Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8604–8608, November 1986
Cell Biology

Photocrosslinking of the signal sequence of nascent preprolactin to the 54-kilodalton polypeptide of the signal recognition particle

(protein secretion/Lys-tRNA analogs/photoaffinity-labeling of elongation factor Tu)

UTE C. KRIEG*, PETER WALTER†, AND ARTHUR E. JOHNSON*‡

*Department of Chemistry, University of Oklahoma, Norman, OK 73019; and †Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448

Communicated by Bruce M. Alberts, July 7, 1986

ABSTRACT **Photoreactive moieties were incorporated into nascent polypeptides in a wheat germ protein-synthesizing system by using a plasmid-derived preprolactin mRNA and a Lys-tRNA analog, N^{ϵ}-(5-azido-2-nitrobenzoyl)-Lys-tRNA (εANB-Lys-tRNA). The presence of the abnormally large amino acid side chains in the nascent chains did not impair function: complete preprolactin chains were synthesized in the absence of the signal recognition particle (SRP), elongation was arrested in the presence of SRP, and SRP-dependent translocation across the membrane of the endoplasmic reticulum and signal peptidase cleavage were observed in the presence of salt-extracted microsomes. Photolysis of elongation-arrested ribosomes resulted in several light- and εANB-Lys-tRNA-dependent crosslinks. By using antibodies specific for each of the proteins, one covalent complex was shown to be a photocrosslink between the preprolactin nascent chain and the 54-kDa protein subunit of SRP. This demonstrates that the N-terminal end of a secretory protein is located adjacent to the SRP in elongation-arrested ribosomes and strongly suggests that the signal sequence is recognized by and binds to the 54-kDa subunit of SRP. The other photocrosslinks involve as-yet-unidentified proteins in the large ribosomal subunit, indicating that this method of incorporating probes provides a powerful approach to examining the environment and interactions of the nascent chain during translation and translocation across the membrane of the endoplasmic reticulum. The Lys-tRNA analog also successfully photoaffinity-labeled the *Escherichia coli* elongation factor Tu (EF-Tu) in the εANB-Lys-tRNA·EF-Tu·GTP ternary complex.**

Protein transport across the membrane of the endoplasmic reticulum, a process complex both in its selectivity and in a mechanical sense, is accomplished by an ordered sequence of interactions that involves both cytoplasmic and membrane-bound multicomponent complexes of macromolecules (1). The critical component in terms of both selectivity and the linking of the initially cytoplasmic structures to the membrane is the signal recognition particle (SRP). SRP appears to function by associating with the signal sequence as it emerges from the translating cytoplasmic ribosome (2). Upon formation of this complex, protein chain elongation is temporarily halted (3) until the SRP–nascent chain–ribosome–mRNA complex interacts with the SRP receptor or "docking protein," an integral part of the endoplasmic reticulum membrane. The direct interaction between the SRP and the SRP receptor elicits the release of the elongation arrest, the initiation of protein translocation across the membrane (4, 5), and the release of SRP from the ribosomal complex (6). However, the specific molecular mechanisms by which SRP identifies polypeptides destined for transport and then facilitates that process remain, for the most part, to be elucidated.

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.

When signal sequences that are rich in leucine are synthesized in the presence of an amino acid analog, β-hydroxyleucine, the interactions between the signal sequences and the SRP are abolished (2, 3, 7). This result suggests that the SRP recognizes and binds directly to the signal sequence, but it is conceivable that the change in the signal sequence structure allosterically alters the SRP interaction with the ribosomal complex. Therefore, knowledge of the relative location of the SRP and the signal sequence would be very useful. If the nascent chain were found to be positioned next to the SRP in the SRP–ribosomal complex, then that would provide evidence for an interaction between SRP and the signal sequence.

The most direct way to identify the macromolecules that are located adjacent to the nascent chain is to determine which molecules can be covalently linked to the nascent chain. To do this most efficiently, one needs to incorporate a chemically or photochemically reactive probe into the nascent chain by using the normal protein synthesis machinery and a functional analog of an aminoacyl-tRNA that has the probe covalently attached to the side chain of the amino acid. Several years ago, we demonstrated that Lys-tRNA could be chemically modified to place a moiety on the side-chain amino group and that the resultant amino acid analog was incorporated into complete globin chains in a reticulocyte lysate (8). A chemically reactive species of this class of analogs, N^{ϵ}-bromoacetyl-Lys-tRNA has since been used successfully to affinity-label ribosomes (9), the prokaryotic elongation factor Tu (EF-Tu) (10), and the corresponding eukaryotic elongation factor (EF-1α) (11).

