(3.4 mg per kilogram of body weight, i.v.) markedly attenuated the response produced by SP, but did not reduce the salivary response produced by acetylcholine (ACh) (Fig. 4). Additionally, the inactive (2*R*,3*R*)-enantiomer did not inhibit the salivary response induced by SP.

The results of in vitro and in vivo studies have demonstrated that CP-96,345 is a potent, competitive, and highly selective antagonist of the SP (NK_1) receptor. Ligand binding studies demonstrated the NK_1-receptor specificity and the enantiomeric selectivity. All of the biological activity was confined to the (2*S*,3*S*)-enantiomer. Functional studies in vitro confirm this receptor specificity and show that CP-96,345 is a reversible and competitive antagonist. Finally, in vivo studies in the rat demonstrate selective antagonism and a lack of agonist activity.

REFERENCES AND NOTES

1. U. S. von Euler and J. H. Gaddum, *J. Physiol. (London)* **72**, 74 (1931); M. M. Chang and S. E. Leeman, *J. Biol. Chem.* **245**, 4784 (1970).
2. S. H. Buck and E. Burcher, *Trends Pharmacol. Sci.* 7, 65 (1986).
3. R. A. Nicoll, C. Schenker, S. E. Leeman, *Annu. Rev. Neurosci.* **3**, 227 (1980).
4. B. Pernow, *Pharmacol. Rev.* **35**, 85 (1983).
5. J. E. Maggio, *Annu. Rev. Neurosci.* **11**, 13 (1988).
6. P. Melchiorri, F. Tonelli, L. Negri, in *Substance P (Nobel Symposium 37)*, U. S. von Euler and B. Pernow, Eds. (Raven, New York, 1977), pp. 311–319; B. Pernow and S. Rosell, *Acta Physiol. Scand.* **93**, 139 (1975).
7. B. Pernow, *Acta Physiol. Scand.* **29** (Suppl. 105), 1 (1953).
8. K. Vogler *et al.*, *Ann. N.Y. Acad. Sci.* **104**, 378 (1963); S. E. Leeman and R. Hammerschlag, *Endocrinology* **81**, 803 (1967).
9. G. Nilsson, T. Hökfelt, B. Pernow, *Med. Biol.* **52**, 424 (1974); T. Hökfelt, J. O. Kellerth, G. Nilsson, B. Pernow, *Science* **190**, 889 (1975); P. W. Mantyh, R. D. Pinnock, C. P. Downes, M. Goedert, S. P. Hunt, *Nature* **309**, 795 (1984); M. D. Womack, A. B. MacDermott, T. M. Jessell, *ibid.* **334**, 351 (1988).
10. D. G. Payan, D. R. Brewster, A. Missirian-Bastian, E. J. Goetzl, *J. Clin. Invest.* **74**, 1532 (1984); H.-P. Hartung, K. Wolters, K. V. Toyka, *J. Immunol.* **136**, 3856 (1986); M. Lotz, D. A. Carson, J. H. Vaughan, *Science* **235**, 893 (1987); A. Perianin, R. Snyderman, B. Malfroy, *Biochem. Biophys. Res. Commun.* **161**, 520 (1989).
11. D. Regoli, G. Drapeau, S. Dion, R. Couture, *Trends Pharmacol. Sci.* **9**, 290 (1988).
12. G. Enberg, T. H. Svensson, S. Rosell, K. Folkers, *Nature* **293**, 222 (1981).
13. CP-96,345 was prepared by reaction of 2-methoxybenzylamine with 2-benzhydryl-3-quinuclidinone [E. J. Warawa, N. J. Mueller, R. Jules, *J. Med. Chem.* **17**, 479 (1974)], stereoselective reduction of the resulting imine, and resolution of the racemic mixture (J. A. Lowe III *et al.*, in preparation).
14. Y.-C. Cheng and W. H. Prusoff, *Biochem. Pharmacol.* **22**, 3099 (1973).
15. R. M. Snider and K. P. Longo, in preparation.
16. O. Arunlakshana and H. O. Schild, *Br. J. Pharmacol.* **14**, 48 (1959).
17. J. W. Constantine, W. S. Lebel, H. A. Woody, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, in press.
18. S. Giuliani *et al.*, *Eur. J. Pharmacol.* **150**, 377 (1988).
19. M. H. Perrone, R. E. Diehl, D. R. Haubrich, *ibid.* **95**, 131 (1983).
20. Y. Takeda and J. E. Krause, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 392 (1989).

