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# Exploring the Interactions Between Signal Sequences and *E. coli* SRP by Two Distinct and Complementary Crosslinking Methods

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## Abstract

Photoaffinity crosslinking comprises a group of invaluable techniques used to investigate in detail a binding interaction between two polypeptides. As the diverse photo crosslinking techniques available display inherent differences, the method of choice will provide specific information about a particular system under study. We used two complementary crosslinking approaches: photo-induced crosslinking of unmodified proteins (PICUP) and benzophenone-mediated (BPM) crosslinking to extensively examine the interaction between the signal recognition particle (SRP) and signal sequences. Signal peptide binding by SRP presents a central puzzle in the protein targeting process because signal sequences must be recognized with fidelity but lack strict primary structural homology. The concurrent use of PICUP and BPM crosslinking to link signal peptides to E. coli SRP allowed us to explore the crosslinking pattern resulting from using different crosslinking chemistries, varying the position of the photoprobe in the hydrophobic core of the signal sequence, and shifting the crosslinking reactive group away from the signal peptide backbone. By PICUP, signal peptides crosslinked exclusively to the NG domain of the SRP protein Ffh, regardless of the position of the reactive residue. Benzophenone-modified amino acids preferentially crosslinked the signal peptide to the C-terminal (M) domain of Ffh. We conclude that signal peptide binding is largely mediated by the M domain. Importantly, our data also indicate intimate, at least transient, contacts between the hydrophobic core of the signal peptide and the NG domain. These results reopen the possibility of a direct involvement of the NG domain in signal sequence recognition.

### Keywords

signal recognition particle; signal peptide; photo crosslinking; PICUP; benzophenone

## INTRODUCTION

Targeting of nascent secretory or integral membrane proteins to the endoplasmic reticulum in eukaryotes or the plasma membranes in prokaryotes is mediated by the signal recognition particle (SRP). SRP specifically recognizes either N-terminal signal sequences or hydrophobic transmembrane regions of newly synthesized proteins,[1–3] directs the ribosome nascent chain (RNC) to the SRP receptor at the membrane and, upon GTP hydrolysis, transfers the RNC to the membrane translocon where the protein is secreted or inserted in the target membrane.[4]



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Signal sequences share general properties, but not primary structure. Their features include a charged N-terminal, a hydrophobic core of 8 to 20 residues, which is believed to be the main discriminating factor among different protein targeting pathways,5 and a less hydrophobic C-terminal region of 6 to 10 residues.6–8

Despite their sequence diversity, signal sequences are universally recognized and bound by SRP. SRP is a ribonucleoprotein particle that in *E. coli* is composed by a single protein Ffh (the homologue of the eukaryotic receptor protein SRP54) and a 4.5S RNA, which serves as a scaffold but is also crucial for SRP function.9–13 This raises the question of what structural details confer upon SRP the ability to selectively recognize and bind such a diverse set of sequences. No atomic structure is available for an SRP-signal sequence/pre-protein complex. Biochemical data (summarized later) have provided an invaluable amount of information about the interaction between SRP and signal sequences, but details of the binding interaction remain unclear. Here, we seek to elucidate the network of interactions that the signal peptide establishes with SRP, more specifically with the individual domains of Ffh.

Ffh is composed of two major domains: NG and M domains. The NG domain is formed by an "N domain," responsible for interaction with the ribosome, and its contiguous GTPase activity-containing "G domain." The C-terminal "M domain" (named "M" because its high density of Met residues) provides the primary (but not the only14) contact to the SRP RNA and contains a deep, highly conserved hydrophobic groove on its surface12 lined with several Met side chain "bristles."15 Both Ffh domains are connected by a flexible linker.

Several lines of study[9,12] led to a model in which the hydrophobic groove of the M domain serves as the binding pocket for the signal peptide. Cryo-EM studies of ribosome-bound *E. coli* SRP16,17 showed electron density that was attributed to the nascent signal sequence. Although both reports modeled the signal sequence inside the hydrophobic groove of the M domain, the two models differ in many aspects of the SRP organization, and a detailed picture of the signal sequence interaction with SRP is challenging at the resolution of the cryo-EM studies (9.1 to 13 Å16 and 16 Å17). The observed density for the NG domain in the cryo-EM study16 implies that this domain is remarkably flexible,16 and its role in signal peptide recognition in the context of the full length SRP cannot be deduced.

Recognition of signal peptides by SRP constitutes a classic archetype of molecular recognition where elucidating the network of interactions established between the two interacting polypeptides is critical. Photo crosslinking constitutes an extraordinarily useful technique for this purpose and has been extensively used, especially to detect interactions refractory to study by other means (for reviews see Refs. 18–21). Particularly, different crosslinking approaches have been applied to study the interaction between signal sequences and SRP in the past: Labeling a RNC with two relatively bulky crosslinkers $N^{\varepsilon}$-(5-azido-2-nitrobenzoyl)-Lys22–24 or 4-(3-trifluoromethyl-diazarino)benzoyl-Lys25,26 allowed the crosslinking of the signal sequences to SRP54. In all cases, the labeled RNC was found attached to the M domain. Intriguingly, by using photo-induced crosslinking of unmodified proteins (PICUP)27,28 a direct interaction between synthetic peptides and Ffh was detected in our laboratory.29

We conjectured that the differences among the crosslinking methods used could explain divergence between the previous reports that crosslink signal peptides to NG or M exclusively. Differences include the use of synthetic signal peptides for PICUP analysis, the different chemistries of the crosslinking methods used, and/or the presence or the position in the sequence of the bulky crosslinker group introduced in the signal peptide.

We sought to explain and reconcile the past results by exploring signal peptide binding by SRP in greater detail using two crosslinking methods: benzophenone-mediated (BPM)[30] crosslinking and PICUP.27,28,29 BPM crosslinking not only relies on different chemistry from

PICUP (Figures 1A and 1B) but also shifts the photo-reactive group away from the peptide backbone: The photo-reactive benzophenone (BP) moiety and the peptide backbone are separated by a spacer arm (Figure 1C). We also explored the context of the bound signal peptide by moving the crosslinking probe through its hydrophobic core for both PICUP and BPM crosslinking. We expected to gain information about the structure of the bound signal peptide as well as to probe the binding site in greater detail.

By comparative application of two inherently different crosslinking methods, we found that the observed crosslinking patterns depend on a combination of factors: the nature and specificity of the photo-reactive group, the length of the linker separating the peptide backbone and photo-reactive group, and the position of the crosslinking moiety in the peptide sequence. Preliminary comparative findings with PICUP and BPM crosslinking were reported in a previous (review) paper.[1] Here, we describe a full and detailed analysis of our results and their implications. These observations provide insights that both help elucidate the detailed mechanism of early steps in protein export and point out the advantages of using complementary crosslinking-methods to detect dynamic interactions.

## MATERIALS AND METHODS

### General

The oligonucleotides used for site-directed mutagenesis and RNA preparation were purchased from Integrated DNA Technologies (Coralville, IA). CM-Sepharose was obtained from Sigma-Aldrich (St. Louis, MO). Fmoc-amino acid derivatives and CLEAR-amide resin for peptide synthesis were purchased from Peptides International (Louisville, KY). Streptavidin-HRP conjugate and Enhanced Chemiluminescence (ECL) kit were from Amersham Biosciences (Piscataway, NJ). Molecular weight markers and biotinylated molecular weight markers for SDS-PAGE were purchased from New England Biolabs (Ipswich, MA) and Cell Signaling (Danvers, MA), respectively. Ni-NTA affinity resin was purchased from Qiagene (Valencia, CA). SDS-PAGE gels were silver-stained using a commercial kit (Bio-Rad, Hercules CA). N-terminal His-tagged *C. perfringens* Perfringolysin O (PFO) was generously provided by Dr. A. P. Heuck.