In this paper, we report the preparation of a functional photoreactive analog of Lys-tRNA and its use as a photoaffinity-labeling probe of the nascent chain interaction with SRP. The discovery of specific photocrosslinks between the nascent chain and proteins associated with both SRP and the large ribosomal subunit confirms that this approach provides an excellent opportunity to examine protein synthesis and secretion from the point of view of the nascent chain.

METHODS

Transfer RNA. Yeast tRNA (Plenum, Hackensack, NJ) was aminoacylated with [^{3}H]lysine as described (10, 11). Modification of the Lys-tRNA was carried out in a darkroom illuminated with a sodium vapor lamp equipped with UV cutoff filters. All subsequent procedures, such as the translation incubations, which involved the photoreactive Lys-tRNA prior to photolysis, were also done in the darkroom. The *N*-hydroxysuccinimide ester of 5-azido-2-nitrobenzoic acid (8.8 mg; Pierce) dissolved in 1.75 ml of dimethyl

Abbreviations: εANB-Lys-tRNA, N^{ϵ}-(5-azido-2-nitrobenzoyl)-Lys-tRNA; SRP, signal recognition particle; EF-Tu, elongation factor Tu; KRM, salt-extracted microsomal membranes; CTAB, cetyltrimethylammonium bromide.

‡To whom reprint requests should be addressed.

sulfoxide was added with stirring at 0°C to a solution of Lys-tRNA (0.75 ml; 55–140 A_{260} units) in 50 mM potassium phosphate (pH 7.0); immediately thereafter 15 μl of 4 M KOH was added to the reaction vessel. After 20 sec, 15 μl of 4 M HOAc was added, followed by 2.5 ml of 2.0 M KOAc (pH 5.0) and 15 ml of cold ethanol. The modified Lys-tRNA was purified by using a benzoylated DEAE-cellulose (Boehringer Mannheim) column: the material that was eluted in buffer A (1.0 M NaCl/0.01 M NaOAc, pH 4.5/0.01 M $MgCl_2$) was discarded, and the material that was eluted in buffer A containing 25% (vol/vol) ethanol was designated εANB-Lys-tRNA. (Unacylated tRNA that required ethanol for elution was removed prior to aminoacylation.) After concentration and dialysis into 1 mM KOAc (pH 5.0), the εANB-Lys-tRNA was stored at −80°C in the dark. The extent of modification of Lys-tRNA was determined by using paper electrophoresis at pH 3.6 for 150 min at 28 V/cm (8).

Translation Components. Wheat germ extract was prepared as described (12) except that the material was chromatographed twice over Sephadex G-25 to minimize the free lysine present and was stored at pH 7.5 in 40 mM Hepes/100 mM KOAc/5 mM $Mg(OAc)_2$/2 mM glutathione/0.1 mM EGTA. Microsomal membranes, salt-extracted microsomal membranes (KRM), and SRP were prepared as detailed elsewhere (13–15). A plasmid, pSPBP4, was constructed by inserting a 950-base-pair (bp) *Nco* I/*Pst* I fragment that codes for preprolactin (derived from pBPRL72; see ref. 16) into the pSP64T vector (17). The *Bgl* II site of pSP64T was converted to a *Nco* I site prior to insertion of the preprolactin fragment by using an *Nco* I linker ACCATGGT so that the AUG codon could be restored in optimal context for translational initiation (18). The preprolactin vector was linearized using *Pst* I endonuclease, and preprolactin mRNA was transcribed as described (19) except that the GTP concentration was raised to 0.5 mM after 90 min to ensure completion of all transcripts.

Translations. Incubations were at 20°C and pH 7.5 in 25 μl of 144 mM KOAc/3.0 mM $Mg(OAc)_2$/0.2 mM spermidine/12 mM triethanolamine/25 μM EGTA/8 μM *S*-adenosylmethionine/0.0024% Nikkol (Nikko Chemicals, Tokyo)/1 mM glutathione containing preprolactin mRNA (50–60 ng), 5.8 pmol of εANB-Lys-tRNA (0.06 A_{260} unit) or 3.0 pmol of Lys-tRNA (0.06 A_{260} unit), and, where indicated, SRP (1.5 μg) and/or KRM (2.5 equivalents; see ref. 2). Wheat germ extract, amino acids, an energy-generating system, placental RNase inhibitor, and protease inhibitors were included as described (12), except that no free lysine was added and methionine was added only when [^{35}S]methionine was absent. When included in the incubation mixture, [^{35}S]-methionine (Amersham, 1 Ci/μmol; 1 Ci = 37 GBq) was present at 1–2 mCi/ml. No tRNA was added other than that which accompanied the εANB-Lys-tRNA or Lys-tRNA because translation was impaired whenever the total added tRNA exceeded 0.1 A_{260} unit per 25-μl of incubation mixture. After 2 min of incubation, further initiation was inhibited with 2 mM 7-methylguanosine 5′-monophosphate.