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Activity and Distribution of Binding Sites in Brain of a Nonpeptide Substance P (NK_1) Receptor Antagonist

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CP-96,345, a nonpeptide substance P antagonist, is selective for the tachykinin NK_1 receptor. The compound binds to a single population of sites in guinea pig brain and potently inhibits substance P–induced excitation of locus ceruleus neurons. CP-96,345 should be a useful tool for studying the action of substance P in the central nervous system.

SUBSTANCE P (SP), AN UNDECAPEPTIDE, is widely distributed in the central and peripheral nervous systems and may function as a neurotransmitter-neuromodulator in a variety of physiological processes (*1*). SP is released from neurons in the midbrain in response to stress (*2*), where it facilitates dopaminergic neurotransmission (*3, 4*), and from sensory neurons in the spinal cord in response to noxious stimuli, where it excites dorsal horn neurons (*5*). In the periphery, release of SP from sensory neurons causes vasodilation and plasma extravasation, suggesting a role in neurogenic inflammation (*6*). Because neurokinin A and neurokinin B elicit effects similar to the effects of SP when they are given in vivo (*3, 7, 8*), it has been difficult to determine whether the various tachykinins are acting through the same or different receptor systems. The discovery of CP-96,345 [(2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo-[2.2.2]octan-3-amine], a selective nonpeptide antagonist at the NK_1 receptor (*9*), will help to elucidate the role of the respective tachykinin receptor subtypes (NK_1, NK_2, NK_3). We present evidence that CP-96,345 is a selective antagonist of NK_1 receptors in mammalian brain.

To obtain a detailed characterization of the receptor-binding properties of CP-96,345 in brain, we radiolabeled the compound (*10*) and carried out binding studies with guinea pig striatal membranes. Incubations were carried out at 22°C for 10 min. The binding was stable over a wide range of pH (6.0 to 8.0) and was fully reversible with the addition of 1 μM unlabeled CP-96,345. Incubation of guinea pig striatal membranes with increasing concentrations of [^{3}H]CP-96,345 (0.01 to 6 nM) revealed high-affinity binding to a single class of receptors [dissociation constant (K_d) ± SEM = 0.22 ± 0.04 nM; maximum binding capacity (B_{max}) ± SEM = 166 ± 5.5 fmol per milligram of protein] with specific binding accounting for 70 to 75% of the total bound radioactivity (Fig. 1). In the same membrane preparation, [^{3}H]SP bound with K_d ± SEM = 1.89 ± 0.31 nM and B_{max} ± SEM = 152 ± 11 fmol per milligram of protein. In competition studies, SP, physalaemin, and the NK_1 receptor–selective analog [methyl-Gly^9, Met(O$_2$)^{11}] SP (*7*) competed with the binding of [^{3}H]CP-96,345 yielding inhibition constant (K_i) values less than 100 nM (Table 1). The lower affinity of the NK_1 tachykinin agonists obtained from the competition experiments with [^{3}H]CP-96,345 is consistent with results obtained from other receptor systems, demonstrating that estimates of agonist affinity are lower when competing against an antagonist rather than an agonist radioligand (*11*). At micromolar concentrations, neurokinin A, neurokinin B, senktide,

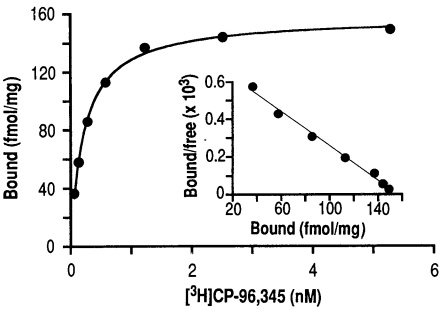

Fig. 1. A saturation isotherm from a single experiment of the binding of [^{3}H]CP-96,345 to a membrane preparation (P_2 fraction) from guinea pig striata. (**Inset**) Scatchard analysis of the binding in Fig. 1. The K_d of 0.22 ± 0.04 nM and a B_{max} of 166 ± 5.5 fmol per milligram of protein represent the mean ± SEM of three experiments run in triplicate. The striata were dissected from male Hartley guinea pigs, and a lysed P_2 fraction was prepared (*16*) and stored at −20°C. Binding studies were initiated by the addition of 25 μl of tissue (75 μg of protein) and 25 μl of buffer or unlabeled CP-96,345 (1 μM final concentration) as the blank to 200 μl of buffer containing ligand (1.0 nM). After a 10-min incubation period, the assay was terminated by rapid filtration over filter paper soaked in 0.01% polyethylenimine and then washed with 5 ml of ice-cold buffer. Radioactivity was measured with liquid scintillation spectrophotometry.

