Communication

Synthetic Signal Peptides Specifically Recognize SecA and Stimulate ATPase Activity in the Absence of Preprotein*

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Although it is known that virtually all exported proteins require a signal peptide, it is not clearly understood how the signal peptide interfaces with the translocation machinery to achieve transport. In this study we document a direct interaction between the signal peptide and SecA, a primary component of the translo- case in Escherichia coli, and show that the signal peptide itself can stimulate SecA-lipid ATPase activity. Using synthetic signal peptides corresponding to the wild type alkaline phosphatase signal sequence and two model sequences, we find that the extent of stimulation of SecA ATPase activity by the different peptides parallels the hierarchy of results found for in vitro function (Izard, J. W., Doughty, M. B., and Kendall, D. A. (1995) Biochemistry 34, 9904–9912). The peptide-induced activity requires a lipid to protein molar ratio of at least 300:1 and liposomes enriched in negatively charged phospholipids. Furthermore, specific binding of the signal peptide to SecA was demonstrated using chemical cross-linking and competition with unlabeled peptides.

The most striking link among all exported proteins is the requirement for a signal peptide. Yet understanding how signal peptide function has been hampered by complexities inherent in all systems. First, signal peptides participate in multiple steps in the transport process and come into contact with a variety of different environments such as the aqueous cytoplasmic milieu, the charged surface of a membrane, and its apolar interior. Second, signal peptides have been implicated in a number of different protein-protein interactions: with their own attached proteins to delay folding, with components of the ribosomal complex, the translocation machinery, and with leader peptidase (1). Few in vitro systems have directly documented and characterized these interactions in a way that can be convincingly related to the biological system. Thus, these fundamental issues remain unanswered. With what components do signal peptides directly interact and what is the mechanistic outcome of these interactions?

For Escherichia coli, it is of particular interest to delineate how a transported protein is recognized by SecA (2, 3) because it functions at the gateway of translocation sites in the inner membrane. This multifunctional protein interfaces with the cytoplasmic chaperone SecB (4) and with nascent preproteins potentially to facilitate targeting. It is also associated with anionic phospholipids (5–7) and the integral membrane translocation complex, SecYEG (5, 8), and is in direct contact with the SecY species (9). At the membrane level, the ATPase activity of SecA appears critical for translocation of the nascent chain, perhaps through successive rounds of binding and release of the preprotein (10). To date, however, specific binding of the signal peptide region of a translocating polypeptide to SecA has only been inferred from studies involving mutations in SecA that relieve signal peptide defects (11), cross-linking with preproteins (7, 12, 13), and modulation of SecA ATPase activity in the presence of both signal peptide and mature regions of exported proteins (5, 14). Furthermore, it has been suggested that signal peptides alone are not substrates for SecA and that stimulation of the ATPase activity also requires the presence of the mature protein (5, 14).

In this study, we employ synthetic peptides corresponding to the signal sequence of alkaline phosphatase (PhoA) and idealized model signal peptides that we have previously characterized with regard to a number of physical parameters (15). Using this approach, we demonstrate that peptides corresponding to functional signal sequences (but not nonfunctional ones) bind SecA and stimulate its ATPase activity in liposomes. Significant activity requires at least a 300-fold molar excess of lipid over SecA and negatively charged phospholipid. However, no mature portion of an exported protein is required for the peptide-induced stimulation.

EXPERIMENTAL PROCEDURES

Materials—All media and culture conditions for the overproduction and purification of SecA from E. coli strain BL21-14 have been described (16). The SecA concentration was determined using the extinction coefficient $E = 0.805 \, \text{mg}^{-1} \text{cm}^{-1}$ at 280 nm or by the Bradford assay at 595 nm (17). ATP, reactive blue 4 agarose, DOPC, DOPG, DOPE, and the octapeptide H\text{LVTLVPEK} were from Sigma. Acetone-precipitated and ether-extracted E. coli phospholipids were from Avanti Polar Lipids, Inc. Alabaster, AL. The photoactivatable cross-linker, APDP, was from Pierce. The synthetic signal peptides (Table I) were synthesized and purified as reported earlier (15). Stock solutions were prepared in dimethyl sulfoxide, aliquoted to avoid repeated freezing and thawing, and stored at $-70 \, ^\circ\text{C}$.