Photoreactions. Translation was halted after 20 min by placing the sample tubes in ice and adding cycloheximide (Sigma) to 1 mM. Samples, located about 5 cm from a lamp, were then photolyzed for 2 min by using a Fotodyne UV 300 transilluminator. After photolysis, 300 μl of 2% (wt/vol) cetyltrimethylammonium bromide (CTAB) was added to a 25-μl translation mixture, followed by 60 μg of yeast tRNA in 300 μl of 0.5 M NaOAc (pH 5.4) (20). After 30 min at 30°C, samples were centrifuged in a Microfuge. The pellets were washed three times in acetone/1 M HCl, 19:1 (vol/vol), and then redissolved in 3% (wt/vol) $NaDodSO_4$/125 mM Tris base by incubation at 65°C for 30 min prior to analysis by $NaDodSO_4$/PAGE.

Immunoprecipitations. CTAB pellets from 100-μl translation incubations were dissolved in 50 μl of 3% $NaDodSO_4$/100 mM Tris base at 65°C for 30–60 min. The samples were boiled for 5 min and then adjusted to contain 20 mM Tris·HCl, 150 mM NaCl, 0.2% $NaDodSO_4$, and 1.0% (vol/vol) Triton X-100. After the pH was adjusted to 7.0–7.5, antiserum or immunoselected IgG (typically 7.5 μg) was added. After an overnight incubation at 4°C, the antibody–antigen complexes were recovered by adsorption for 2 hr at 22°C to protein A-Sepharose.

RESULTS

Preparation of εANB-Lys-tRNA. The method for attaching a small probe, covalently and selectively, to the lysine ε-amino group of Lys-tRNA has been described (8, 9). The same approach was taken to prepare a Lys-tRNA analog with a photoreactive moiety on the lysine side chain. However, dimethyl sulfoxide was used in the reaction solvent to accommodate the increased nonpolarity of the aromatic succinimide ester. Under the given conditions, the extent of labeling at the ε-amino group was high (usually >95%), while the extent of labeling of the α-amino group was low (usually <15%). In general, the recovery of aminoacyl-tRNA specific activity (pmol of lysine per A_{260} unit) exceeded 80% despite the exposure to high pH, and >75% of the total aminoacylated tRNA was εANB-Lys-tRNA.

Unmodified Lys-tRNA will compete with εANB-Lys-tRNA for incorporation into protein and thereby complicate experiments to test the activity of the analog, whereas unacylated tRNA at high concentrations inhibits translation. Consequently, chromatography over benzoylated DEAE-cellulose was used to remove most of the Lys-tRNA (Fig. 1) and 50–75% of the unacylated tRNA. Separation of the εANB-Lys-tRNA from either αANB-Lys-tRNA or α,ε-$(ANB)_2$-Lys-tRNA was not essential because the only photoreactive moieties that will be incorporated into a nascent chain will be the εANB-lysines.

Interaction of εANB-Lys-tRNA with EF-Tu. To ensure that the photoreactive azido group survived the various preparative procedures and that the ε-modified Lys-tRNA was functionally active, we examined its ability to photoaffinity-label *Escherichia coli* EF-Tu. The rate of aminoacyl bond hydrolysis of εANB-Lys-tRNA was reduced in the presence of both EF-Tu and GTP (Table 1), demonstrating that the aminoacyl-tRNA formed a ternary complex with EF-Tu·GTP. Upon photolysis, typically 9–12% of the εANB-Lys-tRNA was crosslinked to EF-Tu·GTP, and this covalent reaction required ternary complex formation (Table 1). Thus, εANB-Lys-tRNA is functionally and photochemically intact, and it successfully photoaffinity-labels EF-Tu.