### Protein Preparation

Wild-type full-length Ffh carrying an N-terminal deca-His tag (His-Ffh wt), and the C406S,S312C mutant (with a Cys introduced within the linker connecting the NG and M domains) were prepared using the pET16bFfh expression plasmid as described previously31 and the generated gene was fully sequenced (Davis Sequencing, Davis, CA). All proteins were purified under native conditions following a two step-protocol involving an ion-exchange purification on CM-Sepharose and affinity chromatography on Ni-NTA resin, as described earlier.29 The DNA corresponding to the His-tagged NG domain of Ffh (residues M1 to V300) was cloned into pET16b. Protein was expressed (in BL21(DE3) pLysS) and purified as previously described for Ffh, except that the CM sepharose purification step was omitted. His-tagged M domain of Ffh (M297 to R453) was expressed and purified as described previously. 29 In all cases, protein concentration was measured by Bradford assay (Bio-Rad, Hercules CA) using as standard an Ffh preparation for which the concentration was determined by amino acid analysis.

### RNA Preparation

A 43-nucleotide fragment of 4.5 S RNA used in all experiments was prepared by standard in vitro transcription techniques using a synthetic deoxyoligonucleotide template, according to the procedure described earlier.[31] RNA was purified by electrophoresis on a 20% polyacrylamide gel under denaturing conditions and recovered from the gel using Elutrap

electroelution system, according to the procedure supplied by manufacturer (Schleicher and Schuell, Keene, NH).

## Peptide Synthesis

Signal peptides based on the alkaline phosphatase signal peptide (PhoA, sequence MKQKKKLALLLLLLLLTPVTKA, see Table I and Figure 1C) were synthesized by solid phase methods on a PerSeptive Biosystems automated peptide synthesizer (Applied Biosystems, Foster City, CA) or a 9050 Plus PepSynthesizer (Millipore, Billerica, MA) using N-α-Fmoc (N-(9-fluorenyl) methoxycarbonyl)-protected amino acids. All peptides were N-terminally biotinylated to render "BioPhoA" peptides by coupling biotin-O-p-nitrophenyl ester (Sigma) to the deprotected resin-bound peptide in DMF in the presence of DIPEA. The biotin tag allows the detection the biotinylated peptides in a film by ECL. The benzophenone (BP) group was introduced into selected positions of the peptide sequence using either of two methods: using the non-natural amino acid 4-benzoylphenylalanine (Bpa) directly during synthesis, yielding set of compounds termed here BioPhoABpa$_n$ where $n$ = 10–14 corresponding to the position of Bpa in the peptide sequence, or by alkylation of the Cys residue in position 10 to 14 with 4-(maleimido) benzophenone (MBP) in HEPES (pH 7.6) in the presence of tris(2-carboxyethyl) phosphine (TCEP, 1.1 mol equiv. relative to the peptide concentration). This second set of PhoA derivatives was termed Bio-PhoAC$_n$-BP where $n$ = 10–14 corresponds to the position of the Cys in the peptide sequence (for details see Table I and Figure 1C). Peptides were cleaved from the resin using a standard cleavage cocktail (88% TFA, 5% water, 5% phenol, 2% tri-isopropylsilane) for 2 h, precipitated with diethyl ether and lyophilized. Peptides were purified by preparative RP-HPLC using a phenyl (25 × 250 mm, 10 μm, 300 Å, Vydac) column and the appropriate water/acetonitrile gradients. The purity of the compounds obtained was checked using analytical phenyl column (4.6 × 150 mm, 5 μm, 300 Å, Vydac), and only fractions with purity greater than 98% were used in the crosslinking experiments. The identity and purity of the compounds were confirmed by mass spectrometry analysis using either a BIFLEX III MALDI-TOF mass spectrometer or an Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Billerica, MA).

In all experiments, 0.1 m*M* signal peptide stock solutions were used, prepared by dilution of 1 mg/ml solutions in 5% DMSO. The concentration of the BP-containing peptides was determined spectroscopically and confirmed by dot-blot analysis using biotin as a probe for ECL detection (Amersham Biosciences, Piscataway, NJ).

## PICUP

PICUP was performed as described.[29] His-Ffh C406S,S312C (see below) and RNA (in a 1:2 ratio) were mixed in buffer containing 15 m*M* HEPES (pH 7.6), 150 m*M* NaCl, 5 m*M* $MgCl_2$ and 10% glycerol and incubated for 10 min in ice. Signal peptide (2-fold excess over protein concentration) was added, and the reaction mixtures were incubated for 10 min at room temperature. In a dark room, $Ru(bpy)_3Cl_2$ was added, followed by ammonium persulfate to final concentrations of 0.125 m*M* and 2.5 m*M*, respectively. The mixtures were illuminated using a flashlight with a 400 nm cut-on filter for 45 s. The reactions were quenched by addition of 2-mercaptoethanol to 5 m*M* and subjected to further analysis (see below), or immediately boiled in 60 m*M* Tris (pH 6.8), 6% w/v SDS, 10% glycerol, 4% 2-mercaptoethanol (gel loading buffer) for SDS-PAGE.

## Chemical Cleavage of Ffh-Signal Peptide Adducts

For reasons that are not entirely clear, digestion of PICUP crosslinked products with V8 protease led to disappearance of the ECL signal. Consequently, after PICUP, chemical cleavage by 2-nitro-5-thiocyanobenzoic acid (NTCB, which cleaves at Cys residues32) was carried out. As wild type Ffh contains only one native Cys residue at position 406, in order to obtain

selectively cleavage of Ffh into the NG and M domains the C406S and S321C double mutant was used.29 S321 is weakly conserved and is located at the end of the linker between NG and M domains.29 Modifications based on the work by Tang and Speicher33 were applied in the final cleavage steps in order to ensure higher cleavage efficiency. After PICUP was performed (see above), 5 m*M* imidazole was added to the reactions followed by 100 µl of a Ni-NTA slurry equilibrated in the reaction buffer supplemented with 5 m*M* imidazol. Ni-NTA binds the His-tagged His-Ffh C406,S321C and separates it from the rest of the reaction components. The mixtures were incubated in ice for 30 min with occasional mixing and applied to a spin column (Promega, Madison WI) in a 1.5-ml microcentrifuge tube. Filtrate was removed by centrifugation, and the pelleted resin was washed five times with 100 µl of 15 m*M* potassium phosphate (pH 7.6), 150 m*M* NaCl, 8 *M* urea, and 5 m*M* imidazole. Protein was eluted with 2 × 60 µl washes of the same buffer supplemented with 200 m*M* imidazole. One half of the eluate was taken for the NTCB cleavage reaction. TCEP in 10 m*M* HEPES (pH 7.6) was added to up to 50 µ*M* from a 10 m*M* stock, and the reaction was incubated at 37°C for 15 min. NTCB was then added to a final concentration of 500 µ*M*, and the reaction was further incubated at 37°C for 15 min, when ammonium hydroxide was added to 1 *M* to induce cleavage. The reaction was carried out at 37°C for 2 to 3 h. Protein fragments were precipitated with trichloroacetic acid (15% final concentration), washed with cold acetone, resuspended in gel-loading buffer and analyzed by (silver stained) SDS-PAGE and ECL.

### BPM Crosslinking

His-Ffh, RNA and BP-containing signal peptide were mixed together as described above for PICUP and irradiated at a distance of ~5 cm with a 365-nm UV lamp at room temperature for 5 min. The samples were then immediately boiled in gel-loading buffer and analyzed by SDS-PAGE or subjected to enzymatic digestion (see below).

