confirmed by indirect immunofluorescence to be $CD3^+$, $CD8^+$, and $CD4^-$. These clones were propagated to large numbers in 12-well plates or 75-$cm^2$ tissue culture flasks by restimulation every 7 to 10 days with autologous CMV-infected fibroblasts and γ-irradiated feeder cells and the addition of 25 to 75 U/ml of IL-2 at 2 and 4 days after restimulation. Written informed consent was obtained from each patient and their bone marrow donor participating in this study after the nature and possible side effects of the study had been fully explained.

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# Peptide Binding by Chaperone SecB: Implications for Recognition of Nonnative Structure

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**The molecular basis for recognition of nonnative proteins by the molecular chaperone SecB was investigated with an in vitro assay based on the protection of SecB from proteolysis when a ligand is bound. The SecB tetramer has multiple binding sites for positively charged peptides. When the peptide binding sites are occupied, the complex undergoes a conformational change to expose hydrophobic sites that bind the fluorescent probe 1-anilinonaphthalene-8-sulfonate. A model is proposed for interaction of nonnative polypeptides with both hydrophilic and hydrophobic sites on SecB.**

Molecular chaperones bind other polypeptides to facilitate protein folding, formation of oligomers, and localization of proteins (*1*). The ability of a given chaperone to recognize many unrelated ligands is remarkable. In *Escherichia coli*, a molecular chaperone, SecB, facilitates the export of proteins by maintaining them in a loosely folded state that is compatible with translocation across the cytoplasmic membrane (*2*, *3*). There is no consensus in primary sequence among the ligands to which SecB binds. Existing evidence supports the idea that selectivity in binding is governed in part by a kinetic partitioning between folding of polypeptides and their association with SecB (*4*). However, what SecB recognizes as a binding site in nonnative polypeptides is still undefined. Evidence presented here indicates that a functional oligomer of SecB has multiple binding sites for positively charged peptides as well as a hydrophobic site that is fully exposed only after the peptide binding sites are occupied.

Previous work (*4*) showed that in vitro SecB [a tetramer of four identical subunits of relative molecular mass ($M_r$) of 16,600 (*5*)] interacts tightly with nonnative proteins without specificity for a particular sequence of aminoacyl residues even though it does not bind the same proteins in their native states. To determine what element is recognized, an assay was established based on the sensitivity to proteolysis of the complex comprising SecB bound to ligand as compared to the sensitivity to proteolysis of the free components. Incubation with proteinase K quantitatively converts free SecB to a form that has lost ~50 residues from the carboxyl terminus (Fig. 1, lanes 1 and 2); in contrast, the free ligand, carboxamidomethylated bovine pancreatic trypsin inhibitor (R·BPTI), is resistant to proteolysis by proteinase K under the same conditions (Fig. 1, lanes 3 and 4). The formation of a complex between SecB and R·BPTI concomitantly renders R·BPTI sensitive and SecB resistant to proteolysis (Fig. 1, lane 5). The presence in the reaction mixture of native BPTI, previously shown not to bind to SecB (*4*), does not protect SecB from proteolysis (Fig. 1, lane 6). Because the concentration of SecB in the assay (0.6 μM) is much greater than the estimated dissociation constant (5 nM) for R·BPTI (*4*), one can use protection from proteolysis to estimate the stoichiometry of the complex as approximately 1 mol of ligand bound to 1 mol of monomeric SecB (Fig. 2A). Thus a tetrameric unit of SecB has multiple peptide binding sites. This assay was used to survey peptides for their ability to bind to SecB. Peptides were considered to be active (see Table 1) if at 15 μM or less they showed at least 50% protection of SecB and inactive (see Table 2) if they afforded no detectable protection of SecB at the highest concentrations tested. The only obvious feature common to all active peptides is their net positive charge.

A quantitative comparison of the interaction of SecB with the various peptides indicates that all are less effective than is R·BPTI (58 residues) in protection of SecB from proteolysis (compare Fig. 2A with Fig. 2, B to D; see Table 1). However, this assay is not suitable for precise determination of affinities since the ligands themselves could be variably susceptible to degradation.