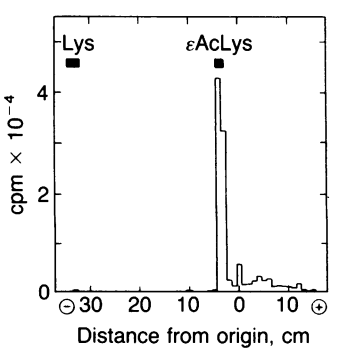

FIG. 1. Composition of an εANB-[^{3}H]Lys-tRNA sample. The electrophoretic analysis shows that this typical preparation contained 80.1% εANB-Lys and 0.9% unmodified lysine, not including lysines at the origin. Based on previous results (8, 9), the radioactivity at +3 to +5 cm is α,ε-$(ANB)_2$-Lys, while that at the origin is still covalently attached to tRNA. Lysine and N^{ε}-acetyllysine (εAcLys) marker locations were visualized by using ninhydrin.

Table 1. Photoaffinity labeling of EF-Tu

Incubation components	Aminoacyl bond protection	Fraction crosslinked	Light-dependent crosslinking, %
Complete	0.93	0.104	9.4
−light	0.95	0.010	0
−*P-e*Prv	0.59	0.063	5.3
−PK	0.59	0.040	3.0
−EF-Tu	0.59	0.017	0.7
−EF-Tu/PK	0.62	0.007	0

Incubation mixtures (175 μl) contained 1.2 nmol of EF-Tu (21), 25 pmol of εANB-[^{3}H]Lys-tRNA, 3.5 units of pyruvate kinase (PK; Sigma), 50 mM Hepes (pH 7.6), 100 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM phospho*enol*pyruvate (*P-e*Prv), and 10 μM GDP, except where indicated. After preincubation (10 min at 37°C) to convert GDP to GTP, εANB-Lys-tRNA was added to samples and incubated for 5 min at 0°C to allow ternary complex formation. Protection is expressed as the fraction of cold acid-insoluble cpm remaining after incubation at 37°C for 90 min (10). Crosslinking is expressed as the fraction of the initial cold acid-insoluble radioactive material that was hot acid-insoluble (10) after a 2-min photolysis (see *Methods*). Data shown are from a typical experiment.

At high concentrations, EF-Tu·GDP associates with aminoacyl-tRNA (22, 23), and the relatively high extent of covalent reaction with EF-Tu observed in the absence of pyruvate kinase or phospho*enol*pyruvate (Table 1) was probably caused by photocrosslinking in εANB-Lys-tRNA·EF-Tu·GDP ternary complexes.

Incorporation of Lysine Analog into Protein. A translation system was programmed with a mRNA that was prepared by *in vitro* transcription of a plasmid encoding preprolactin. The ribosomes accepted εANB-Lys-tRNA as a substrate, and the amino acid analog was incorporated into complete preprolactin chains (Fig. 2, lane 1). These chains were translocated and correctly processed into prolactin in the presence of SRP and KRM (Fig. 2, lane 3). Equally important, elongation was arrested in the presence of SRP (Fig. 2, lane 2). Thus, the presence of the modified lysine residues in the nascent chain does not prevent the signal sequence-dependent and SRP-mediated arrest of protein synthesis. Salt-extracted membranes in the absence of added SRP were diminished in their translocation activity (Fig. 2, lane 4), indicating that the translocation was SRP-dependent. The membrane preparation used in this experiment was not entirely free of SRP, as indicated by the band of prolactin visible in lane 4.

Photocrosslinking of Nascent Chains. Translations were carried out in the presence of [^{35}S]methionine to facilitate the detection of crosslinks. To maximize the homogeneity of the samples prior to photolysis, translations were pulsed[§] by the addition of a cap analog to inhibit initiation. To enrich for nascent chains, we precipitated peptidyl-tRNA by the addition of CTAB at the end of some incubations. This procedure removed most of the complete chain preprolactin and other translation products (compare lanes 1 and 2 in Fig. 3 *A* and *B*) without greatly affecting the recovery of arrested fragments (compare lanes 3 and 4 in Fig. 3 *A* and *B*) and thereby reduced the background considerably.

When samples that contained εANB-Lys-tRNA were photolyzed, new radioactive species were formed, both in the presence (compare lanes 3 and 4 in Fig. 3*B*) and in the absence (compare lanes 1 and 2 in Fig. 3*B*) of SRP. These new bands on the gel presumably resulted from the covalent crosslinking of radioactive nascent chains to proteins present in the ribosomal or ribosome–SRP complexes. In contrast, there

[§]Translations have been termed "pulsed," rather than the widely-used "synchronized," because initiation events are not coincident in the incubation, and individual ribosomes will not traverse the mRNA at the same rate.