### Enzymatic Cleavage of Ffh-Signal Peptide Adducts

Protease digestion was performed using V8 endopeptidase. Reaction mixtures containing 2.5 µ*M* His-Ffh, 5 µM RNA fragment, and 2.5 µ*M* BP-containing signal peptide were subjected to crosslinking as described above and next incubated with V8 protease at 20:1 (w/w) protein:enzyme. The enzymatic digestion was carried out at 25°C for 0 or 2 h in the dark, and products were separated by SDS-PAGE. The result was visualized by silver staining of the SDS-PAGE gels. Simultaneously, the products from an identical gel were blotted to PVDF membranes, and detected by ECL (see below). For the time-course digestion experiments, the same procedure was repeated but the incubation with V8 was carried out for 0, 2, and 18 h.

### Detection of Ffh Fragments Crosslinked to Signal Peptide

Protein fragments were electro-blotted from SDS-PAGE gels onto nitrocellulose or polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica MA) using semidry or wet blotting system (Bio-Rad, Hercules CA) respectively. Membranes were then incubated for 1 h in 5% milk in phosphate-buffered saline (pH 7.2) containing 0.05% Tween-20 (PBS-T), washed extensively with PBS-T, and incubated for 1 h in PBS-T containing a 1:1500 dilution of streptavidin-HRP conjugate. Biotin-labeled fragments were detected by ECL. The intensities of the bands or spots in the ECL films were analyzed using ImageJ software (Wayne Rasband, ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2006). Briefly, a square or circle was drawn around the band or spot (respectively) to be quantified and the intensity of the spot was calculated by Image J. For each experiment, the same area was measured for all the spots involved in order to compare the relative intensities. The background was subtracted for each series of spots by quantifying the signal intensity of the same area in a blank part of the film next to the spot to be quantified.

### Ni-NTA Pull-Down Assay

To a 100 μl mixture of 1 μ*M* His-tagged protein (Ffh, M domain of Ffh, NG domain of Ffh or PFO) and 1 μ*M* RNA in buffer containing 15 m*M* HEPES (pH 7.6), 150 m*M* NaCl, 5 m*M* $MgCl_2$ and 10% glycerol, 2 μ*M* of the appropriate biotin-labeled signal peptide were added and the mixes were incubated 15 min at room temperature. At this point, some tubes were supplemented with either nonlabeled $PhoAC_{23}$ as binding competitor or KRRLamB (solubility-enhanced variant of the signal peptide of the outer membrane protein LamB, sequence MITLRKRRKLPLAVAVAAGVMSAQAMA) as nonbinding competitor to a final concentration of 20 μ*M*. The reactions were incubated for additional 15 min and 30 μl of a Ni-NTA resin suspension pre-equilibrated 1:1 in reaction buffer were added. The mixtures were incubated for 30 min at room temp with occasional mixing, and the resin and supernatant were separated using spin columns by centrifugation as described previously. The pelleted resin was washed three times with 100 μl of reaction buffer, and protein was eluted with 40 μl of the same buffer supplemented with 200 m*M* Imidazole. Twenty microliter of the eluate were then applied to a mixed cellulose or PVDF membrane and biotin-containing fractions were detected using ECL as described.[29]

## RESULTS

### Modified Signal Peptides Retain SRP Binding Ability and Specificity

We used synthetic, modified signal peptides to probe the interaction with in vitro reconsituted *E. coli* SRP by BPM crosslinking and PICUP. In all cases, the signal peptides were modified with biotin at the N-terminus as a tag detectable by enhanced chemiluminescence. For crosslinking experiments involving Bpa, the signal peptides contain Bpa at positions 10 to 14 of the hydrophobic core ($BioPhoABpa_n$ $n$ = 10–14; see Table I and Figure 1C). For PICUP the signal peptides contain a Cys residue that was moved along its hydrophobic core ($BioPhoAC_n$ $n$ = 10–14; see Table I and Figure 1C), and for BPM crosslinking the same Cys-containing signal peptides were modified with MBP to render the BP-modified signal peptides ($BioPhoABP_n$ $n$ = 10–14; see Table I and Figure 1C). The model signal sequence used here (PhoA, see Table I) has been shown previously to interact efficiently with Ffh.[29,34] However, introduction of a bulky BP group into the middle of the functionally important hydrophobic core of a signal peptide may interfere with the affinity of SRP for the signal peptide. In order to test whether the modified signal peptides retain the ability to bind SRP, whether this binding is specific, and whether the introduction of a crosslinker at some positions perturb binding but not others, a pull-down binding assay was performed.[29] By this method, coelution of the biotin-labeled signal peptide with in vitro reconstituted SRP (containing His-tagged Ffh and the 43-nt RNA fragment) from Ni-NTA resin is indicative of efficient binding. Binding to reconstituted SRP was observed for all three sets of peptides (Figure 2A); binding of the BP-modified peptides was weaker than that of unmodified peptides, requiring a longer ECL exposure time to see signals of comparable intensity. In the same experiment the specificity of binding was examined using binding and nonbinding competitors. Nonbiotinylated PhoA signal peptide with a Cys in position 23 ($PhoAC_{23}$) was used as a binding competitor for all the signal peptides assayed. As a nonbinding competitor, we used nonbiotinylated KRRLamB signal peptide, as its considerably lower hydrophobicity leads to a lower affinity for SRP than that of the modified PhoA peptides used here.[29] Despite the somewhat reduced binding affinity of the BP-modified peptide series, specificity of binding was confirmed for all signal peptides studied, as all could be competed by a 10-fold excess of unlabeled binding competitor. No significant diminution of binding was observed for any of the substituted positions upon addition of the unlabeled nonbinding competitor (Figure 2B. Only the experiments using signal peptides with probe at positions 11 and 14 are shown as an example). Thus, we conclude that it is valid to use all sets of peptides to explore the signal sequence binding site.

In order to test whether there is preferential binding of the separate Ffh domains to modified versus unmodified signal peptides, we performed pull-down binding assays with each individual domain using BioPhoAC[11] (biotinylated PhoA peptide containing Cys at position 11) and Bio-PhoABpa$_{11}$ (biotinylated PhoA peptide containing Bpa at position 11) as model signal peptides (Figure 2C). We selected this set of peptides with reactive probe at position 11 as it showed a good level of crosslinking using both BPM crosslinking and PICUP, and at the same time it was not the peptide that yielded the highest or the lowest crosslinking intensity in either case.

Although both NG and M domains can bind either version of the signal peptide, the binding affinities appear to differ: the NG domain showing a modest preference for the unmodified signal peptide, and the M domain, by contrast, shows a preference for the modified version. Importantly, binding to both of these signal peptides is significantly stronger to full-length Ffh than to either of the separate domains.

## BPM Crosslinking Occurs to the M Domain of Ffh

SRP was crosslinked to both families of BP-containing signal peptides (BioPhoAC$_n$-BP and BioPhoABpa$_n$) and subjected to by V8 proteolysis. V8 protease is known to generate, under conditions of limited digestion, two main Ffh fragments corresponding to the NG and M domains.[23,29] On the other hand, V8 is not expected to cleave the signal peptides as they do not contain any Glu or Asp. Biotinylation of the signal peptides at the N-terminus combined with V8 partial digestion provides a fast and sensitive tool to asses which Ffh domain the signal peptide crosslinks. Figure 3A depicts the Ffh fragments generated by partial digestion with V8 after BPM crosslinking showing BioPhoAC$_{10}$-BP as a representative example. In Figure 2B, upper-right panel, two bands can be observed for the NG domain in the silver-stained SDS-PAGE, revealing the presence of two nearby V8 digestion sites.

For all BP-modified peptides a band corresponding to the M domain crosslinked to signal peptide was clearly visible (~18 kDa) in the ECL film together with the band of crosslinked His-Ffh (~55 kDa) and its oligomers (above 140 kDa) (Figure 3A, right panel and Figure 3B, bottom right panel). No band corresponding to crosslinked NG-domain (~35 kDa) was observed (see Figure 3A as this gel was run for an adequate time to clearly show the area around 30–40 kDa).