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Table 1. The effect of CP-96,345, tachykinins, and tachykinin analogs on the binding of [^{3}H]CP-96,345 (1.0 nM) to guinea pig striatal membranes. Competition studies were carried out with five to seven concentrations of unlabeled drug. Values are from one to five experiments performed in triplicate. Haloperidol, carbachol, propanolol, desipramine, prazosin, naloxone, clonazepam, and mepyramine at 10 μM caused insignificant displacement of [^{3}H]CP-96,345. (Me, methyl.)

Competitor	K_i ± SE
CP-96,345	0.20 ± 0.02 nM
[D-Pro^2-D-Trp^{7,9}]SP	1,300 nM
[D-Arg^1-D-Pro^2-D-Trp^{7,9}]SP	1,600 nM
Spantide	751 nM
SP	75 ± 20 nM
Physalaemin	83 ± 13 nM
[MeGly^9-Met(O$_2$)^{11}]SP	94 ± 30 nM
Neurokinin A	>10,000 nM
Neurokinin B	>1,000 nM
Senktide	>10,000 nM
Eledoisin	>10,000 nM

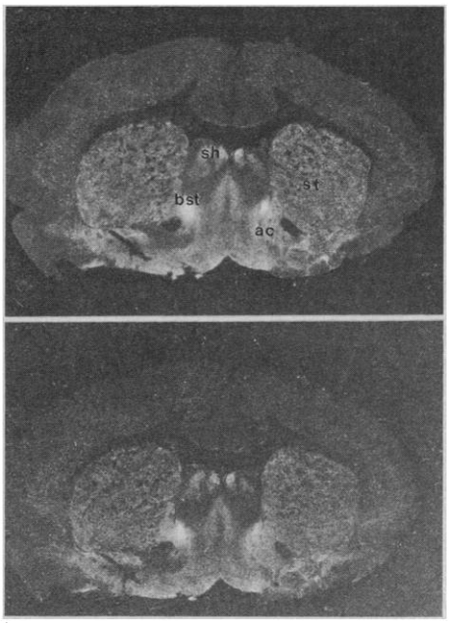

Fig. 2. Comparison of [^{3}H]CP-96,345 (top) and [^{3}H]SP (bottom) binding in the guinea pig at the level of the striatum. Guinea pig brains were frozen in isopentane (−20°C), sectioned at 20-μM thickness, thaw-mounted onto gelatin-coated slides, and stored at −65°C. Sections were incubated for 15 min at room temperature in 50 mM tris-HCl, 5 mM $MnCl_2$, and 0.02% bovine serum albumin, and adjusted to pH 7.3, then incubated for 30 min at 4°C in buffer containing 2.0 nM [^{3}H]CP-96,345 or the same buffer but with the addition of chymostatin (2 μg/ml), bacitracin (40 μg/ml), leupeptin (4 μg/ml), and 2.0 nM [^{3}H]SP. Nonspecific binding was defined by the addition of 5 μM SP. The sections were washed twice in ice-cold buffer for 5 min each time, dipped briefly in distilled water, and dried under a stream of air. All sections were placed in standard x-ray cassettes, apposed to tritium-sensitive Ultrofilm (LKB) for 1 month, and developed by standard photographic procedures. (Abbreviations are ac, nucleus accumbens; bst, bed nucleus stria terminalis; sh, septohippocampal nucleus; and st, striatum.)

and eledoisin displaced less than 50% of [^{3}H]CP-96,345 binding (Table 1). In addition, the binding of [^{3}H]CP-96,345 was stereoselective; the (2*R*,3*R*)-enantiomer was at least three orders of magnitude less potent at displacing [^{3}H]CP-96,345 binding. Thus, in guinea pig striatum, [^{3}H]CP-96,345 exhibits stereoselective, high-affinity binding to a site that is pharmacologically similar to the NK_1 receptor and labels a population of receptors with a B_{max} similar to that obtained with [^{3}H]SP.