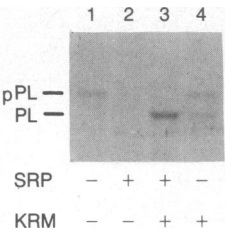

FIG. 2. Functional activity of εANB-[^{3}H]Lys-tRNA. Translation reactions (60 min, not pulsed) were not exposed to light and contained SRP and KRM as indicated. After precipitation in trichloroacetic acid, the proteins in the 15-μl samples were separated by PAGE in $NaDodSO_4$ using 10–15% gradient gels (2, 14). The tritium-labeled translation products were visualized by fluorography. pPL, preprolactin; PL, prolactin.

was no difference in the irradiated and nonirradiated translation incubations that contained unmodified Lys-tRNA (Fig. 3*C*). Thus, the formation of the photocrosslinks was mediated by the aryl azide incorporated into nascent chains.

When SRP was present, three prominent photoproducts with molecular mass values of 62, 49, and 46 kDa (Fig. 3*B*, lane 4, arrows) were visible in fluorographs of the gel. The formation of these covalent adducts was light-dependent, probe-dependent, and stimulated by the presence of SRP. The small number of different crosslinked species indicates that nascent chains that are arrested in their elongation by SRP can form covalent complexes with only a defined and limited subset of the protein components of the SRP–ribosome complex.

In the absence of SRP, many new radioactive species were found in the gel following photolysis (Fig. 3*B*, lane 2). These photoadducts were heterogeneous in terms of their nascent chain content because SRP was not present to arrest elongation. Also, in the longer preprolactin nascent chains, photoreactive lysine analogs were located at positions other than in the signal sequence and, therefore, were exposed to different crosslinking targets.

Sedimentation Analysis. To identify the source of the

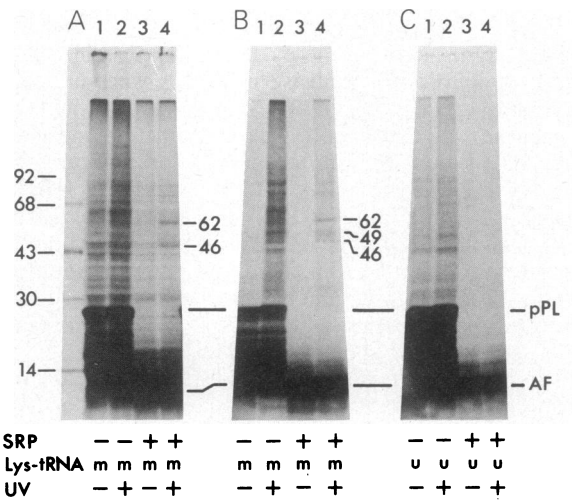

FIG. 3. Photocrosslinking of nascent and SRP-arrested nascent preprolactin (pPL) chains. Pulsed translation incubations were photolyzed as shown and contained [^{35}S]methionine, unmodified Lys-tRNA (u) or εANB-Lys-tRNA (m), and, where indicated, SRP. Prior to PAGE in $NaDodSO_4$, samples (15 μl in *A* and 25 μl in *B* and *C*) were precipitated by the addition of either trichloroacetic acid (*A*) or CTAB (*B* and *C*). Fluorographs of the 10–20% gradient gels are shown. Molecular sizes are shown in kDa, and the locations of the 62-kDa, 49-kDa (this usually appears as a doublet), and 46-kDa photocrosslinked species are indicated. AF, arrested fragment.

proteins that had been crosslinked to the nascent chain, the SRP–ribosome complexes were dissociated with puromycin and high salt (24) and then sedimented to separate the SRP and the 40S and 60S ribosomal subunits from each other. The 49-kDa and 46-kDa photocrosslinked species were found to be associated with the large ribosomal subunit (fractions 11 and 12 in Fig. 4*B*), while the 62-kDa photocrosslinked material sedimented at a much slower rate (fractions 2–4 in Fig. 4*B*). Immunoprecipitations of the gradient fractions with serum raised against prolactin showed that the 49-kDa band (as well as the 62-kDa band; see below) contained epitopes of prolactin (Fig. 4*C*). Thus, the 49-kDa covalent complex appears to originate from photocrosslinking the nascent chain to a protein that cosediments with the large ribosomal subunit. The presence of the IgG heavy chain in the gels prevented detection of the 46-kDa band (Fig. 4*C*). No radioactivity was found in the fractions containing the small ribosomal subunit, indicating that the nascent chain is associated with the large ribosomal subunit (cf. ref. 25).