Binding sites on SecB apparently interact most effectively with positively charged peptides when they are flexible. Thus, somatostatin (Fig. 2D) and the defensins, HNP-1 (Fig. 2C), NP-1, and NP-5, were more active in protecting SecB when disulfide bonds that stabilize their structures were reduced with dithiothreitol (DTT). The inclusion of DTT in the assay with other peptides that do not contain disulfides had no effect. In addition, the zinc finger domain of ADRI was two- to threefold more effective in the protection assay when the peptide was free of $Zn^{2+}$ as compared to the $Zn^{2+}$-bound structure.

The observed protection from proteolysis has at least two possible explanations. Interaction of a peptide with SecB might directly exclude proteinase K from the sensitive site through steric hindrance; alternatively, binding of the ligand might induce a conformational change in SecB that results in protection of the sensitive site. The notion that a conformational change is involved is supported by the observation that SecB, which is sensitive to proteolysis at low ionic strength, can be rendered completely resistant to proteolysis even in the absence of ligand if the ionic strength is increased to between 100 and 150 mM with either NaCl or KCl (Fig. 3) or if 10 mM magnesium acetate or 1 mM $CaCl_2$ is pre-

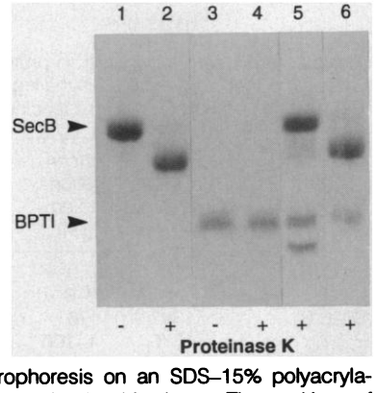


**Fig. 1.** Proteolysis as an assay of ligand binding. Incubation mixtures (0.4 ml, 10 mM Hepes, pH 7.6) contain purified proteins as follows: lanes 1 and 2, SecB (4 μg) purified as described (*14*); lanes 3 and 4, R·BPTI (1 μg); lane 5, SecB (4 μg) and R·BPTI (1 μg); and lane 6, SecB (4 μg) and native BPTI (2 μg). The mixtures were incubated on ice either with (+) or without (−) proteinase K (0.3 μg/ml) for 20 min. Proteolysis was terminated by addition of trichloroacetic acid (final concentration 10%), and the precipitates were collected by centrifugation. The pellets were washed once with acetone and suspended in SDS–gel electrophoresis sample buffer. After incubation at 100°C for 5 min, the samples were analyzed by electrophoresis on an SDS–15% polyacrylamide gel. Only the relevant portion of the Coomassie blue–stained gel is shown. The positions of monomeric SecB (16,600 $M_r$) and BPTI (6,500 $M_r$) are indicated.

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sent. SecB has an isoelectric point of 4 (3), so it is likely that at low ionic strength negative charges on SecB keep regions of the polypeptide in an open, protease-sensitive conformation. High ionic strength or neutralization of charges on SecB by the binding of ligands or divalent cations would allow the formation of a tighter structure. Circular dichroic spectra of SecB either at high ionic strength or with ligand bound show more structure than corresponding spectra at low ionic strength or with the binding site unoccupied (6).

If the peptide binding sites are negatively charged and do not have a strong preference for particular side chains, then polymers of basic amino acids might be readily accommodated. Indeed, low concentrations of poly-L-Lys (poly-Lys) or poly-L-Arg (poly-Arg) are effective in protection of SecB (Fig. 4, A and B; poly-Arg gives a similar result) whereas polymers of Glu (5 μM of $M_r$ 18,000; Sigma) and poly(ProGlyPro) (20 μM of $M_r$ 5,300; Sigma) showed no protection. One mole of poly-Lys ($M_r$ 42,000, ~260 residues) protects ~18 mol of monomeric SecB (Fig. 4A). Thus each Lys polymer binds four or five SecB oligomers with ~14 Lys residues available for each binding site. A Lys polymer of 15 residues also efficiently protects SecB (Fig. 4B). On a molar basis a much higher concentration of $(Lys)_{15}$ polymer is required to protect SecB relative to the concentration of 42-kD poly-Lys (0.7 μM versus 0.017 μM for half-maximal effect); however, when the concentration of the polymers is expressed as micrograms per milliliter, the smaller polymer is less effective by only a factor of 2 (Fig. 4, A and B). Each $(Lys)_{15}$ likely occupies one peptide binding site with an affinity similar to that of each separate site within the larger polymer. Polymers of Lys containing seven or fewer residues showed no protection, whereas $(Lys)_8$ gave 50% protection at ~2.5 μM and $(Lys)_9$, $(Lys)_{10}$, $(Lys)_{12}$, and $(Lys)_{15}$ all gave 50% protection at concentrations between 0.7 and 1.1 μM. Among the peptides surveyed the shortest that were effective in rendering SecB resistant to proteolysis contained 14 or 15 resi-