The autoradiographic localization of [^{3}H]CP-96,345 binding in sections of guinea pig brain was consistent with its selective binding to the NK_1 receptor. A heterogeneous pattern of specific binding was obtained that closely matched the distribution of [^{3}H]SP binding observed in adjacent sections. A moderate density of [^{3}H]CP-96,345 and [^{3}H]SP binding sites was present in the striatum and nucleus accumbens with dense labeling located in the bed nucleus of the stria terminalis and septohippocampal nucleus (Fig. 2). Dense binding was also obtained in the amygdalohippocampal area, central and cortical nuclei of the amygdala, habenula, periventricular nucleus, olfactory bulb, and in the ependymal cells lining the cerebral ventricles. Moderate levels were seen in the olfactory tubercle, hypothalamus, superficial layer of the superior colliculus, medial geniculate, subiculum, locus ceruleus, several sensory trigeminal nuclei, and in the central gray. Little displaceable binding could be demonstrated in cortex, hippocampus, cerebellum, and most thalamic nuclei. The overlapping distribution of [^{3}H]CP-96,345 and [^{3}H]SP labeling in guinea pig brain is in accord with the autoradiographic distribution of NK_1 receptors described in (*12*).

Initial functional characterization of the actions of CP-96,345 in the central nervous system used extracellular recordings from locus ceruleus neurons in slices of guinea pig brainstem (*13*) and focused on the ability of CP-96,345 to antagonize SP-induced responses in vitro. SP is a potent excitant of locus ceruleus neurons both in vitro and in vivo (*14*). When we applied SP (10 to 1000 nM) in the perfusate, it induced rapid and reversible increases in the firing rate of spontaneously active locus ceruleus neurons (Fig. 3A). SP (1,000 to 10,000 nM) produced a fivefold maximal increase in firing rate (from 0.37 ± 0.03 Hz to 1.9 ± 0.14 Hz, n = 18). A 5- to 10-min wash with control medium returned the firing rate to baseline. Repeated applications of SP did not result in signifi-

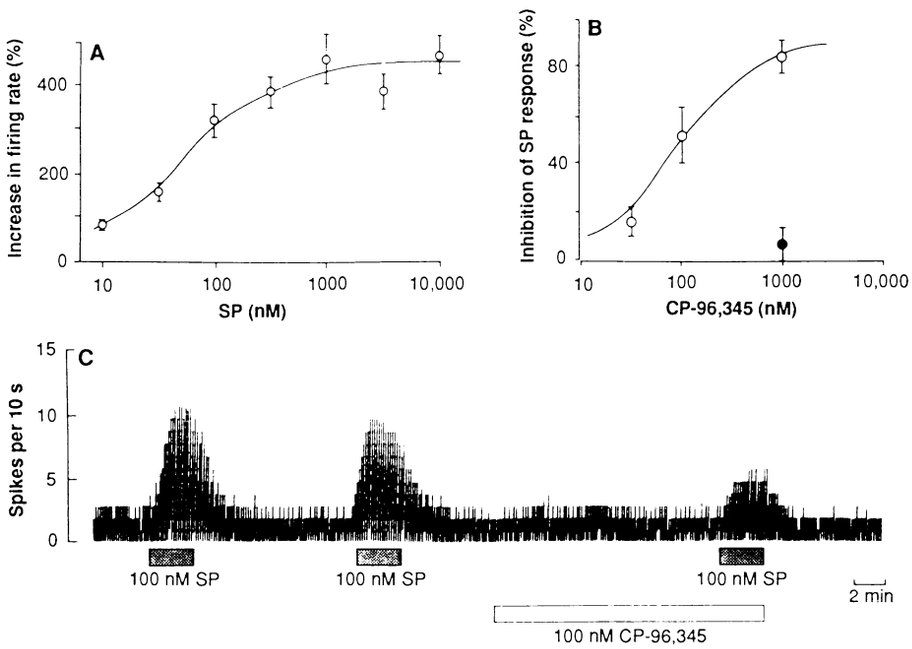

Fig. 3. The effect of SP and CP-96,345 on the firing rate of locus ceruleus neurons in vitro. (**A**) Dose-response relation of bath-applied SP on increases in firing of spontaneously active locus ceruleus neurons. The mean firing rate of the neurons included in these studies was 0.31 ± 0.02 Hz (46 cells total; mean ± SEM from 3 to 20 cells for each point). (**B**) CP-96,345 (○) inhibited increases in firing rates of locus ceruleus neurons induced by 100 nM SP with an IC_{50} of approximately 90 nM (mean ± SEM from 3 to 7 cells for each point). Mean inhibition of SP response by the (2*R*,3*R*)-enantiomer of CP-96,345 (●). (**C**). The excitatory effects of SP on a locus ceruleus neuron and the antagonist action of CP-96,345 on the SP-induced response. The bars indicate periods in which the medium flowing through the recording chamber was switched to drug-containing medium. CP-96,345 (100 nM) reduced the SP-induced excitation in this neuron by 59%. In this and other experiments, the antagonist effects of CP-96,345 on SP-induced responses were slow to wash out.

cant tachyphylaxis of the response. Antagonist experiments were performed on responses induced with 100 nM SP, a concentration that produced a submaximal response.