The amount of M domain bound to signal peptide (ECL signal, Figure 3A right panel and Figure 3B bottom-right panel) is very small compared to the amount of signal peptide crosslinked to undigested Ffh. The difference is most likely because the accessibility of the primary digestion site (the linker between M and NG domains) by V8 is impaired in some way after crosslinking, perhaps because of direct involvement of the linker in signal peptide binding.[16]

Figure 3C shows the kinetics of digestion of signal peptide crosslinked SRP by V8. In the Coomassie blue-stained gels (where the crosslinked products are not detectable) as well as in the ECL film (where the crosslinked products are detected), the same bands are present throughout the digestion, suggesting that the kinetics of digestion by V8 after crosslinking may be retarded, but that no alternative sites are cleaved. Under these circumstances we expect the NG domain to be detectable if it bound signal peptide. Still, the area in the ECL film between 30 and 42 kDa where the signals for crosslinked NG domain should be detected if present, does not show any signal after 3 h or even 18 h of V8 digestion. The lack of bands is readily apparent in Figure 3A, right panel, where the SDS-PAGE gel has been run long enough to extensively separate the 30 and 40 kDa bands in the MW marker. The most plausible explanation for the bands seen around 30 kDa in the ECL films is that they arise from fragments of Ffh containing the M domain.

The intensities of the signals corresponding to the M domain are dependent on the position of the photo probe (Figure 3B, bottom right panel and Figure 4). The intensity increases from that of BioPhoAC$_{10}$-BP signal peptide and reaches a maximum value for BioPhoAC$_{12}$-BP going back to the minimum intensity for BioPhoAC$_{14}$-BP. Remarkably, the same pattern of crosslinking to the M domain of Ffh is observed when the set of BioPhoABpa$_n$ peptides is used (not shown).

### Signal Peptides Crosslink to the NG Domain of Ffh by PICUP

In order to explore the spatial arrangement of the signal sequence-binding site in SRP and also investigate the role of the NG domain in signal sequence-binding, we crosslinked using the PICUP method. As mentioned before, this method keeps the reactive group in the signal peptide close to the peptide backbone avoiding the introduction of large photo-activable probes into the interaction interface. Furthermore, the chemistry underlying this crosslinking method is intrinsically different from BPM crosslinking (see Figures 1A and 1B and Discussion).

Signal peptide variants with Cys residues in position 2, 10–14, or 23 and reconstituted SRP were subjected to PICUP followed by NTCB cleavage at Cys321 (introduced to enable chemical cleavage[29] between the NG and M domains). The silver-stained SDS-PAGE gel (Figure 5, upper panel) shows the separation of NG and M domain of Ffh by NTCB cleavage. Figure 5 (bottom panel) shows the ECL signals of PICUP. Strikingly, all signal peptides exclusively crosslink to the NG-domain irrespective of the position of the Cys in the sequence. No crosslinking to M domain is observed even when the ECL film was exposed for 12 h. The intensity of NG-domain signal shows some variability depending on the position of the reactive Cys in the peptide sequence. This trend is roughly the inverse of the one noted before for BP-modified signal peptides: Stronger binding to the NG domain is observed in the case of peptides for which BPM crosslinking was weaker (compare Figure 3B bottom right panel and Figure 5B bottom panel).

## DISCUSSION

Signal sequence recognition and binding by the signal recognition particle are the first events in a cascade of processes that precede the delivery of the nascent secretory or transmembrane protein to the plasma membrane (or endoplasmic reticulum membrane), where it is inserted or translocated. Although signal sequences share common features, notably a core of hydrophobic residues with preceding basic residues, they differ extensively in their primary structure. This raises the question of what structural details confer upon SRP the ability to selectively recognize and bind such a diverse set of sequences. The lack of a direct structural understanding underlines the need for a detailed study of signal peptide interactions with SRP.

The M domain was first identified as the signal sequence-binding domain of SRP54[23] and the NG domain was suggested to be an enhancer of the affinity of SRP for signal sequences.[23,26,35] In previous work, we postulated a significant role for the NG domain in signal peptide binding.29 In the present study, to follow up these observations and to reconcile seemingly disparate models from earlier data, we used one model signal peptide and two photo-cross-linking methods: PICUP27,28 and BPM[30] crosslinking. Our results revealed that the crosslinking patterns between signal peptide and Ffh strongly depend on the nature of the chosen crosslinking method. BPM crosslinking attached the signal peptide preferentially to the M domain. This result agrees with the results obtained by other groups that used crosslinking strategies where the signal sequence (as part of the RNC) contained a relatively bulky reactive head.[22–26]

Crosslinking to the M domain was observed regardless of the length of the linker (BioPhoAC$_n$-BP vs. BioPhoABpa$_n$) or the position of the probe in the hydrophobic core of the

signal peptide. In this set of experiments, we detected an influence of the position of the photoprobe on the crosslinking efficiency, and the trend in crosslinking efficiencies is consistent with a helical conformation of the signal peptide upon binding to SRP, as had been anticipated.[16,17,36,37] However, more positions in the peptide sequence need to be tested to confirm the binding conformation more rigorously.

Interestingly, when PICUP was performed, the signal peptide attached to the NG domain of Ffh regardless of the position of the reactive group. The position of the Cys (most probable nucleophile for attack of the radical in PICUP) within the hydrophobic core of the signal peptide did not have significant influence on the crosslinking efficiency. Small variations noted could reflect different accessibility or relative orientation of the particular Cys towards reactive group on the Ffh surface.

The domain-specific crosslinking observed here could be caused by a number of factors: the nature of the chemistry involved in the crosslinking and the available reactive groups at the binding surface, the polarity of the reactive groups involved in the crosslinking, the time scale of the crosslinking reaction compared to the interaction time scale, the distance between the peptide backbone and the reactive group (along with the reactive group size), and preferential binding of the modified signal peptides to the M domain.

In BPM crosslinking (Figure 1A), UV light leads to the generation of a BP diradical which can abstract a hydrogen from an electron-rich σ bond, targeting crosslinking to nearby C—H with facile hydrogen abstraction (e.g., hydrophobic residues like Met, Leu or Val).[30] Additionally, in the BP-modified peptides the reactive radical intermediate is shifted away from the peptide backbone by 6 or 12 Å, which allows scanning more of distant interactions between components of the peptide-protein complex. During BPM crosslinking, the diradical species formed can only attack geometrically accessible C—H bonds.30 The reactant and substrate may spend enough time at the optimal distance for interaction which constitutes the main source of specificity for the reaction.

The observed preferential crosslinking of the BP-modified peptides to the M domain can be also biased by the abundance of Met residues in this part of the Ffh. As mentioned before, the M domain contains a hydrophobic cleft lined with Met side chains, which is proposed to be the signal peptide-binding site.[12,15] The flexibility of the Met side chains was argued to account for the ability of SRP to bind signal peptides that are very diverse in their primary structure. In the case of the experiments presented here, the fact that Bio-PhoAC$_n$-BP and BioPhoABpa$_n$ peptides can be accommodated by the M domain and generate the same pattern of crosslinking efficiencies can be explained in terms of the plasticity of the binding site conferred by the Met side chains. Introduction of the bulky BP reactive group in the signal peptide, appears to alter the nature of interaction between signal peptide and SRP and may prevent the NG from binding efficiently to the modified signal peptides. This explanation would account for the lack of BPM crosslinking to NG domain and is supported by our direct binding assays (Figure 2C). The ability of the M domain to both bind and be crosslinked by the BP-modified signal peptides supports a plastic binding mode to this domain and a longer-lived complex.

