Mapping the Signal Sequence-binding Site on SRP Reveals a Significant Role for the NG Domain*

We present evidence that the signal recognition particle (SRP) recognizes signal sequences via the NG domain on the SRP54 protein subunit. Using a recently developed cross-linking method (Fancy, D. A., and Kodadek, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6020–6024; Correction (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1317), we find that signal peptides cross-link to the Escherichia coli SRP protein Ffh (the homologue of the mammalian SRP54 subunit) via the NG domain. Within the NG domain, the cross-linking site maps to the ras-like C-terminal subdomain termed the G domain. This result stands in contrast to previous studies, which concluded based on nascent chain cross-linking that the signal sequence bound to the adjacent M domain. As independent evidence of a direct binding interaction between the NG domain and the signal sequence, we find that the NG domain of Ffh binds signal peptides as an isolated entity. Our results suggest that the NG domain forms a substantial part of the binding site for the signal sequence.

It has been known for many years that proteins are routed out of the cytoplasm to other compartments of the cell by specific targeting sequences (1). Secretory proteins are directed out of the cell by a signal sequence found at the amino terminus of their peptide chain. These signal sequences consist of an amino-terminal region (the n region) enriched in basic amino acids, followed by a stretch of hydrophobic amino acids between 6 and 15 amino acids in length (the h region) and ending with a short stretch of less hydrophobic but generally uncharged amino acids (2, 3). Beyond these shared overall characteristics, the composition of the signal sequence is highly variable.

In mammalian cells, the signal recognition particle (SRP)1 makes the first interaction with the signal sequence to guide a protein into the secretory pathway (for review, see Ref. 4). The SRP, a ribonucleoprotein complex consisting of six protein subunits and a single RNA molecule, selectively directs ribosomes synthesizing secretory and membrane proteins to the endoplasmic reticulum membrane. The SRP54 protein subunit of the SRP binds to the exposed signal sequence, causing a halt in translation referred to as elongation arrest, which is mediated by the SRP9 and SRP14 subunits. Ultimately, the SRP releases the nascent polypeptide chain into the translocon at the endoplasmic reticulum membrane upon interaction, in a GTP-dependent manner, with its membrane-resident receptor, Sro.

The binding of signal peptides to the SRP is not only a biologically essential process but is also of great interest from a structural biology perspective. High fidelity in the selection of proteins for secretion demands that the SRP recognize the features of signal sequences that uniquely distinguish them from random segments of non-secretory proteins. It is therefore highly probable that the binding of signal sequences to SRP represents a novel mode of protein-peptide interaction. As an essential first step in understanding this binding process, the site on the SRP54 protein where the signal sequence binds must be identified. The SRP54 protein consists of two domains, termed the NG domain and the M domain. Cross-linking studies have implicated the C-terminal M domain as the primary binding site for signal sequences (5–7). As for the adjacent NG domain, various lines of evidence have supported the idea that it has an important role in signal-sequence recognition (6, 8, 9), but it is yet to be confirmed that it binds directly to the signal sequence.

A recent crystal structure of a bacterial homologue of the SRP54 subunit, termed Ffh, revealed a hydrophobic groove in the M domain dominated by a long, flexible conserved loop termed the finger loop (10). This groove was proposed to be the signal sequence-binding site. Unfortunately, there is relatively little experimental data with which to evaluate this attractive hypothesis. To confirm whether the groove truly represents the binding site will require that the precise segments of the M domain that bind the signal sequence be identified.

To map the segments of Ffh comprising the signal peptide-binding site, we used the recently developed PICUP (photo-induced cross-linking of unmodified proteins) cross-linking method (11, 12). The PICUP method relies on photolisys of a metal complex (such as tris(2,2′-bipyridyl)-ruthenium(II) chloride hexahydrate), which photo-oxidizes the metal. This activated metal complex is believed to extract an electron from an aromatic side chain to create a radical species that can then react with a variety of groups, such as another aromatic residue or a nucleophilic amino acid. Since this method forms a linkage between two proteins without the intervention of a spacer arm, it is a more precise means to map the signal peptide-binding site than the cross-linking approaches that have been used previously in the SRP system.