CP-96,345 (32, 100, or 1000 nM) antagonized SP-induced excitation of locus ceruleus neurons (Fig. 3, B and C), reducing the firing rate increases produced by 100 nM SP in 14 of 14 neurons recorded from 13 slices. The median inhibition concentration (IC_{50}) for the antagonism of SP responses by CP-96,345 was 90 nM. CP-96,345 (32 to 1000 nM) alone did not significantly change baseline firing rates of locus ceruleus neurons (control, 0.37 ± 0.08 Hz; CP-96,345, 0.36 ± 0.07 Hz, n = 14). The (2*R*,3*R*)-enantiomer of CP-96,345 did not have significant activity at 1000 nM, reducing the excitatory response to 100 nM SP by less than 10% in the two neurons examined. An NK_1-specific action of CP-96,345 was indicated by the compound's inability to antagonize the excitations produced by the NK_3-specific agonist senktide (*15*). Excitations induced by 1 nM senktide, which elicited an excitatory effect equivalent to 100 nM SP, were inhibited 5% by 1000 nM CP-96,345 in three of three neurons. Thus, the ability of CP-96,345 to antagonize SP-elicited responses but not the excitant effects of senktide indicates that locus ceruleus neurons have multiple tachykinin receptors and that the excitatory effects of SP are mediated primarily by NK_1 receptors.

CP-96,345 is a nonpeptide compound that acts as a potent and selective NK_1 antagonist in brain. Its pharmacological characteristics make it an important tool for elucidating the physiological significance of NK_1 receptors. In addition, CP-96,345 may provide the means for determining the usefulness of an NK_1 receptor antagonist as therapy for disorders of the nervous system.

REFERENCES AND NOTES

1. C. J. Helke, J. E. Krause, P. W. Mantyh, R. Couture, M. J. Bannon, *FASEB J.* **4**, 1606 (1990); B. Pernow, *Pharmacol. Rev.* **35**, 85 (1983).
2. M. J. Bannon *et al.*, *Brain Res.* **381**, 393 (1986); A. Y. Deutch *et al.*, *Peptides* **6**, 113 (1985).
3. R. B. Innis, R. Andrade, G. K. Aghajanian, *Brain Res.* **335**, 381 (1985).
4. J. Davies and A. Dray, *ibid.* **107**, 623 (1976); P. J. Elliott, C. B. Nemeroff, C. D. Kilts, *ibid.* **385**, 379 (1986).
5. M. Otsuka and M. Konishi, *Nature* **264**, 83 (1976); T. L. Yaksh, T. M. Jessell, R. Gamse, A. W. Mudge, S. E. Leeman, *ibid.* **286**, 155 (1980); J. L. Henry, *Brain Res.* **114**, 439 (1976); M. Randic and V. Miletic, *ibid.* **128**, 164 (1977).
6. J. M. Lunberg, E. Brodin, X. Hua, A. Saria, *Acta Physiol. Scand.* **120**, 217 (1984); D. G. Payan, *Annu. Rev. Med.* **40**, 341 (1989); F. Lembeck and P. Holzer, *Naunyn-Schmeideberg's Arch. Pharmacol.* **310**, 175 (1979).
7. D. Regoli, G. Drapeau, S. Dion, P. D'Orleans-Juste, *Life Sci.* **40**, 109 (1987).
8. Y. Takano, Y. Takeda, K. Yamada, H.-O. Kamiya, *ibid.* **37**, 2507 (1985).
9. R. M. Snider *et al.*, *Science* **251**, 435 (1991).
10. A brominated precursor (2*S*, 3*S*)-*cis*-2-[bis(4-bromophenyl)methyl]-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine, was provided to C. N. Filer and co-workers at New England Nuclear. Tritium-bromine exchange yielded [$^3H_2$]CP-96,345 of 99.4% chemical purity and 97.2% radiochemical purity with a specific activity of 48.1 Ci/mmol (J. A. Lowe III *et al.*, in preparation).
11. M. Titeler, R. A. Lyon, K. H. Davis, R. A. Glennon, *Biochem. Pharmacol.* **36**, 3265 (1987).
12. T.-V. Dam and R. Quirion, *Peptides* **7**, 855 (1986).
13. Brainstem slices (thickness 350 μM) containing locus ceruleus were obtained from 250- to 350-g male Hartley guinea pigs [T. M. Egan *et al.*, *J. Physiol. (London)* **345**, 477 (1983)]. Guinea pigs were anesthetized with halothane and decapitated, and the brains were quickly removed into ice-cold oxygenated medium (124 mM NaCl, 5 mM KCl, 1.25 mM $NaH_2PO_4{\cdot}H_2O$, 26 mM $NaHCO_3$, 10 mM D-glucose, 2 mM $MgSO_4{\cdot}7H_2O$, and 2 mM $CaCl_2$). One slice at a time was placed on a nylon net in the recording chamber in which the slice was completely submerged in continuously flowing medium (~1 ml/min) at 33°C. We applied drugs by switching the perfusing medium to the appropriate solution. Extracellular action potentials from spontaneously active neurons were recorded with 0.9% saline-filled glass pipettes (10 to 20 MΩ). Locus ceruleus noradrenergic neurons were identified by virtue of their position in the slice, their slow, steady firing rates, and their biphasic action potentials. When possible, we added 25 nM clonidine to the perfusion medium at the end of an antagonist experiment to confirm the noradrenergic nature of the neuron.
14. P. G. Guyenet and G. K. Aghajanian, *Brain Res.* **136**, 178 (1977); H. J. Cheeseman, R. D. Pinnock, G. Henderson, *Eur. J. Pharmacol.* **94**, 93 (1983).
15. U. Wormser *et al.*, *EMBO J.* **5**, 2805 (1986).
16. E. G. Gray and V. P. Whitaker, *J. Anat.* **96**, 79 (1962).