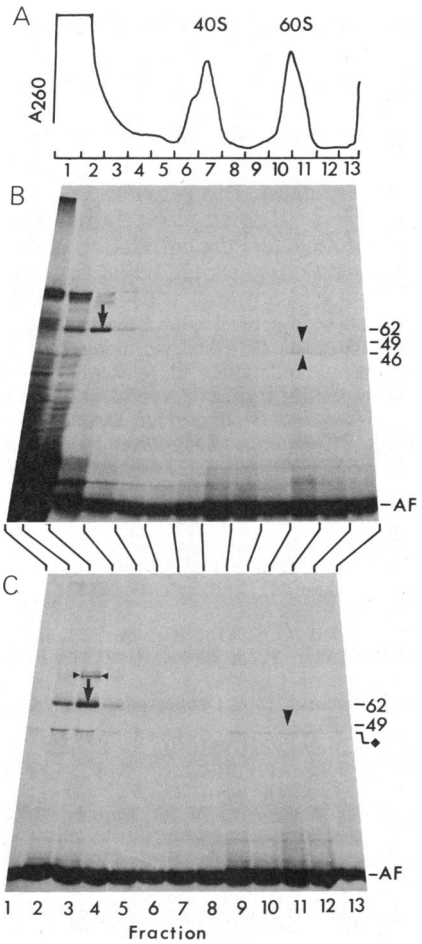

FIG. 4. Sedimentation analysis of photoproducts. A pulsed translation incubation (300 μl) containing SRP and [^{35}S]methionine was photolyzed; adjusted to contain 1 mM puromycin, 0.5 M KCl, 2 mM $Mg(OAc)_2$, and 50 mM triethanolamine (pH 7.5); and incubated at 0°C (15 min) and then at 37°C (10 min) to dissociate the SRP–ribosome complexes (24). Samples were then layered onto 13-ml 5–20% (wt/vol) linear sucrose density gradients in 0.5 M KCl/5 mM $MgCl_2$/50 mM triethanolamine, pH 7.5 and sedimented at 20°C and 40,000 rpm for 165 min in a Beckman SW 40 rotor. Gradients were fractionated by using either a Buchler Auto Densi Flow II or an Isco fractionator. (*A*) Absorbance profile of a sample after centrifugation. The protein content of each 1-ml gradient fraction was examined after acid precipitation by using $NaDodSO_4$/PAGE either before (*B*) (0.3 ml of each fraction) or after (*C*) immunoprecipitations with anti-prolactin serum (United States Biochemical, Cleveland, OH; 2 μl of serum per 0.6 ml of fraction). The location of the large ribosomal subunits in the fluorographs of the 10–20% gels (*B* and *C*) is slightly different from their location in the absorbance profile (*A*) because the absorbance profile and fluorographs are from different experiments. Molecular sizes are shown in kDa. The arrow in *B* and *C* marks the location of the 62-kDa photocrosslinked species. The small arrowheads indicate unidentified photocrosslinked material that cosediments with SRP. The diamond in *C* indicates the position of the IgG heavy chain front, which is important to localize because its presence obscures the detection of bands in this region. The 49-kDa band is only faintly visible (arrowhead), whereas the 46-kDa band may be completely obscured. AF, arrested fragment.

The sedimentation rate of the material containing the 62-kDa photocrosslink was consistent with its being associated with SRP (Fig. 4). Therefore, a photolyzed translation incubation was sedimented long enough to resolve the 11S SRP from ribosomal subunits and peptidyl-tRNA. The 62-kDa material was found to sediment at the same rate as purified SRP (Fig. 5). Hence, the radioactively labeled and photoreactive nascent chain appears to react covalently with a component of SRP to form a 62-kDa covalent adduct.

Two minor, higher molecular mass species were also immunoprecipitated with anti-prolactin (Fig. 4*C*); the origin of this material has not yet been determined.

Identification of the Nascent Chain-SRP Crosslink. SRP-arrested nascent chains are ≈70 amino acids long and have a molecular mass of 8 kDa (3). This suggested that the 62-kDa band constituted a covalent complex formed by photocrosslinking the arrested preprolactin fragment containing εANB-Lys to the 54-kDa protein of SRP. This presumption was confirmed by the demonstration that the 62-kDa material was immunoprecipitable by antibodies specific for either one of the covalently linked polypeptides, prolactin and the 54-kDa SRP protein (Fig. 6, lanes 2–4). When these antibodies were added to control samples (nonirradiated samples or translations lacking SRP), they failed to immunoprecipitate a 62-kDa species (Fig. 6, lanes 6–9).