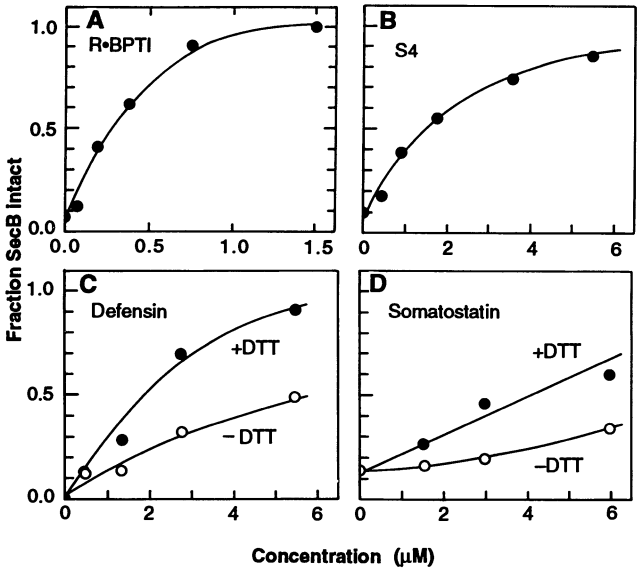


**Fig. 2.** Binding of (**A**) R•BPTI, (**B**) S4, (**C**) defensin, and (**D**) somatostatin to SecB. The assay was carried out as described in the legend to Fig. 1. Data were quantified by densitometric scanning of the stained gels with a Helena Laboratories Quick Scan R & D. When included in the incubation, DTT was 2 mM.

**Table 1.** Peptides that protect SecB from proteolysis (*17*). Protection of SecB was determined as described in the legends to Figs. 1 and 2. $P_\beta$ is from BPTI; S1, S1b, and S4 are from HIV gp41; and the zinc finger is from ADR1.

| Peptide | $M_r$ (daltons) | Concentration for 50% protection (μM) | Sequence |
|---|---|---|---|
| $P_\beta$ | 1680 | 6 | RYFYNAKAGLCQTF |
| S1 | 2400 | <3 | VIEVVQGAYRAIRHIPRRIR |
| S1b | 2856 | <3 | DRVIEVVQGAYRAIRHIPRRIRQG |
| S4 | 2751 | 1 | NNNTRKSIRIQRGPGRAFVTIGKIG |
| Melittin | 2848 | 3.5 | GIGAVLKVLTTGLPALISWIKRKRQQ-$NH_2$ |
| Zinc finger (no $Zn^{2+}$) | 3592 | <4 | RSFVCEVCTRAFARQEHLKRHYRSHTNEK |
| Defensin HNP-1 | 3444 | 2 | ACYCRIPACIAGERRYGTCIYQGRLWAFCC |
| Defensin NP-1 | 3893 | <3 | VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR |
| Defensin NP-5 | 3547 | <3 | VFCTCRGFLCGSGERASGSCTINGVRHTLCCRR |
| Somatostatin | 1638 | 4 | AGCKNFFWKTFTSC |
| Mastoparan | 1479 | 12 | INLKALAALAKKIL-$NH_2$ |

**Table 2.** Peptides that showed no protection at the concentrations tested (*17*). Protection of SecB was determined as described in the legends to Figs. 1 and 2. S026-B is from T4 lysozyme; $P_\alpha$ is from BPTI; GCN4-p1 and Fos-p1 are leucine zippers; Ac, acetyl.