Methionine residue can also play another crucial role in the applied crosslinking method. It has been shown that the BP photoprobe has pronounced selectivity for Met side chains comparing to other reactive amino acids[38–40] which may limit the precision of its application in assays concerning spatial proximity and organization of interacting molecules. In our case this feature of Met residues may influence the observed crosslinking selectivity of the modified BioPhoA peptides towards the M domain, despite the binding affinity for modified peptides.

The chemistry of PICUP (Figure 1B) relies upon the formation of direct linkages between two residues.[27,28] In the first step of the process, light excitation of a tris(2,2′-bipyridyl) dichlororuthenium(II) leads to the formation of a reactive radical that will preferentially form at side chains that offer stabilization through aromatic or neighboring-group effects (typically, Tyr, His, or Met). If none of these side chains are near the Ru(III) oxidizer, it may abstract an electron from a less reactive side chain or react with the solvent. [27,28] Once the protein radical is formed, it will be attacked by a neighboring nucleophile. The signal peptides used here contain Lys and Cys residues, both considered as candidate nucleophilic residues to participate in PICUP.[27,28] The side chain sulfhydryl group of a Cys residue is a stronger nucleophile at the pH used for crosslinking and can be then considered the more likely candidate for crosslinking. In PICUP, which is also called a “zero-length crosslinking” method, a covalent bond is created directly between interacting molecules thus allowing the capture of an intimate contact and serving potentially as a more precise way to map binding sites, as PICUP crosslinks will form only where groups with the appropriate reactivity reside. Importantly, the crosslinking reaction occurs very fast (≤1 s) allowing detection of very dynamic protein-protein interactions.[41]

Despite the complexity of interpreting the crosslinking patterns, we can draw several conclusions. We argue that interactions between the signal peptide and the NG domain are captured using PICUP because of the spatial requirements of the reaction, where only short-distance interactions between the side chains of interacting peptides/proteins that are close to each other can be observed.[27,28] Additionally, compared to the reaction that occurs during BPM crosslinking, PICUP takes place more rapidly[41] so that interactions between NG domain and the hydrophobic core of the signal peptide that are transient could be captured by PICUP but not by BPM crosslinking. On the other hand, the polarity of the Cys residue or the lack of correct spatial requirements for PICUP to occur may prevent this residue from crosslinking to the M domain binding pocket

It is interesting to consider our data in light of the mechanism of the SRP targeting cycle, emphasizing the importance of the NG domain in the regulation of the entry and/or release of the nascent chain to the M domain.

As mentioned previously, before a direct interaction between signal sequences and the NG domain was demonstrated, [29] it had been shown that the NG domain enhances the efficiency of signal sequence binding.[23,26,35] Alkylation of Cys residues in the NG domain of SRP inhibits signal sequence binding, and this inhibition can be relieved upon proteolytic removal of the alkylated NG domain, suggesting that this domain can influence access of the signal sequence-binding site in the M domain.[26] Interestingly, in our direct binding assays signal peptides bound to full-length Ffh/RNA much more efficiently than to either of the separate domains (Figure 2C). This observation strongly argues that the architecture of the binding site is intact only in the context of both domains.

These ideas put the signal peptide in the role of allosteric modulator of domain interactions in SRP. Zopf et al.[24] indeed proposed many years ago that the NG domain directly stabilizes the binding of the signal sequence to the M domain, and moreover, that association between the M domain and the signal sequence would influence the positions of NG relative to M domain. The signal sequence could then be locked into the binding site. In alternative, more indirect mechanism, the NG domain may allosterically change the conformation of the M domain such that it would then bind signal sequences more tightly. Signal peptide binding may trigger conformational changes in both NG and M domains, leading to the opening of the finger loop, which otherwise protects the hydrophobic cleft on the M domain, allowing binding of the hydrophobic part of the signal peptide (in this case, along with the BP photo-probe).

These sorts of conformational changes paint a picture of SRP as a truly dynamic molecular machine. Indeed, several recent findings point to a fundamental role for interdomain communication in the SRP-mediated delivery of a RNC to the translocon. In this process, the flexibility of the linker connecting both NG and M domains is essential, allowing rearrangements between the domains[35,42,43] and playing an active role in the regulation of signal peptide binding and/or release. In addition, the communication between NG and M domains of Ffh is intimately related to the multiple roles of the SRP RNA. The catalytic activity of the RNA is responsive to signal sequence binding to SRP,[9,10,44,45] and is linked to the conformational changes in the M domain of Ffh. It has been proposed that the RNA may coordinate the interaction of the SRP and the SR with ribosome recruitment and transfer to the translocon.[10,46]

Our observations are consistent with a two-domain model for signal peptide recognition, and emphasize the intrinsic dynamics of the process. Importantly, the inherently different nature of the interactions between signal peptide and NG domain and signal peptide and M domain were revealed by exploiting the power of two different photo crosslinking approaches.

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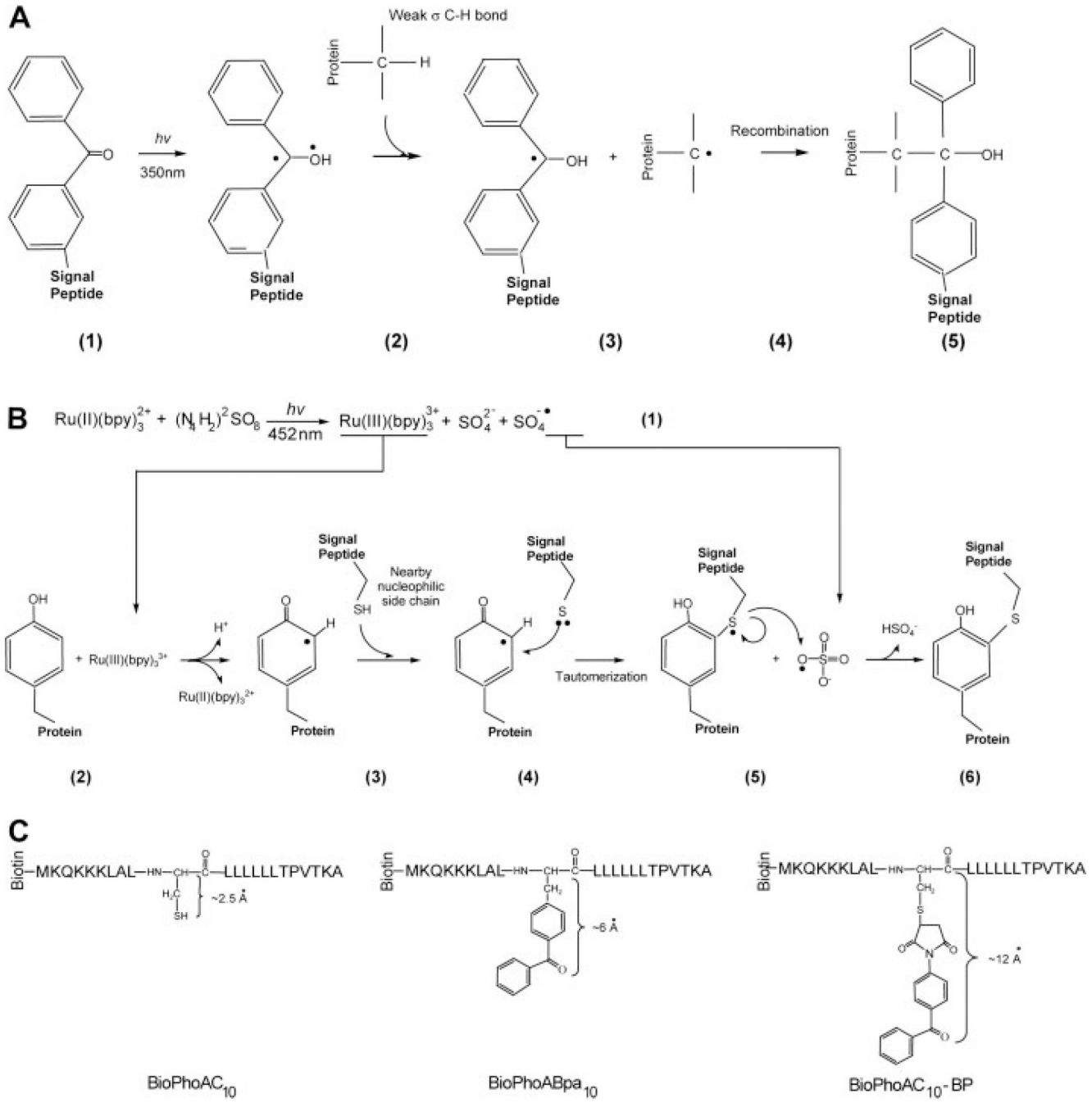