Surprisingly, we found the PICUP method cross-links signal peptides to the Escherichia coli SRP exclusively via the NG...
domain. As independent evidence that the signal sequence interacts with the NG domain of Ffh, we also find that the NG domain binds efficiently and specifically to signal peptides as an isolated entity. Overall, we conclude that the NG domain interacts extensively with the signal peptide.

EXPERIMENTAL PROCEDURES

Protein, Peptide, and RNA Preparation—All of the experiments described in this paper used Ffh prepared as described previously using the expression plasmid pET16bFfh (13). At a late stage during this work, mutations of two weakly conserved residues were discovered in the pET16bFfh plasmid (K28E, K342R; Lys28 is 28% conserved in an alignment of 150 ng sequences and on the surface of the N domain; Lys342 is 13% conserved and in the finger loop). To verify that the two mutations do not significantly alter the structure of Ffh, CD thermal melts were used to compare the stability of the pET16bFfh-derived protein with authentic wild-type Ffh. No significant difference between the thermal melts of the two proteins is detectable. As evidence that the binding interaction of signal peptides with pET16bFfh-derived Ffh detected by PICUP cross-linking is not an artifact caused by the presence of the two mutations, it was found that the authentic Ffh lacking the two mutations also cross-links efficiently to signal peptides by the PICUP method (data not shown). Finally, as evidence that the pET16bFfh-derived Ffh recognizes the signal sequence in a functionally relevant manner, it was observed that the protein could be reconstituted into the mammalian SRP to yield a chimeric SRP able to arrest elongation of secretory proteins.2

Ffh and the mutants E89C, E277C, S321C/C406S, and Y140Q/Y148F/S523C/C406S were purified as described previously (15). The N-terminal deca-histidine tagged M domain (15) and the NG domain construct (14) were prepared as described previously.

The biotin-labeled, leucine-enriched alkaline phosphatase signal peptide was synthesized by solid phase methods on a PerSeptive Biosystems automated peptide synthesizer using Nα-Fmoc (N-(9-fluorenyl) methoxycarbonyl)-protected amino acids. The label was placed on the N terminus by coupling biotin-O-p-nitrophenyl ester (Sigma) to the deprotected resin-bound peptide at pH 8. The peptide was purified by reverse-phase HPLC on a phenyl column maintained at 55 °C. Biotin-labeled molecular weight markers were obtained from PerkinElmer Life Sciences.

All of the experiments described in this paper used a 43-nucleotide fragment of 4.5S RNA that has been described previously (15). This fragment encompasses the binding site for Ffh (16) and the region of SRP RNA most highly conserved among different organisms (17). A slightly larger region of 4.5S RNA, encompassing just six additional non-conserved nucleotides, believed to be Watson-Crick base paired, is slightly larger region of 4.5S RNA, encompassing just six additional non-conserved nucleotides, believed to be Watson-Crick base paired, is sufficient to complement depletion of full-length 4.5S RNA (18).

PICUP Cross-linking—The general cross-linking protocol (11) involved mixing protein, RNA, and signal peptide at the desired concentrations in 15 mM potassium phosphate buffer, pH 7.6, 150 mM NaCl, and 10% glycerol (buffer C). In a darkened room, Ru(bipy)3Cl2 was then added from a 25 mM stock solution, to final concentrations of 0.125 mM and 2.5 mM, respectively. Reaction volumes ranging from 20 to 500 μl were used. The mixture was then immediately illuminated under a flashlight with a 400 nm cut-on filter for 30–45 s. The reaction was then flash-frozen in liquid nitrogen for later analysis or immediately boiled in 60 mM Tris, 6% w/v SDS, 10% glycerol, pH 6.8 (loading buffer) for analysis by SDS-PAGE.

To detect biotin-labeled fragments, SDS-PAGE gels were electroblotted onto nitrocellulose membranes, and the membrane was then incubated for 1 h in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and 5% w/v non-fat dry milk (blocking buffer). After extensive washing with PBS-T, the membrane was incubated for 1 h in PBS-T containing a 1:1000 dilution of streptavidin-HRP conjugate (Amersham Biosciences). After further washing of the membrane, chemiluminescent detection of the biotin label was undertaken following the procedure in the Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences).