| Peptide | $M_r$ (daltons) | Concentration tested (μM) | Sequence |
|---|---|---|---|
| Bradykinin | 1060 | 9 | RPPGFSPFR |
| M•K bradykinin | 1320 | 2–8 | MKRPPGFSPFR |
| S026-B | 1397 | 70 | Ac-SLNAAKSELDKAIG-$NH_2$ |
| $P_\alpha$ | 1670 | 1–100 | NNFKSAEDCMRTAGGA |
| Glucagon | 3483 | 1–7 | HSEGTFTSDYSKYLDSRRAQDFVQWLMNT |
| GCN4-p1 | 3992 | 3–50 | Ac-RMKQLEDKVEELLSKNYHLENEVARLKKLVGER |
| Fos-p1 | 4609 | 0.4–40 | Ac-CGGLTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAAY |

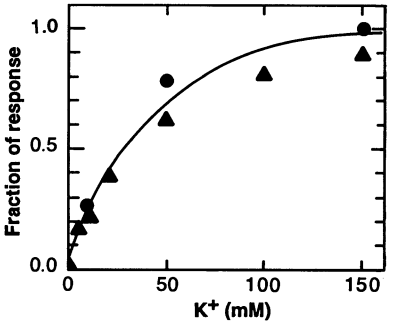


**Fig. 3.** Effect of ionic strength on SecB. The protease assay (●) was carried out as described in legends to Figs. 1 and 2. ANS fluorescence (▲) was measured with a Shimadzu RF-540 fluorescence spectrophotometer with excitation at 370 nm and emission at 472 nm. The cuvette contained 0.6 μM SecB and 15 μM ANS in 10 mM Hepes, pH 7.6, held at 5°C. The ionic strength was increased by additions of potassium acetate (●) or potassium chloride (▲). The maximal response in the protease assay corresponds to complete protection of SecB, and in the fluorescence assay it corresponds to an increase of 5 units (U) of fluorescence (addition of SecB in 10 mM Hepes, pH 7.6, causes no increase in ANS fluorescence, which was 9 U).

dues. This length may be near the limit in size since mastoparan (14 residues) showed the weakest binding and bradykinin (9 residues) did not show any activity despite its net charge of +2. Shorter polymers of poly-Lys may be more effective because they are highly charged.

Poly-Lys was shown to interact with SecB at the physiological peptide binding site by its ability to compete for binding with R·BPTI, which in turn has been shown previously to compete with a natural ligand of SecB, maltose binding protein (MBP) (4). As described above, R·BPTI complexed with SecB is clipped to a distinct fragment whereas free R·BPTI is not (Fig. 1, lanes 4 and 5). Thus the appearance of the fragment indicates that R·BPTI is in the binding site. When SecB was incubated with poly-Lys ($M_r$ 42,000) before the addition of R·BPTI or if poly-Lys was added to SecB simultaneously with R·BPTI, the R·BPTI was resistant to degradation (Fig. 5). The concentration of poly-Lys required to compete with R·BPTI for binding is the same as that needed to render SecB resistant to protease. To eliminate the possibility that indirect effects explain the resistance of R·BPTI to proteolysis, it was shown that poly-Lys does not inactivate proteinase K; the protease degrades other sensitive substrates if they are included in the assay. Neither does poly-Lys inactivate SecB in a nonspecific way since at the same concentrations used for the competition experiment poly-Lys does not affect the ability of SecB to block the folding of MBP as assessed in the previously described fluorescence assay (4).

These binding studies indicate that SecB oligomers have multiple sites that preferentially bind to positively charged stretches of polypeptides. It is reasonable to expect that SecB also has hydrophobic binding sites since during protein export in vivo SecB binds polypeptides that are not tightly folded and such ligands would have hydrophobic regions accessible for interaction with SecB. The fluorescent compound 1-anilinonapthalene-8-sulfonate (ANS), which binds to hydrophobic clusters of aminoacyl residues, was used to search for the putative hydrophobic sites on SecB. When this probe is bound, the intensity of fluorescence increases and the emission maximum is shifted from ~520 nm to shorter wavelengths (7). In this study, neither SecB nor the free ligands (with the exception of poly-Arg) showed any significant binding of ANS. However, upon adding a ligand to SecB (Fig. 6) a hydrophobic site is exposed as evidenced by an increase in fluorescence intensity of ANS and a shift in the maximum of emission to 472 nm. The same emission maximum was seen for ANS that was bound to SecB in complexes with all of the ligands investigated, that is, S4, R·BPTI, mastoparan, poly-Lys ($M_r$ 41,000 and 2,000), and poly-Arg. The intensity of fluorescence seen at saturation of SecB with the peptide ligands varied from two- to threefold higher than the fluorescence of free ANS. Under similar conditions (low ionic strength) the intensity of fluorescence seen with poly-Lys or poly-Arg as the ligand was tenfold higher than that of free ANS. However, this fluorescence induced by the polyamino acids decreased to the same level as seen with peptide ligands when 150 mM KCl and 5 mM magnesium acetate were added to the buffer. The fluorescence of the complex between SecB and the peptide ligand S4 was not significantly affected by the addition of salt (a decrease of 30% was observed). The binding of the negatively charged ANS to poly-Arg and poly-Lys involves electrostatic as well as hydrophobic interaction (8); thus at low ionic strength in the case of the amino acid polymers the fluorescence may reflect the binding of ANS both to SecB and to the polyamino acids, whereas at high ionic strength the binding may be primarily to SecB.