**FIGURE 1.**

Proposed mechanisms for BPM crosslinking and PICUP. A: Chemistry of BPM crosslinking. According to Dorman and Prestwich,[30] absorption of a photon at ~350 nm by the BP (1) (here attached to the signal peptide), results in promotion of one electron from a nonbonding $sp^2$ -like n-orbital on oxygen to an antibonding σ*-orbital of the carbonyl group. The electron-deficient oxygen n-orbital is electrophilic and interacts with weak C—H σ bonds (2) (here a C—H bond in Ffh) resulting in hydrogen abstraction. The generated radicals (3) readily recombine (4) to form a new C—C bond (5), linking the signal peptide and SRP. The diradicaloid species can only attack geometrically accessible C—H bonds. B: Proposed mechanism for PICUP,[27,28] using the reaction between a Tyr in one of the reacting proteins

(here Ffh) and free Cys in the other reacting protein (here the signal peptide) as examples. $Ru(II)bpy_3^{2+}$ is photolyzed in the presence of ammonium persulfate generating Ru(III) and sulfate radical (1). Ru(III) oxidizes Tyr (2) and the resulting radical would proceed to crosslink two associated proteins. In the case of the reaction with the free Cys of the signal peptide, the SH nucleophilic group could attack the radical to provide a heteroatom-arene linkage [(3) and (4)]. An H atom must be lost to form stable products, and the sulfate radical produced during Ru(III) formation could play a role in this step (5). At the end of the process, SRP and the signal peptide would be crosslinked (6). C: Schematic representation of the signal peptides used for crosslinking. Only one of each of the sets used is shown, with the reactive groups (Cys or BP) introduced at position 10 of the signal peptide. The approximate distances between the crosslinking reactive groups and the peptide backbone are indicated.

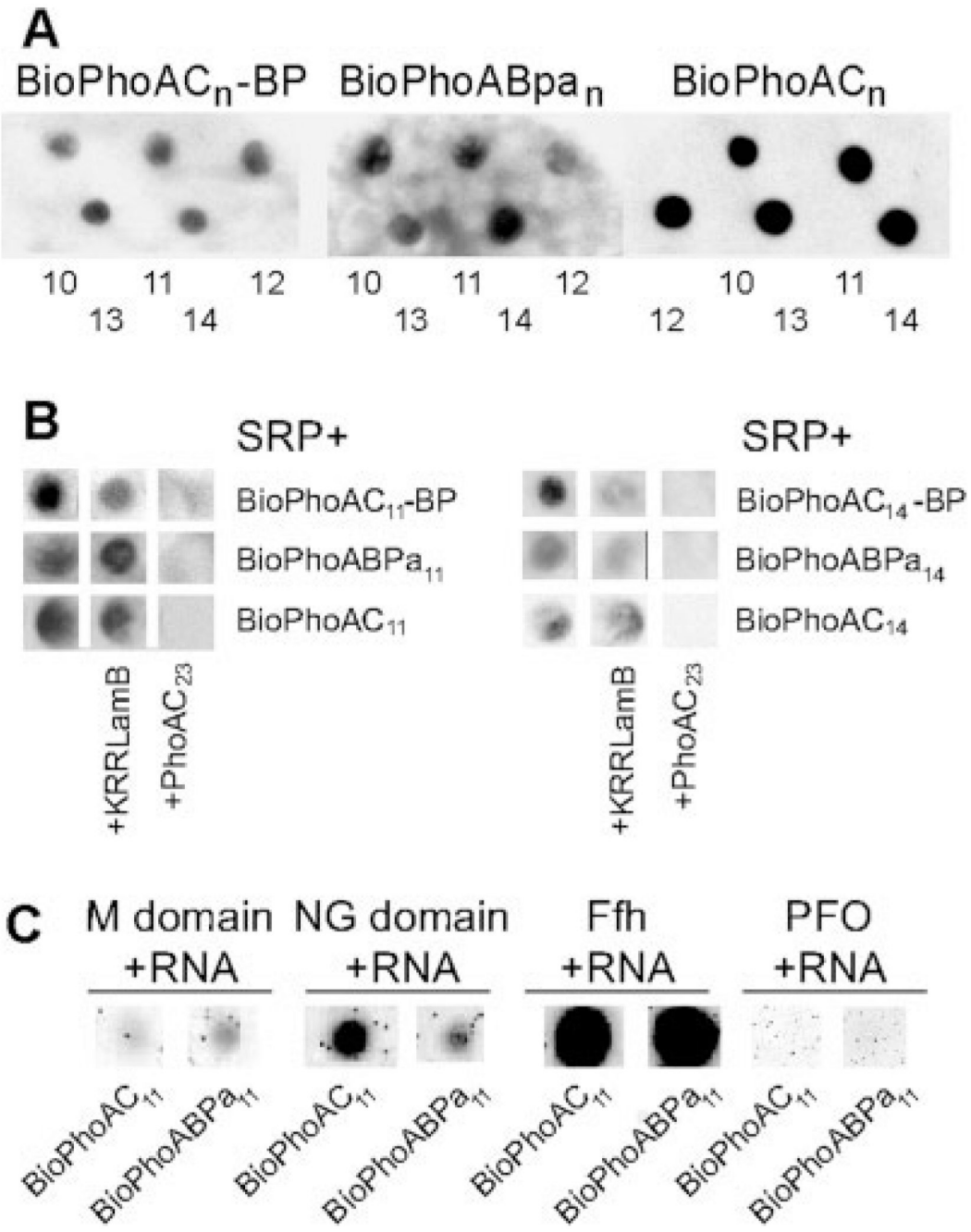


**FIGURE 2.**

Binding between in vitro reconstituted SRP and signal peptides by pull-down assays. ECL film detecting biotinylated signal peptides are shown. A: Binding between SRP and BioPhoAC$_n$-BP, BioPhoABpa$_n$ and BioPhoAC$_n$ $n$ = 10–14. Position of the reactive probe in the signal peptide sequence is indicated at the bottom of each panel. B: Addition of excess unlabeled PhoAC$_{23}$ (20 μ*M*) competes the labeled signal peptide off the SRP binding site. Excess unlabeled KRRLamB signal peptide (20 μ*M*) has a minor effect. For simplicity, only the reaction between SRP and the signal peptides with reactive probes at positions 11 and 14 are shown. All the signal peptides tested for each group (BioPhoAC$_n$-BP, BioPhoABpa$_n$ and BioPhoAC$_n$ sets) showed similar results. C: Binding of Bio-PhoABPa$_{11}$ and BioPhoAC$_{11}$ to

His-tagged M domain, His-tagged NG domain, and His-tagged Ffh (all in the presence of RNA). We used His-tagged PFO + RNA in the presence of biotinylated signal peptides as a negative control. In the absence of labeled signal peptide, SRP, NG domain plus RNA, or M domain plus RNA did not show any appreciable signal (not shown).