SecA ATPase Assay—ATPase assays were conducted essentially as described (5, 16) with the following modifications. Enzyme activity was measured in 50 mM HEPES-KOH, pH 7.0, containing 30 mM KCl, 30 mM KH\text{Cl}, either 0.5 mM or 5 mM magnesium acetate, 1 mM DTT, 4 mM ATP, BSA (0.5 mg/ml), typically 40–320 $\mu$g/ml SecA, and 40–320 $\mu$g/ml phospholipids in a total reaction volume of 50 $\mu$l. Small unilamellar vesicles were formed by sonication in 1 mM DTT using a Branson sonifier; iced samples were subjected to brief pulses to obtain optical clarity. All reaction components were added to acid-washed, glass tubes on ice, the reactions were initiated by the addition of ATP, and incubation was at 37 $^\circ\text{C}$ for 40 min. The amount of inorganic phosphate released was determined as described previously (18). The peptide-stimulated SecA-lipid ATPase activity is given as the difference in the rate of ATP hydrolysis in the presence and absence of peptide.
The hydrophobic core regions of the peptides are represented in bold face, and the amidated carboxyl termini are designated by the NH2 groups attached to the cysteine residues.

Ability to efficiently transport alkaline phosphatase in vivo.

E. coli wild type alkaline phosphatase signal sequence.

Cross-linking Reaction—The amino-terminal group of the wild type peptide was radiolabeled with [3H]acetic anhydride in N-methylpyrrolidine essentially as described by Seligman (19). The cross-linking of APDP to the labeled peptide was carried out as described by the manufacturer with minor modification. A Kwik Sep polycrylamide 1800 desalting column (Pierce) was used for final purification.

SecA (160 pmol) in the ATPase assay buffer was incubated at 37 °C for 40 min with the APDP-labeled, tritiated, wild type peptide and E. coli phospholipid (1.2 mg/ml) in the dark. Competition experiments were carried out similarly except that a 10-fold excess of cold peptide was also present. Subsequently, photostimulation was accomplished by UV illumination for 5 min. Cross-linked complexes were analyzed by SDS-PAGE and liquid scintillation counting of solubilized gel slices.

RESULTS AND DISCUSSION

The synthetic peptides used in this study include one corresponding to the wild type sequence of the alkaline phosphatase signal peptide and two that are model signal sequences, each containing a carboxyl-terminal cysteine for incorporation of a photoaffinity label (Table I). The latter two peptides (3K7L and 1K2L) were designed to have simple sequences while maintaining the prominent physical characteristics of E. coli signal peptides in general. They differ from each other by containing different proportions of leucine and alanine residues to represent functional and nonfunctional core regions, respectively, as determined previously using in vivo analysis (15). These two peptides also differ in amino-terminal charge (+1 versus +3), but this results in no observable difference in transport efficiency in vivo.

To demonstrate binding of synthetic signal peptides to SecA, several challenges had to be considered. Previous studies indicated that SecA needed to be associated with lipids to produce an active form and potentially to produce the conformation which recognizes the signal peptide (5, 6). In addition, we needed to be able to distinguish specific binding to SecA from nonspecific binding of the peptides to either SecA or lipid. These problems were addressed, in part, using SecA ATPase activity as a functional indicator of signal peptide binding in vivo. These initial experimental conditions, maximum stimulation of SecA-lipid ATPase by the functional 3K7L and wild type alkaline phosphatase signal peptide; 1K2L. Each data point represents an average of duplicate assays from at least two separate experiments.

The effects of lipid concentration and composition on the 3K7L peptide-stimulated SecA ATPase activity are shown in Fig. 2. Keeping the concentration of SecA and peptide constant, we observed a direct dependence of the peptide-induced SecA ATPase activity on lipid concentration. At concentrations less than 80 μg/ml lipid the effect of the peptide was somewhat inhibitory whereas above 120 μg/ml lipid (corresponding to a lipid to SecA molar ratio of about 300:1) the peptide was stimulatory. The ATPase activity continues to increase up to 320 μg/ml lipid (corresponding to a lipid to SecA molar ratio of 900:1), the highest concentration used in this study. The trend was similar for DOPC:DOPG (3:1) and DOPC:DOPG (3:1) and slightly enhanced for lipids extracted from E. coli (PE:PG:cardiolipin about 3:1:0.5). These findings have several implications. First, it underlines the requirement that SecA be sufficiently ensconced in lipid to form an ATPase active form (6). It does not necessarily mean that the lipid is needed to produce the signal peptide binding form, only that our means of assaying binding requires it. Second, it provides an explanation as to why signal peptide stimulation of SecA ATPase activity was obscured previously. We estimate that in earlier work (5) the lipid to SecA ratio was below the threshold required to see an effect of the signal peptide alone and that the overall lipid to protein ratio was further reduced by high concentrations of peptide. That SecA is so sensitive to relative lipid concentration, particularly in small liposomes with a high radius of curvature, is not surprising given its size (102 kDa) and its pronounced integration into the bilayer (6, 21, 22). Third, that the peptide-induced ATPase activity is enhanced (rather than