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A Kinetic Partitioning Model of Selective Binding of Nonnative Proteins by the Bacterial Chaperone SecB

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An in vitro assay for the interaction of SecB, a molecular chaperone from *Escherichia coli*, with polypeptide ligands was established based on the ability of SecB to block the refolding of denatured maltose-binding protein. Competition experiments show that SecB binds selectively to nonnative proteins with high affinity and without specificity for a particular sequence of amino acids. It is proposed that selectivity in binding is due to a kinetic partitioning of polypeptides between folding and association with SecB.

MOLECULAR CHAPERONES ARE proteins whose general function is to ensure the correct interactions within and between other polypeptides (*1*). Chaperones are involved in the assembly of oligomeric proteins (*2, 3*), in ensuring that certain proteins fold correctly (*4*), in facilitating protein localization (*3, 5*), and in the prevention of formation of proteinaceous aggregates during physiological stress (*6*). Since a single chaperone commonly interacts with a number of different polypeptides, how a chaperone specifically recognizes and binds to a target is an important and intriguing question.

SecB, an oligomeric molecular chaperone in *E. coli*, is involved in export of a subset of unrelated proteins (*7–9*). SecB not only enhances the efficiency of delivery of the precursors to the membrane (*10*), most likely through specific interaction with SecA (*11*), but also is responsible for maintaining the proteins in a loosely folded state so that they remain competent for translocation from the cytoplasm across the cytoplasmic membrane to their final destination in either the periplasmic space or in the outer membrane (*9–11*). The interaction of cytoplasmic SecB with precursor maltose-binding protein (MBP), one of the proteins that depends on SecB for efficient export, occurs early in the export pathway in vivo (*7*) but does not involve recognition of the amino-terminal leader sequence [(*12–14*); see (*15*) for an opposing view]. SecB binds the precursor polypeptide at sites that are distinct from the leader sequence (*16*). The studies described here indicate that precursor MBP contains no unique sequence of amino-acyl residues that serves as a hallmark for binding of SecB. SecB bound tightly to all nonnative polypeptides tested even though it did not interact with any native protein. It seems that SecB has little selectivity for the sequence of amino-acyl residues.

If SecB recognizes all nonnative structures, how are proteins that are to be exported distinguished from those that are to remain in the cytoplasm? We propose that selectivity results from a kinetic partitioning. The pathway a polypeptide takes in vivo depends on the rate of its folding relative to the rate of its association with SecB. Proteins that rapidly fold are precluded from binding, whereas the presence of a leader,

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