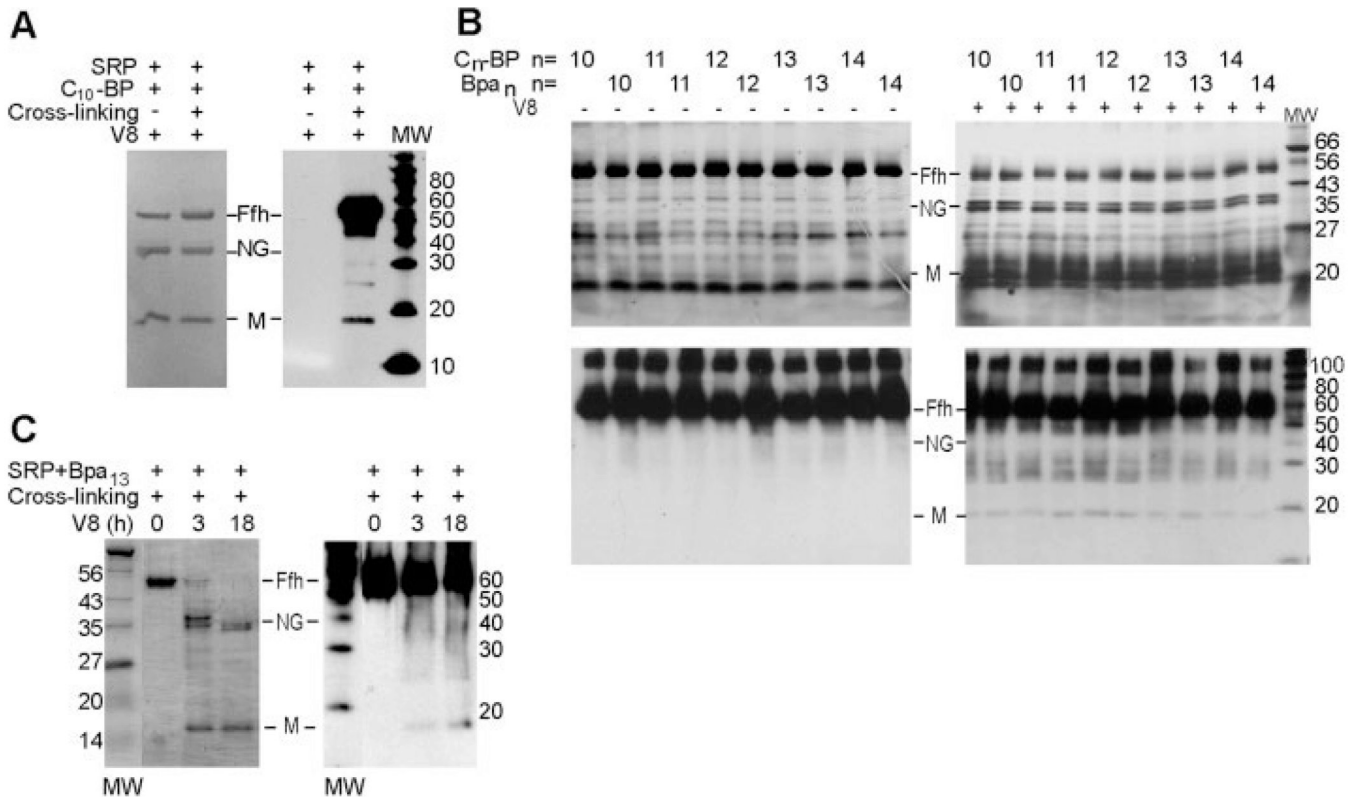


**FIGURE 3.**

Detection of BPM crosslinking products. A: Coomassie blue-stained SDS-PAGE gel (left panel) and ECL film (right panel) of the Ffh fragments generated by partial digestion with V8 for 2 h after BPM crosslinking between in vitro reconstituted SRP and BioPhoAC$_{10}$-BP, as a representative example of the signal peptide group. B: Silver stained SDS-PAGE gel (top panel) and ECL developed film (bottom panel) of the Ffh fragments generated before and after partial digestion with V8 for 3 h, following BPM crosslinking. BioPhoABpa$_n$ and BioPhoAC$_n$-BP sets of peptides were used, where the BP group was moved along the peptide covering positions 10 to 14. Positions of bands corresponding to undigested Ffh, NG and M domain are indicated. For simplicity, "BioPhoA" is omitted from the peptide names in the Figure (i.e., C$_n$-BP stand for BioPhoAC$_n$-BP). "V8" is used to denote the reactions that were digested with V8. C: Time course of V8 digestion after crosslinking between SRP and BioPhoABpa$_{13}$ visualized by Coomassie-blue stained SDS-PAGE (left panel) and ECL film (right panel). In all panels, "MW" indicates the lane with the molecular weight markers and the corresponding mass of each band is indicated (in kDa).

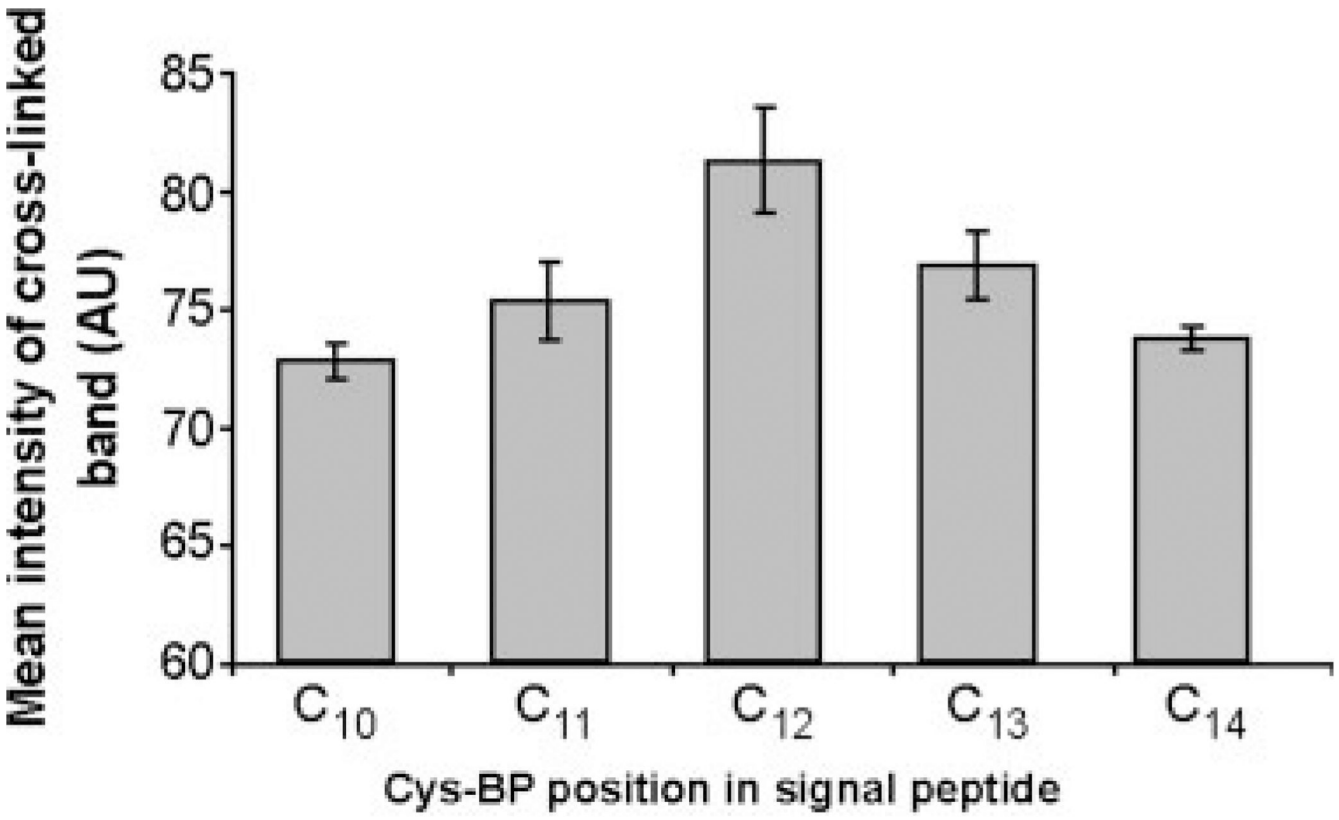


**FIGURE 4.**

Quantification of the ECL signal corresponding to M domain of Ffh crosslinked to BP-containing signal peptides. The intensities of the bands corresponding to the M domain in Figure 3B (bottom-right panel) were quantified for the BioPhoAC$_n$-BP sets of peptides and plotted (in arbitrary units) against the position of the BP probe in the peptide. Two independent experiments were quantified, the intensities averaged, and the range shown as error bars.