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FIG. 1. Effects of synthetic signal peptides on E. coli SecA-lipid ATPase activity. The reactions were performed in 50 mM HEPES-KOH, pH 7, containing 30 mM KCl, 30 mM NH4Cl, 5.0 mM Mg(OAc)2, 1 mM DTT, 4 mM ATP, BSA (0.5 mg/ml), SecA (40 μg/ml), and DOPC:DOPG (molar ratio 1:1), 500 μg/ml. All other conditions were as described under “Experimental Procedures.” ○, 3K7L; □, wild type alkaline phosphatase signal peptide; ●, 1K2L. Each data point represents an average of duplicate assays from at least two separate experiments.
diminished) at higher lipid content argues that the stimulation is due to a direct effect of the peptide on SecA as opposed to a secondary effect on SecA due to lipid perturbation as peptide partitions into the bilayer. In the latter case, we would have expected the effect to be diminished when more lipid is used, effectively diluting the SecA and peptide concentrations.

Several studies have pointed to a role for negatively charged phospholipids in protein translocation in *E. coli* using both *in vivo* (23) and *in vitro* (5, 7, 24) systems for analysis. The dependence of the signal peptide-stimulated SecA ATPase activity on the amount of the anionic lipid, PG (relative to PE), is shown in Fig. 3. Indeed, for the 3K7L peptide, SecA ATPase activity was enhanced as the PG content of the liposomes was increased up to a molar fraction of 0.4, which corresponds to a PE:PG molar ratio of 3:2. No such effect was observed with the nonfunctional 1K2L peptide. Interestingly, optimum activity occurs over a fairly narrow range of PG content (PG molar fraction of 0.4 to 0.6). The increased activity observed with PG may reflect enhanced association of the positively charged peptides with the liposomes and/or may be due to a preferred SecA conformation produced by the presence of sufficient anionic lipids. Unfavorable charge repulsion of neighboring PG molecules among themselves to produce nonbilayer structures (25) or of PG and the anionic SecA could account for the reduced stimulation observed at very high PG levels.

Chemical cross-linking analyses further indicate that the functional signal peptides are interacting with SecA in a direct and specific manner. Incubation of *H*-labeled wild type peptide carrying the photoaffinity label APDP, with liposomes and SecA, followed by UV photoactivation, resulted in substantial cross-linking of the radiolabeled peptide to SecA (Fig. 4). When the experiment is repeated in the presence of a 10-fold excess of unlabeled wild type peptide (carrying neither the [3H]acetyl nor APDP groups), 40% of the labeled peptide is displaced and not cross-linked. This is approximately half of the displacement theoretically expected from an optimal competition between identical species or precisely the amount expected if the labeled peptide has an affinity for SecA that is only twice that of the unlabeled one. To have achieved such high levels of displacement in our experiments is quite remarkable considering that the labels render the peptide more hydrophobic than its unlabeled counterpart and because the illumination and separation of bound ligand from free is not instantaneous. Therefore, covalent complex formation of the labeled peptide and SecA can continue, precluding an “off” state during which the unlabeled peptide could continue to compete. In contrast, excess 1K2L or a control peptide with the sequence HVLTPVEK displaced only 10% and 9%, respectively, of the labeled wild type peptide. Furthermore, APDP-mediated cross-linking of the wild type peptide to SecA in the absence and presence of lipid is very similar (10, 877 cpm and 9, 494 cpm, respectively) indicating that the lipid does not alter the extent of association of the peptide with SecA.

These studies are the first to demonstrate that the signal peptide region alone can bind SecA and stimulate ATPase activity. This does not mean that the mature region does not also bind SecA, only that it is not required for the signal peptide interaction. During the initial association of the preprotein with the membrane, SecA might act as a funnel, in the metaphorical sense. It makes sense that the signal peptide binds SecA and would do so with higher affinity, relative to the mature region, because it may well be the first part of the preprotein that is exposed to translocation sites. This initial interaction can then serve to localize and concentrate the pre-
protein at the translocon for subsequent translocation steps, which may include interactions with other regions of the preprotein.

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