NTCB Cleavage of Ffh-signal Peptide Adduct—A 500-μl cross-linking reaction was set up in a 1.5-ml microcentrifuge tube containing 1 μM Ffh, 2 μM RNA, and 2 μM biotin-labeled signal peptide. After a 45-s illumination, the reaction was quenched by adding β-mercaptoethanol to 5 mM and imidazole was added to 5 mM. 30 μl of a Ni-NTA suspension was added and the mixture incubated on ice for 30 min with occasional mixing. The mixture was then filtered through a spin column (Promega, Madison, WI) in a 1.5-ml microcentrifuge tube, and the filtrate was removed by centrifugation. The pelleted resin was washed three times with 100 μl of 15 mM potassium phosphate, 150 mM NaCl, 8 M urea, 5 mM imidazole, pH 7.6 and then eluted with 140 μl of the same buffer supplemented with 200 mM imidazole. After adding dithiotreitol to 1 mM, NTCB was added to 60 μl of the eluate to 5 mM, and the reaction incubated at 37 °C overnight. 1 M NaOH was then added to increase the pH to 10, and the reaction was incubated at 37 °C for 4 h. Protein was precipitated with trichloroacetic acid, redissolved in sample buffer, and analyzed by SDS-PAGE.

Ni-NTA Pull-down Assay—To a 100-μl mixture of 1 μM His-tagged protein, 2 μM RNA, and 0.2–1.3 μM biotin-labeled signal peptide in buffer C supplemented with 5 mM imidazole and 20 μl of a Ni-NTA resin suspension (pre-equilibrated 1:1 in buffer C) was added. After 30 min on ice with occasional mixing, the mixture was filtered through a spin column in a 1.5-ml microcentrifuge tube, and the filtrate was removed by centrifugation. The pelleted resin was washed three times by resuspending in 100 μl of buffer H supplemented with 5 mM imidazole. His-tagged proteins were eluted with 40 μl of buffer H supplemented with 200 mM imidazole, and 10 μl of the eluate was then filtered through a nitrocellulose membrane (Millipore, HAWP 0.45 μm pore size). To detect biotin label the membranes were treated in the same way as membranes containing protein transferred from SDS-PAGE gels by electroblotting. Protein in the residual (30 μl) eluate was precipitated with trichloroacetic acid for SDS-PAGE analysis.

Solvent Accessibility Calculations—Solvent accessibility calculations were undertaken within the Frosstat module of the InsightII program (Accelrys, Inc., San Diego, CA) using van der Waals radii for the protein atoms and a solvent radius of 1.4 Å.

RESULTS

Cross-linking of Signal Peptides to Ffh—Ffh in complex with a 43-nucleotide 4.5S RNA fragment is efficiently cross-linked by the PICUP method to a modified version of the alkaline phosphatase signal peptide carrying a biotin label, termed here PhoA9L1A (Fig. 1). This signal sequence has been shown previously to interact efficiently with Ffh (20). Evidence that the observed cross-linking depends on a specific binding interaction with the signal peptide comes from the observation that the signal peptide cannot be cross-linked to cellular retinoic acid-binding protein (CRABP), glutathione-S-transferase, lysozyme, or CRABP (cellular retinoic acid-binding protein).

FIG. 1. The PICUP method cross-links signal peptides efficiently to Ffh. An N-terminally biotin-labeled, leucine-enriched variant of the alkaline phosphatase signal peptide, MKQKKK-IALLLLLLLTPVTKAC-NH2, was cross-linked to Ffh in complex with a 43-nucleotide 4.5S RNA fragment. The biotinylated Ffh-signal peptide adduct is detectable by SDS-PAGE after electroblotting onto a membrane and probing the membrane with streptavidin-HRP conjugate. By comparison, the PICUP method yields little, if any cross-linked product when Ffh is substituted with glutathione-S-transferase, lysozyme, or CRABP (cellular retinoic acid-binding protein).