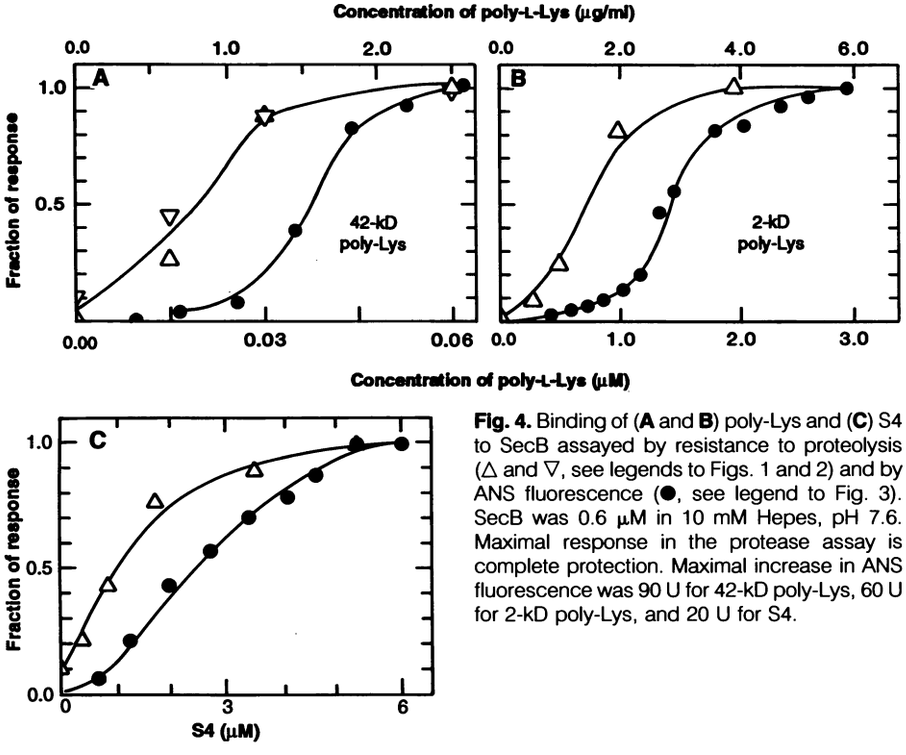


**Fig. 4.** Binding of (**A** and **B**) poly-Lys and (**C**) S4 to SecB assayed by resistance to proteolysis (△ and ▽, see legends to Figs. 1 and 2) and by ANS fluorescence (●, see legend to Fig. 3). SecB was 0.6 μM in 10 mM Hepes, pH 7.6. Maximal response in the protease assay is complete protection. Maximal increase in ANS fluorescence was 90 U for 42-kD poly-Lys, 60 U for 2-kD poly-Lys, and 20 U for S4.

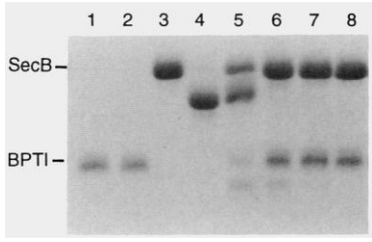


**Fig. 5.** Competition between R·BPTI and poly-Lys for binding to SecB. The assay is carried out as described in the legend to Fig. 1. Incubation mixtures were as follows: R·BPTI without (lane 1) or with (lane 2) proteinase K; SecB without (lane 3) or with (lane 4) proteinase K; SecB and R·BPTI with proteinase K (lane 5); and proteinase K with SecB, RBPTI, and 42-kD poly-Lys at 0.03 μM (lane 6), 0.06 μM (lane 7), and 0.12 μM (lane 8), respectively. When present, SecB was 0.6 μM, R·BPTI was 0.37 μM, and proteinase K was 0.3 μg/ml.

