterized modifications. Only by further experimental investigation can the nature of the modifications, as well as their potential functional and physiological significance, be determined.

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