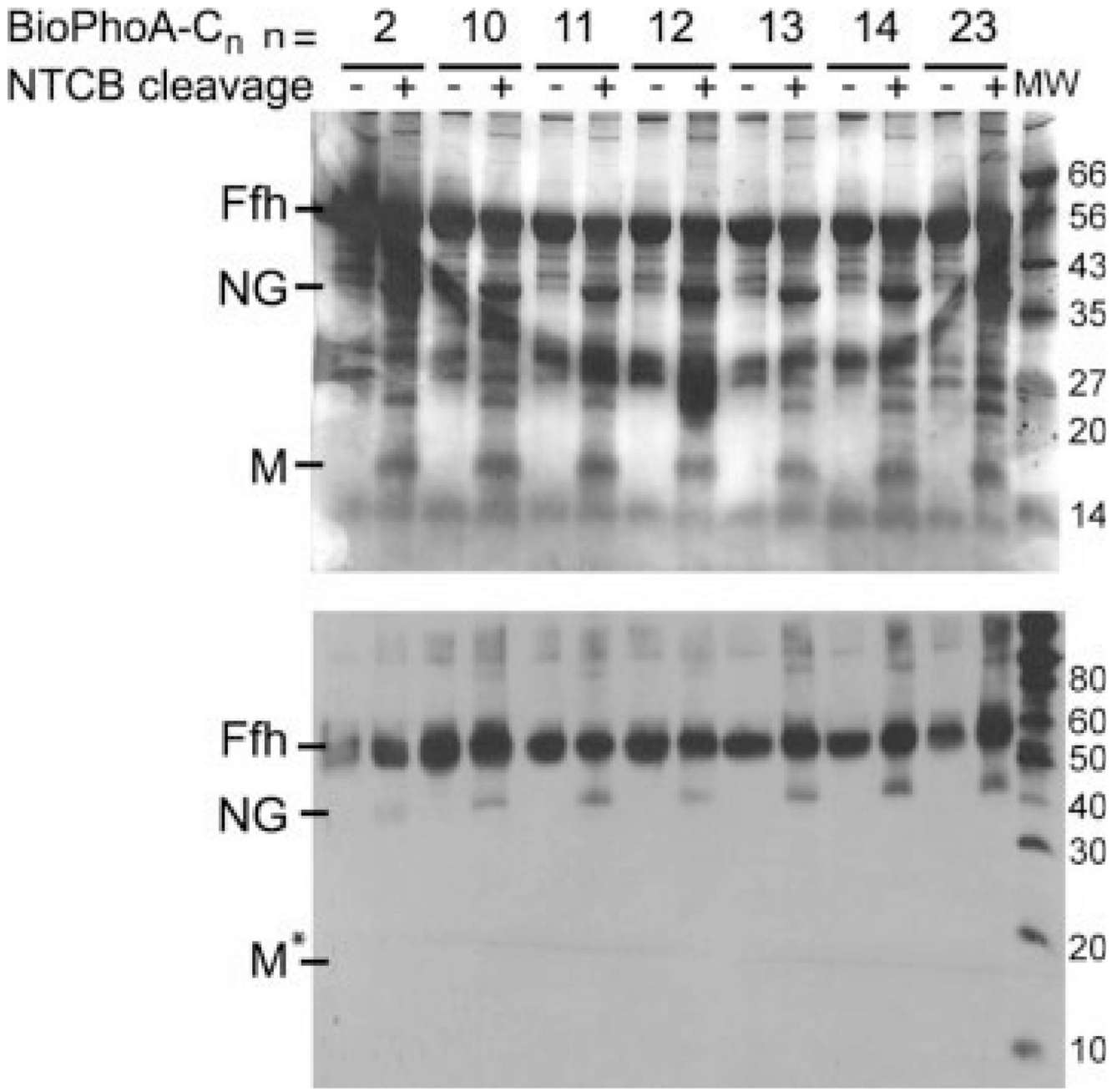


**FIGURE 5.**

Detection of PICUP products. Silver-stained SDS-PAGE gel (top panel) and ECL developed film (bottom panel) of the Ffh fragments generated by NTCB cleavage after PICUP between in vitro reconstituted SRP and BioPhoAC$_n$ peptides where a Cys residue was moved along the peptide in positions 10 to 14. The positions of bands corresponding to noncleaved Ffh, NG and M domain are indicated by an arrow. An asterisk indicates the position where the band corresponding to the M domain would be located. “MW” indicates the lane with the molecular weight marker and the corresponding mass of each band is shown (in kDa).

**Table I**

Sequences of the Signal Peptides Used in this Study

| Peptide | Sequence |
|---|---|
| BioPhoA | Biotin-MKQKKKLALLLLLLLLTPVTKA-$NH_2$ |
| $BioPhoAC_2$ | Biotin-MCKQKKKLALLLLLLLLTPVTKA-$NH_2$ |
| $BioPhoAC_{10}$ | Biotin-MKQKKKLAL**C**LLLLLLTPVTKA-$NH_2$ |
| $BioPhoAC_{11}$ | Biotin-MKQKKKLALL**C**LLLLLTPVTKA-$NH_2$ |
| $BioPhoAC_{12}$ | Biotin-MKQKKKLALLL**C**LLLLTPVTKA-$NH_2$ |
| $BioPhoAC_{13}$ | Biotin-MKQKKKLALLLL**C**LLLTPVTKA-$NH_2$ |
| $BioPhoAC_{14}$ | Biotin-MKQKKKLALLLLL**C**LLTPVTKA-$NH_2$ |
| $BioPhoAC_{23}$ | Biotin-MKQKKKLALLLLLLLLTPVTKA**C**$NH_2$ |
| $BioPhoABpa_{10}$ | Biotin-MKQKKKLAL(**Bpa**)LLLLLLTPVTKA-$NH_2$ |
| $BioPhoABpa_{11}$ | Biotin-MKQKKKLALL(**Bpa**)LLLLLTPVTKA-$NH_2$ |
| $BioPhoABpa_{12}$ | Biotin-MKQKKKLALLL(**Bpa**)LLLLTPVTKA-$NH_2$ |
| $BioPhoABpa_{13}$ | Biotin-MKQKKKLALLLL(**Bpa**)LLLTPVTKA-$NH_2$ |
| $BioPhoABpa_{14}$ | Biotin-MKQKKKLALLLLL(**Bpa**)LLTPVTKA-$NH_2$ |

The positions indicated in bold were replaced by Cys to create the $BioPhoAC_n$ set of peptides or Bpa to create the $BioPhoABpa_n$ series. The $BioPhoAC_n$-BP series of peptides was created with the $BioPhoAC_n$ set of peptides by alkylation of the free Cys with 4-maleimido-benzophenone.