H. Bernstein, personal communication.

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lysozyme. CRABP and lysozyme both contain more tryptophan and tyrosine residues than Ffh, and several are significantly surface-exposed; these residues are considered the most likely to participate in the PICUP cross-linking reaction (Refs. 11 and 12, and see below). Therefore, these control proteins should be competent to form cross-links by the PICUP method were it able to capture transient, nonspecific interactions. In addition, the CRABP construct contains the same deca-histidine tag that is present on the N-terminus of the Ffh construct used in all of our cross-linking experiments. The lack of cross-linking to CRABP argues that the formation of cross-links to Ffh is not an artifact caused by the attached deca-histidine tag.

**Signal Peptides Cross-link to the NG Domain of Ffh**—Ffh was mutatet to incorporate sites for specific cleavage of the signal peptide-Ffh adduct with the reagent NTCB (5-nitro-2-thiocyanobenzoic acid), which specifically cleaves X-Cys bonds (21). To enable selective cleavage into fragments encompassing the NG domain and the M domain, the cysteine in the M domain of wild-type *E. coli* Ffh (Cys406) was mutated to serine, and a cysteine was introduced into the linker between the two domains by mutating Ser321 to cysteine. A serine is likely to be well tolerated at position 406 because serine is found frequently at this position (in 50 out of an alignment of 60 SRP54 sequences). NTCB cleavage of the S321/C406S mutant yields fragments of the expected sizes for the NG and M domains (Fig. 2A, panel i). Surprisingly, ECL detection after cross-linking reveals the presence of the biotinylated signal peptide in an adduct with the NG domain (Fig. 2A, panel ii). A fragment corresponding to the M domain-signal peptide adduct is not detectable, even at high loading levels.

As noted above, tyrosine and tryptophan are believed to have the highest capacity to form cross-links in the PICUP protocol, followed by histidine and then phenylalanine (11, 12). Ffh lacks tryptophans and contains only two tyrosines, which are in the NG domain. However, the preferential cross-linking to the NG domain does not depend on the presence of the two tyrosines as the signal peptide still cross-linked to the NG domain after both tyrosines were replaced within the context of the S321/C406S mutant (Fig. 2B). The preferential cross-linking to the NG domain is therefore not caused by the distribution of tyrosines on Ffh. We conclude that there must be phenylalanine or histidine residues suitably positioned with respect to the bound peptide to form cross-links. Both the NG and M domains contain several Phe and His residues that could form cross-links. In fact, isolated M domain constructs, with or without deca-histidine tags, are able to cross-link biotinylated signal peptide using the PICUP method, albeit weakly. But in the context of intact Ffh, the major cross-linked product was the NG domain/signal peptide adduct, arguing that the NG domain plays a role in binding the signal peptide.

**Binding of Signal Peptides to the Isolated NG Domain**—Further evidence that the preferential cross-linking to the NG domain reflects a major role for this domain in binding the signal sequence was provided by a direct binding assay, which revealed that the isolated NG domain has the capacity to bind signal peptides in the absence of the M domain. A pull-down assay, monitoring co-elution of the biotin-labeled signal peptide with N-terminally deca-histidine-tagged protein from Ni-NTA resin, was used to assess binding of the biotin-labeled signal peptide to Ffh and to a construct encompassing the NG domain (Met-Lys324). We found that the biotin-labeled peptide co-elutes from the resin with the His-tagged protein in the case of both the NG domain and Ffh (Fig. 3). As evidence of the specificity of this interaction, the biotin-labeled peptide does not co-elute from the resin with deca-histidine-tagged cellular retinoic acid binding protein. Interestingly, an N-terminal deca-histidine-tagged construct encompassing the M-domain (residues 297–453 of Ffh) also did not show appreciable signal peptide binding under the same conditions. With a 6-fold increase in the concentration of biotin-labeled peptide, binding to the M domain is observable, but the level of biotin label co-eluted from the Ni-NTA resin is still less than that observed under the same conditions with Ffh and the NG domain (data not shown).