DISCUSSION

A unique class of modified aminoacyl-tRNAs has been utilized to place photosensitive probes in the nascent chain obtained by translation of preprolactin mRNA. The *in situ* generation of the nascent chain ensures that the ribosome and other macromolecules will be in the proper orientation during any examination of nascent chain environment and interactions. Elongation arrest of nascent preprolactin carrying the

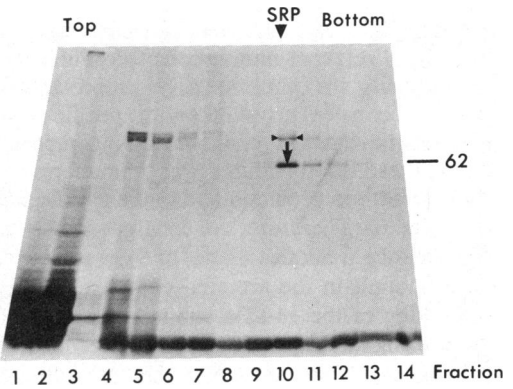

FIG. 5. Crosslinking of nascent chain to SRP. A pulsed translation incubation (100 μl) containing [^{35}S]methionine and SRP was photolyzed and treated as described in Fig. 4. The sample was sedimented in the SW 40 rotor at 40,000 rpm for 20 hr at 4°C and analyzed by $NaDodSO_4$/PAGE without immunoprecipitation. The position of purified SRP centrifuged in a parallel gradient is indicated. The arrow marks the position of the 62-kDa material, while the small arrowheads indicate minor bands of SRP-associated nascent chains. Most of fraction 3 was lost during work-up.

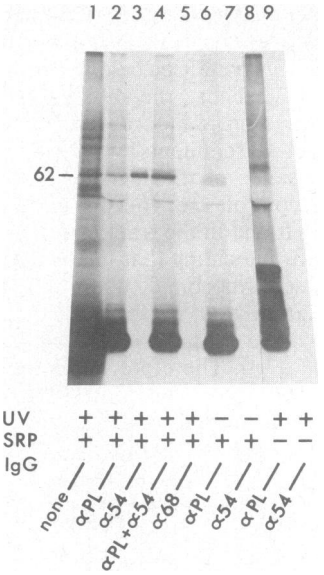

FIG. 6. The 62-kDa photoadduct contains epitopes of both prolactin and the 54-kDa polypeptide of SRP. Translation mixtures containing SRP where indicated and [^{35}S]methionine were precipitated in CTAB and analyzed by immunoprecipitation and NaDodSO$_4$/PAGE. The material in lanes 2–9 was immunoprecipitated from 100-μl samples using the immunoselected antibodies indicated, while the material in lane 1 from a parallel 25-μl sample was analyzed directly by PAGE. Immunoselected antibodies to prolactin and to the 54-kDa and 68-kDa polypeptides of SRP (αPL, α54, and α68, respectively) were prepared as described (26). A fluorograph of a 10–20% gradient gel is shown. The IgG heavy chains again interfered with the detection of the 46- and 49-kDa photoadducts.

modified lysine was effected by SRP after about 70 amino acids had been polymerized. This placed probes at positions 4 and 9 of preprolactin, the only lysine locations in the first 70 amino acids of preprolactin (16). While positively charged amino acids are one of the few consensus features of signal sequences, we have demonstrated that replacement of these lysines by neutral or even negatively charged amino acids did not affect either proper signal sequence recognition by SRP or translocation (P. Garcia, M. Inouye, and P.W., unpublished data). Also, a few eukaryotic signal sequences do not have positively charged amino acids at their amino terminus. Therefore, it is likely that the alteration of the signal sequence introduced by the incorporation of the εANB-Lys residues (which lack a positive charge) does not significantly perturb its physiologically relevant interactions (see Fig. 2).

Upon photolysis, the photoreactive preprolactin signal sequence formed covalent crosslinks with specific proteins of the SRP–ribosome–nascent chain–mRNA complex (Figs. 3–6). One of these photoadducts was immunoprecipitated with antibodies both to prolactin and to the 54-kDa polypeptide of SRP (Fig. 6). Therefore, we conclude that a lysine in the signal sequence (residues 4 and/or 9) is adjacent to the 54-kDa SRP subunit in the arrested complex. The specific photocrosslinking of the 54-kDa subunit also strongly suggests that the signal sequence at the N-terminal end of nascent chains is recognized by and binds to the 54-kDa protein of the SRP. We have demonstrated previously that the 54-kDa protein is part of an SRP domain that contains the signal-recognition function (14).