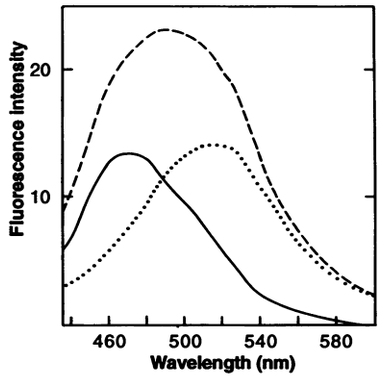


**Fig. 6.** Detection of conformational change by ANS fluorescence. Fluorescence measurements were made as described in the legend to Fig. 3. The emission spectra shown are for ANS (15 μM) and SecB (0.6 μM), dotted line; for ANS, SecB with S4 (5.4 μM), dashed line; and the difference spectrum, solid line.

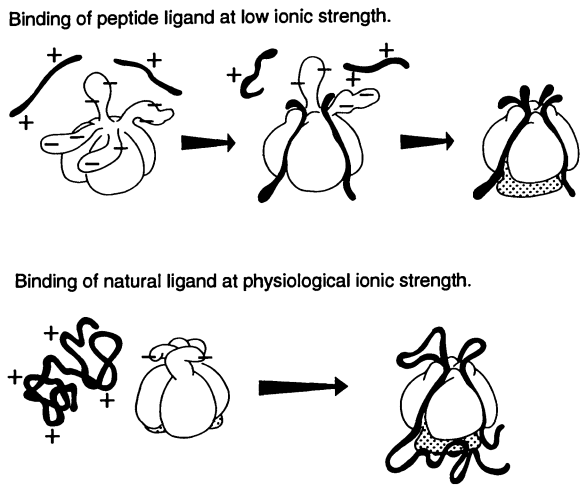


**Fig. 7.** A model of interaction of nonnative polypeptides and SecB. See text for discussion.

In these studies it is not possible to know definitively whether the ANS binds to the ligand, to the SecB, or to a site comprising residues from both. In an experiment of similar design, Martin *et al.* (9) showed that ANS binds the chaperonin groEL in complex with either dihydrofolate reductase or rhodanase. They assumed that the ANS is bound to the ligand and concluded that groEL stabilizes the molten globule state of the polypeptides as a means to facilitate folding. In the case of the ligand-SecB complex, the interpretation that at least part of the enhancement of ANS fluorescence is due to ANS binding directly to a hydrophobic patch on SecB is supported by several lines of evidence. First, even in the absence of ligand, increasing the concentration of salt causes SecB to bind up to 25% of the amount of ANS that is bound in the presence of ligands (Fig. 3). Second, it is not clear that the short peptides (14 to 25 residues) and Lys polymers (8 residues) that induce ANS binding could themselves form a hydrophobic patch sufficiently large to bind ANS. Even though larger Lys polymers are known to bind ANS when they are in the β conformation (8), at the concentration of poly-Lys used here to form complexes with SecB, the poly-Lys alone, even when it was converted to the β structure (10), did not show detectable binding of ANS. A third argument that ANS binds to SecB and not to the bound ligand comes from a comparison of the protease protection and the ANS fluorescence assays (Fig. 4). If ANS bound to the ligand directly when it bound SecB, then the curves representing peptide binding (assessed by protection of SecB from proteolysis) and the increase in ANS fluorescence should be coincident; however, ANS binding does not directly follow peptide binding but is shifted to higher concentrations of ligand. These results are consistent with the interpretation that simultaneous occupation of multiple peptide binding sites is required to induce a conformational change that in turn exposes an ANS binding site on SecB.

If the model is correct that simultaneous occupation of binding sites on SecB induces exposure of a hydrophobic patch, since binding is readily reversible as demonstrated previously (4) and shown here by the competition between poly-Lys and R·BPTI (Fig. 5), then addition of excess SecB to a preformed complex should decrease the amount of ANS bound. Indeed, after addition of SecB to complexes containing as ligand poly-Lys, R·BPTI, or S4, the fluorescence decreased to the expected level as calculated from the final ratio of ligand to SecB.