As evidence that the signal peptide is not binding to multiple, nonspecific sites on either protein, the binding of the labeled signal peptide can be competed with 10-fold excess of unlabelled signal peptide (Fig. 3B, points 2 and 5). A similar excess of the LamB signal peptide, which, owing to its considerably lower hydrophobicity, should bind to Ffh with lower affinity than the alkaline phosphatase-based peptide used here (22), was not able to compete for binding of the labeled peptide (Fig. 3B, points 3 and 6).

**Mapping the Cross-linking Site to the G Domain**—The NG domain consists of two subdomains, with a helical bundle at the
N terminus termed the N domain adjacent to the ras-like G domain (23). Although the N domain has been suggested to interact with the signal sequence (8), we found by using NTCB cleavage at an engineered cysteine that the cross-linking site maps to the G domain. A cysteine was introduced into the linker between the N and G domains by mutation of Glu89 to cysteine. Upon cleavage of the adduct between the E89C mutant of Ffh and the biotinylated signal peptide, a biotin-labeled fragment was observed with an SDS-PAGE mobility of between 37.5 kDa and 46.5 kDa, which fits with the molecular mass of the Cys89=Gly405 fragment linked to the signal peptide (1 μM protein, 0.2 μM signal peptide) in the presence of Ni-NTA resin and 2 μM of a 4.5S RNA fragment. After extensive washing of the resin, the bound His-tagged protein was eluted using buffer containing 200 mM imidazole, and the eluate was filtered through a nitrocellulose membrane. Biotin label on the membrane was visualized as in Fig. 1 by probing the membrane with streptavidin-HRP conjugate. The positions on the membrane where the eluates from the various binding reactions were filtered are marked with arrows. SDS-PAGE gel analysis of the eluates from the binding reactions, shown on the right, confirms a similar quantity of each protein is rescued from the Ni-NTA resin. Binding was monitored as described in A. All binding reactions contain 1 μM protein, 2 μM 4.5S RNA fragment, and 1.25 μM biotin-labeled PhoA9L1A. Addition of excess unlabeled PhoA9L1A (15 μM, points 2 and 5) competes off binding of the labeled peptide. By comparison, excess unlabeled KRRLamB signal peptide (sequence MMITLKRKKRKLPLAVAVAAGVMSAQAMA) has a minor effect (points 3 and 6, 25 μM KRRLamB added).

The cross-linking site therefore maps to the G domain. To further define the segment of the G domain containing the cross-linking site, a cysteine was introduced at residue 277 and using the cysteine-specific cleavage agent NTCB, the cross-linking site can be localized to between residues 89 and 276, in the G domain. NTCB cleavage of adduct from PICUP cross-linking of biotin labeled signal peptide to wild-type Ffh and two mutants incorporating an additional cysteine, E89C and E277C. Biotin-labeled fragments were detected as in the legend to Fig. 1 and are identifiable based on their mobility on SDS-PAGE. B and D, Coomassie Blue staining of SDS-PAGE gels verifies that the E89C and E277C mutants are cleaved into the expected fragments based on the positions of the introduced cysteines.

DISCUSSION

The work described in this paper provides the first definitive evidence that the NG domain interacts directly with the signal sequence. Other studies had suggested that the NG domain had a role in signal sequence recognition (6, 8, 9) but left uncertain whether it directly interacted with the signal sequence or whether it enhanced signal sequence binding by stabilizing the fold of the adjacent M domain. Using the PICUP cross-linking system, signal peptides have been shown here to cross-link to the NG domain of Ffh but to only a very much weaker extent to the M domain. Second, Ffh has been shown to bind signal sequences even after removal of the M domain. These two observations point to major involvement of the NG domain in the binding of the signal sequence.