The photocrosslinking of the preprolactin nascent chain to proteins in the large ribosomal subunit is probably also mediated by εANB-lysines at positions 4 and/or 9. However, since the carboxyl terminus of the arrested fragment has not been determined precisely and may be heterogeneous, it is possible that εANB-lysines incorporated at positions 72 and/or 78 (16) may generate or contribute to the photocrosslinks observed with ribosomal proteins (46 and 49 kDa in Fig. 4).

It is important to note that the methods detailed in this report provide a very powerful approach to investigating the interactions and environment of the nascent chain before, during, and after its translocation through the endoplasmic reticulum membrane. This is evidenced by the successful photoaffinity labeling both of SRP (Figs. 3–6) and of specific, as yet unidentified, proteins in the ribosomal 60S subunit (Fig. 4). We also have incorporated successfully a large fluorescent probe, N^{ε}-fluoresceinthioacetyllysine, into complete globin chains in a reticulocyte lysate (S. K. Shore and A.E.J., unpublished data). This provides us with the opportunity to examine several points along the nascent chain with complementary techniques; the correlation of spectroscopic data with crosslinking data should be particularly useful.

While this work was in progress, we became aware of a similar study by Kurzchalia *et al.* (27), and we thank Tom Rapoport for communicating their results prior to publication. We thank W. Hansen and L. Lauffer for a gift of preprolactin plasmid; D. Miller for a gift of EF-Tu; and S. Shore for assistance in preparing εANB-Lys-tRNA. This work was supported by National Institutes of Health Grants GM 26494 (to A.E.J.) and GM 32384 (to P.W.). P.W. is a recipient of support from the Chicago Community Trust/Searle Scholars Program.

1. Walter, P., Gilmore, R. & Blobel, G. (1984) *Cell* **38**, 5–8.
2. Walter, P., Ibrahimi, I. & Blobel, G. (1981) *J. Cell Biol.* **91**, 545–550.
3. Walter, P. & Blobel, G. (1981) *J. Cell Biol.* **91**, 557–561.
4. Gilmore, R., Walter, P. & Blobel, G. (1982) *J. Cell Biol.* **95**, 470–477.
5. Meyer, D. I., Krause, E. & Dobberstein, B. (1982) *Nature (London)* **297**, 647–650.
6. Gilmore, R. & Blobel, G. (1983) *Cell* **35**, 677–685.
7. Hortin, G. & Boime, I. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1356–1360.
8. Johnson, A. E., Woodward, W. R., Herbert, E. & Menninger, J. R. (1976) *Biochemistry* **15**, 569–575.
9. Johnson, A. E. & Cantor, C. R. (1980) *J. Mol. Biol.* **138**, 273–297.
10. Johnson, A. E., Miller, D. L. & Cantor, C. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3075–3079.
11. Johnson, A. E. & Slobin, L. I. (1980) *Nucleic Acids Res.* **8**, 4185–4200.
12. Erickson, A. H. & Blobel, G. (1983) *Methods Enzymol.* **96**, 38–50.
13. Walter, P. & Blobel, G. (1983) *Methods Enzymol.* **96**, 84–93.
14. Siegel, V. & Walter, P. (1985) *J. Cell Biol.* **100**, 1913–1921.
15. Walter, P. & Blobel, G. (1983) *Methods Enzymol.* **96**, 682–691.
16. Sasavage, N. L., Nilson, J. H., Horowitz, S. & Rottman, F. M. (1982) *J. Biol. Chem.* **257**, 678–681.
17. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
18. Kozak, M. (1984) *Nature (London)* **308**, 241–246.
19. Hansen, W., Garcia, P. D. & Walter, P. (1986) *Cell* **45**, 397–406.
20. Gilmore, R. & Blobel, G. (1985) *Cell* **42**, 497–505.
21. Miller, D. L. & Weissbach, H. (1974) *Methods Enzymol.* **30**, 219–232.
22. Johnson, A. E., Janiak, F., Dell, V. A. & Abrahamson, J. K. (1986) in *Structure, Function, and Genetics of Ribosomes*, eds. Hardesty, B. & Kramer, G. (Springer, New York), pp. 541–555.
23. Pingoud, A., Block, W., Wittinghofer, A., Wolf, H. & Fischer, E. (1982) *J. Biol. Chem.* **257**, 11261–11267.
24. Blobel, G. & Sabatini, D. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 390–394.
25. Bernabeu, C. & Lake, J. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3111–3115.
26. Walter, P. & Blobel, G. (1983) *J. Cell Biol.* **97**, 1693–1699.
27. Kurzchalia, T. Z., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H. & Rapoport, T. A. (1986) *Nature (London)* **320**, 634–636.