Two possible physiological roles for the hydrophobic site on SecB immediately come to mind. The site might be involved in interaction with other components of the export machinery or it might function in ligand binding. During export, SecB interacts with at least one other component, SecA, a protein involved in translocation of polypeptides through the cytoplasmic membrane. Since the affinity of SecA for SecB is higher if a precursor polypeptide is bound (11), the hydrophobic site might play a role in binding SecA. Alternatively, physiological ligands, which are loosely structured polypeptides, could interact at both the hydrophilic and hydrophobic sites on SecB. Supporting the notion that the ANS binding site on SecB would be occupied by a portion of a natural polypeptide ligand is the observation that a complex formed between SecB and unfolded mature MBP displays less ANS fluorescence (13 units) than the sum of the ANS fluorescence seen with the separate components [26 units: 17 units for the folding intermediate of MBP and 9 units for SecB at high ionic strength; see (12) for details]. It seems that interactions between hydrophobic patches on SecB and on MBP exclude binding of ANS.

A detailed study of the chaperone BiP revealed a binding site that selects for aliphatic residues (13). This site on BiP might correspond functionally to the hydrophobic site on SecB. The peptide binding site on SecB as defined by the protease assay does not seem to have any preference for hydrophobic residues since copolymers of Lys and Leu or Lys and Val were twofold less effective than was poly-Lys. SecB might have a third type of binding site that prefers negatively charged peptides. Such sites would not necessarily be detected by the assays described here. Gierasch and colleagues (14), using a nuclear magnetic resonance method, the measurement of transferred nuclear Overhauser effects, have shown that upon binding to GroEL a peptide ligand adopts an α-helical conformation whereas the same ligand binds to DnaK in an extended conformation. Our data provide no information on the conformational state of ligands bound to SecB.

A reasonable scenario for the interaction of SecB and ligands is presented in Fig. 7. In low ionic strength SecB is in an open conformation with a region comprising the carboxyl-terminus accessible to protease. The binding of positively charged peptides or an increase in ionic strength changes the conformation of SecB, rendering it resistant to protease and partially exposing the hydrophobic site. At physiological ionic strength SecB is in this conformation with the hydrophobic site partly exposed even if no ligand is bound. Saturation of the binding sites at either low or high ionic strength induces a further conformational change that fully exposes the hydrophobic site, which is then available to bind hydrophobic regions of polypeptide ligands. Exposure of the hydrophobic site only after initial interaction with a ligand would be advantageous because if SecB always displayed a hydrophobic patch it would tend to aggregate. The existence of two sites with different binding requirements would allow the chaperone to have high selectivity for nonnative proteins even though each site demonstrates broad specificity. SecB might transiently interact with flexible loops of native proteins, but recognition of a polypeptide as nonnative would require subsequent occupation of the hydrophobic site on SecB by regions of the polypeptide ligand that would not be accessible in the native state. The same mechanism can be invoked to explain why direct recognition of the leader cannot account for the high selectivity in vivo for binding of precursors to SecB during protein export (15). Even though the leader, which is positively charged, might fill one of the peptide-binding sites the interaction would not be stable unless a second site were filled. The leader slows the folding of the precursor (16), thereby ensuring that the necessary

regions of the polypeptide are available to fill the sites.

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17. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.


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# Phosphorylation-Independent Modulation of L-Type Calcium Channels by Magnesium-Nucleotide Complexes


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**Free magnesium ions and magnesium-nucleotide complexes can exert opposite effects on many fundamental cellular processes. Although increases in the intracellular concentration of magnesium ions inhibit the L-type calcium current in heart cells, magnesium–adenosine triphosphate complexes (MgATP) would be expected to increase the current by promoting channel phosphorylation. Rapid increases in the intracellular concentration of MgATP induced by flash photolysis of caged magnesium or caged ATP resulted in enhanced calcium current. The increase in calcium current was not prevented by blocking phosphorylation, revealing a previously unrecognized direct regulatory action of the magnesium-nucleotide complex.**


Cellular processes as diverse as transcription, energy metabolism, and excitability are regulated by changes in the intracellular concentrations of either free magnesium ions ($Mg^{2+}$) or Mg-nucleotide complexes (*1*), with the two forms often exerting opposite effects on ion channels (*2*, *3*). Increases in the concentration of intracellular $Mg^{2+}$ in the millimolar range inhibit L-type calcium current ($I_{Ca}$) (*4*).