A central role for the NG domain in signal sequence recognition contrasts with the dogma, built up from previous cross-linking studies and reinforced by structural observations (15, 24), that the M domain contains the primary binding site for signal sequences (6, 8, 9). Previous cross-linking studies have not detected cross-linking of the signal sequence to the NG domain. In these studies cross-linking was mediated either by 4-(3-trifluoromethyl)-benzoyl (5, 6) or by 5-azido-2-nitrobenzoyl groups (7) attached to the ε-amo group of a lysine in the n region of the signal sequence. The 4-(3-trifluoromethyl)-benzoyl group has been shown to insert efficiently into OH bonds (25) and therefore threonine and serine are good candidates to react with this reagent. By contrast, the PICUP method relies upon the formation of direct linkages between two aromatic residues or between an aromatic residue and a nucleophile (11, 12). The signal peptide lacks aromatic residues but does contain lysine and cysteine residues, both of which are considered as candidate nucleophile residues to participate in
PICUP cross-linking (11, 12). The cysteine sulfhydryl is a stronger nucleophile at the pH used for cross-linking and therefore can be considered the more likely candidate for cross-linking.

A notable difference between the aziridino/azido based methods and the PICUP method is in the length of the "spacer arm" linking the signal peptide to Ffh. With the former methods, the length of the spacer arm is at least 5 Å, whereas the PICUP method forms direct linkages between amino acid side chains. In addition, the two methods are likely to differ with regard to the type of residues that are cross-linked. Finally, the method used here is most likely to form cross-links to the cysteine in the c region of the signal peptide. By contrast, the aziridino/azido based method forms cross-links exclusively to the n region of the signal sequence. Overall then, there are a number of reasons why the two methods will sample spatially distinct regions of the signal peptide-binding site. This may explain why the two different approaches detected cross-linking to different domains of Ffh.

The observed preferential cross-linking to the NG domain and the fact that the isolated NG domain binds signal peptides invite analysis of the structure of the NG domain in order to identify candidate signal peptide-binding sites. Two conditions should be met by these sites: They should present clusters of exposed conserved hydrophobic amino acids that can bind the conserved hydrophobic cores of signal sequences, and they should be close enough to histidines (or possibly phenylalanines) to account for the cross-linking to a Cys in the signal peptide c region. Within the G domain segment that cross-links the signal sequence, there are two tyrosines (residues 140 and 184), four histidines (residues 126, 193, 206, and 261), and six phenylalanines (residues 21, 156, 157, 180, 216, 233, and 269). Our mutagenesis results show clearly that Ffh does not require tyrosines to cross-link to the signal peptide. Hence, one of the histidines or phenylalanine residues must be in proximity to the bound signal sequence.

We examined the surface of the NG domain by visual inspection of space-filling models of the Thermus aquaticus Ffh NG domain-GDP complex (PDB entry 2NG1; Ref. 26). Side chains of residues corresponding to positions in an alignment of 60 SRP54 sequences where a hydrophobic residue type is more than 70% conserved are shown in yellow. All residues in the N domain are colored blue, residues in the I-box are colored green. The green and red regions of the G domain encompass the signal sequence cross-linking site. In orange is the C-terminal part of the G domain, which was not cross-linked to the signal peptide. GTP is shown in cyan. Clusters of conserved hydrophobic residues that stand out on visual inspection of the model are depicted, together with the area of nonpolar atoms exposed by the cluster on the surface of the NG domain. All residue numbering corresponds to the T. aquaticus Ffh sequence.

FIG. 5. Identification of clusters of conserved hydrophobic residues on the surface of the NG domain. Space-filling models are shown of the T. aquaticus NG domain-GDP complex (PDB entry 2NG1; Ref. 26). Side chains of residues corresponding to positions in an alignment of 60 SRP54 sequences where a hydrophobic residue type is more than 70% conserved are shown in yellow. All residues in the N domain are colored blue, residues in the I-box are colored green. The green and red regions of the G domain encompass the signal sequence cross-linking site. In orange is the C-terminal part of the G domain, which was not cross-linked to the signal peptide. GTP is shown in cyan. Clusters of conserved hydrophobic residues that stand out on visual inspection of the model are depicted, together with the area of nonpolar atoms exposed by the cluster on the surface of the NG domain. All residue numbering corresponds to the T. aquaticus Ffh sequence.