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In contrast, addition of adenosine triphosphate (ATP) to the intracellular solution reduces the rate of irreversible decline (or "run-down") of $I_{Ca}$ in dialyzed cells (*5*) and increases $I_{Ca}$ when endogenous ATP production is inhibited (*6*). To clarify the regulatory roles of $Mg^{2+}$ and MgATP, we used flash photolysis of caged $Mg^{2+}$ (*7*) or caged ATP (*8*) to rapidly raise cytosolic $Mg^{2+}$, MgATP, or both from submicromolar concentrations to concentrations of several hundred micromolar. We found that L-type $Ca^{2+}$ currents are increased by Mg-nucleotide complexes through a phosphorylation-independent mechanism, possibly by allosteric interaction.

Dimethoxy (DM)-nitrophen is a photolabile chelator that binds either $Ca^{2+}$ or $Mg^{2+}$ (*7*). We used $Ca^{2+}$-loaded DM-nitrophen to determine the efficiency of photolysis in our system by monitoring the concentration of free intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) (*9*). In a whole cell–clamped myocyte loaded with caged $Ca^{2+}$ (in the absence of $Mg^{2+}$), depolarizing pulses elicited small $[Ca^{2+}]_i$ transients (Fig. 1A). A flash of ultraviolet light abruptly raised the baseline $Ca^{2+}$ signal. Two subsequent flashes evoked little further increase in basal $[Ca^{2+}]_i$, suggesting that photolysis was 80 to 90% complete after one flash. To use DM-nitrophen as caged $Mg^{2+}$, the cytoplasm was equilibrated with pipette solutions which included ATP, magnesium, DM-nitrophen, and 1,2-is(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) to maintain $[Ca^{2+}]_i$ at subnanomolar levels. Under these conditions, no change in $[Ca^{2+}]_i$ was detected in response to depolarizing pulses or to flashes (Fig. 1B).

To verify that $Mg^{2+}$ was in fact released by photolysis of caged $Mg^{2+}$, we used a bioassay that exploited the capacity of cytoplasmic $Mg^{2+}$ to block outward currents through inwardly rectifying $K^+$ channels ($I_{K1}$) (*10*, *11*). Depolarization to +20 mV elicited a large outward current before photolysis, but the subsequent release of $Mg^{2+}$ induced inward rectification (Fig. 1C). The results verify that $[Mg^{2+}]_i$ reached concentrations sufficient to block outward current through the $K^+$ channels (1.7 to 30 μM) (*10*), consistent with our estimate that photolysis increased $[Mg^{2+}]_i$ from 0.06 to 58 μM in this experiment (*11*).

Having confirmed that $Mg^{2+}$ can be released from caged $Mg^{2+}$ without concomitant changes in $[Ca^{2+}]_i$, we examined the effect of $Mg^{2+}$ release on $I_{Ca}$. Currents were stable until exposure to a flash (Fig. 2A), after which the amplitude of $I_{Ca}$ transiently decreased and then increased to a steady value approximately twice that during the control period. A second flash induced a similar biphasic effect resulting in further augmentation of $I_{Ca}$. Subsequent flashes had no effect. The large $Mg^{2+}$-induced increase in $I_{Ca}$ was not associated with detectable alterations in the kinetics of the current, as confirmed by examination of representative records before and after the flashes (Fig. 2A). In six cells studied under identical conditions, $I_{Ca}$ increased from 1328 ± 266 (SEM) to 2073 ± 290 pA ($P < 0.05$). Current through L-type $Ca^{2+}$ channels was also enhanced by release of $Mg^{2+}$ when $Ba^{2+}$ replaced $Ca^{2+}$ as the charge carrier (Fig. 2B). This result provides additional evidence that the response does not involve changes in $[Ca^{2+}]_i$ (*12*), nor does it require $Ca^{2+}$ occupancy of the pore, in contrast to the phenomenon of $Ca^{2+}$-de-