Six clusters exposing more than 50 Å² of non-polar surface were identified (Fig. 5); the largest cluster comprises 133 Å². Estimates of the surface area buried on proteins upon binding to peptide ligands range from ca. 400 to over 1000 Å² (27). About half the buried area in protein-protein interactions on average is hydrophobic (28), suggesting that the observed patches on the NG domain are smaller than would be expected for signal peptide-binding sites, particularly given the likely reliance of signal sequence binding on hydrophobic core interactions. The insufficient hydrophobicity of the surface of the NG domain strongly suggests that it undergoes a conformational change to bind the signal peptide. Our previous studies have in fact implied that signal peptides induce conformational changes in the NG domain (13). Among the identified clusters, a conformational change that increases the hydrophobic surface area it exposes can be envisaged most easily for site 2. Movement of the C-terminal helix away from the N domain can be considered a feasible conformational change given that the helix is flanked by a disordered loop and the disordered NG-M domain linker. Solvent accessibility calculations on a modified version of the structure in which the C-terminal helix has been physically separated from the rest of the molecule reveal that a movement of the C-terminal helix may increase the exposed nonpolar surface available for interaction up to a maximum of 475 Å².

Site 2 is also a plausible binding site in terms of the availability of residues on the surface of Ffh able to form cross-links with the signal peptide. There is an exposed histidine residue (His138, corresponding to Gly 126 in T. aquaticus Ffh, 77% accessible) within a 20- to 33-Å distance from each of the residues in site 2 (based on Ca-Ca distance). There is also a phenylalanine (Phe123, corresponding to Tyr121 in T. aquaticus Ffh, 25% accessible) within 13–25 Å of the residues in site 2. The residues in the cluster should interact primarily with the h region of the signal peptide. The candidate cross-linking cysteine in the c region of the signal peptide is located seven residues downstream of the h region. It is possible for the cysteine to become cross-linked to a residue within 24 Å of where the h region binds if the c region adopts an extended conformation.
Given the evidence from cross-linking and the pull-down assay that the signal sequence binds to the NG domain, together with the previously observed cross-linking of signal sequences to the M domain, it can be concluded that the signal peptide-binding site is in proximity to the interface between the two domains. Defining how the two domains interact, an issue left uncertain by the crystal structure of T. aquaticus Ffh (10), is therefore essential for a full understanding of how SRP binds signal peptides.

In addition to providing a new perspective on signal-sequence recognition, the binding of the signal sequence to the NG domain has important implications with regard to how the GTP-dependent binding of the SRP receptor mediates release of the signal sequence. The binding site for the SRP receptor is presumably to a large extent on the NG domain of the SRP, as two studies have shown that the isolated Ffh NG domain can interact with its receptor (29, 30). To clarify how the receptor mediates signal sequence release, the precise locations of the binding sites on the NG domain for both the receptor and the signal sequence must now be determined. Two possibilities can be considered: 1) The receptor may influence signal peptide binding at a distant site in an allosteric fashion. 2) Alternatively, the two binding sites (for receptor and for signal sequence) may be sufficiently close to each other for receptor binding to sterically occlude the interaction of the signal peptide with its binding site. That the two binding sites may be in proximity to each other is supported by the signal peptide cross-linking site mapping to the G domain, which, in a recently published model of the Ffh/PtsY complex, encompasses the majority of the receptor binding site (31).

The observation that the GTPase domain of Ffh binds the signal sequence also invites the speculation that other proteins that bind to both nucleotide and hydrophobic peptides, such as molecular chaperones and the translocase subunit SecA, similarly use nucleotide binding-domains to recognize their substrate peptides. Consistent with this, a recently published study has shown that a truncated version of SecA composed largely of one of its two ATPase domains binds signal peptides efficiently (32).

In summary, we have shown that Ffh uses the NG domain to recognize signal peptides. Ffh does not require the M domain to bind signal peptides, and signal peptides have been found to cross-link to Ffh exclusively via the NG domain, two observations which support a major role for the NG domain in signal sequence binding. Our data also reveal an unexpected role for the ras-like G domain in signal sequence recognition. Future attempts to decipher how the SRP recognizes signal sequences must pay great attention to the role of the NG domain.

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