Restoration of Viral Immunity in Immunodeficient Humans by the Adoptive Transfer of T Cell Clones

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The adoptive transfer of antigen-specific T cells to establish immunity is an effective therapy for viral infections and tumors in animal models. The application of this approach to human disease would require the isolation and in vitro expansion of human antigen-specific T cells and evidence that such T cells persist and function in vivo after transfer. Cytomegalovirus-specific CD8$^+$ cytotoxic T cell (CTL) clones could be isolated from bone marrow donors, propagated in vitro, and adoptively transferred to immunodeficient bone marrow transplant recipients. No toxicity developed and the clones provided persistent reconstitution of CD8$^+$ cytomegalovirus-specific CTL responses.

Cytomegalovirus (CMV) infection is not associated with a significant clinical illness in immunocompetent individuals but represents a major cause of morbidity and mortality in immunodeficient hosts, such as patients undergoing bone marrow or solid organ transplantation and those with the acquired immunodeficiency syndrome (AIDS) (*1*). After primary CMV infection, normal individuals develop CD8$^+$ CTLs that recognize CMV Ag presented in association with class I major histocompatibility complex (MHC) molecules (*2*) and maintain an immunodominant CD8$^+$ CTL response specific for structural virion proteins that are presented rapidly after viral entry (*3*). These CTLs are capable of lysing CMV-infected cells without the requirement for viral gene expression, thus potentially providing prompt control of viral reactivation in hosts with latent infection (*3*). In allogeneic bone marrow transplant (BMT) recipients, recovery of CD8$^+$ CMV-specific CTL responses confers protection from the development of CMV disease, and CMV pneumonia is observed only in patients who have not reconstituted detectable CMV-specific CTLs (*4*). At day 40 after transplant, 65% of BMT recipients are deficient in CD8$^+$ CMV-specific CTL responses, and these patients are at high risk for developing fatal CMV pneumonia (*4*). The essential role for CTLs in protective immunity to CMV is supported by studies in a murine CMV model, in which the adoptive transfer of CD8$^+$ CTLs to immunosuppressed mice conferred protection from otherwise lethal viral challenge (*5*). The development of methods to isolate and propagate human CMV-specific CTL clones in vitro has made it possible to evaluate the potential for reconstituting protective immunity in immunocompromised BMT patients at risk for life-threatening CMV disease by the adoptive transfer of CMV-specific T cell clones derived from the MHC identical bone marrow donor (*3*, *6*).

For this study, CD3$^+$, CD8$^+$, and CD4$^-$ CMV-specific CTL clones were generated from three CMV seropositive bone marrow donors and propagated in vitro for 5 to 12 weeks before adoptive transfer. All clones used in therapy were representative of the class I MHC–restricted immunodominant protective CTL response, as demonstrated by rapid lysis of autologous cells infected with CMV despite blockade of new viral gene expression by the RNA synthesis inhibitor actinomycin D (Table 1) (*3*). The CTL clones were administered to the BMT recipient by intravenous infusion each week for four consecutive weeks in escalating cell numbers beginning 28 to 35 days after the BMT. The cell doses of 3.3×10^7 cells/m^2, 1×10^8 cells/m^2, 3.3×10^8 cells/m^2, and 1×10^9 cells/m^2 of body surface area were each comprised of one to ten clones. During T cell transfer the individuals continued to receive, as prophylaxis for graft-versus-host disease (*7*), immunosuppressive therapy that consisted of either cyclosporine A alone (patients 6025 and 6131) or cyclosporine A and prednisone (patient 6032). Recipients were monitored during and after the T cell infusions for evidence of toxicity.

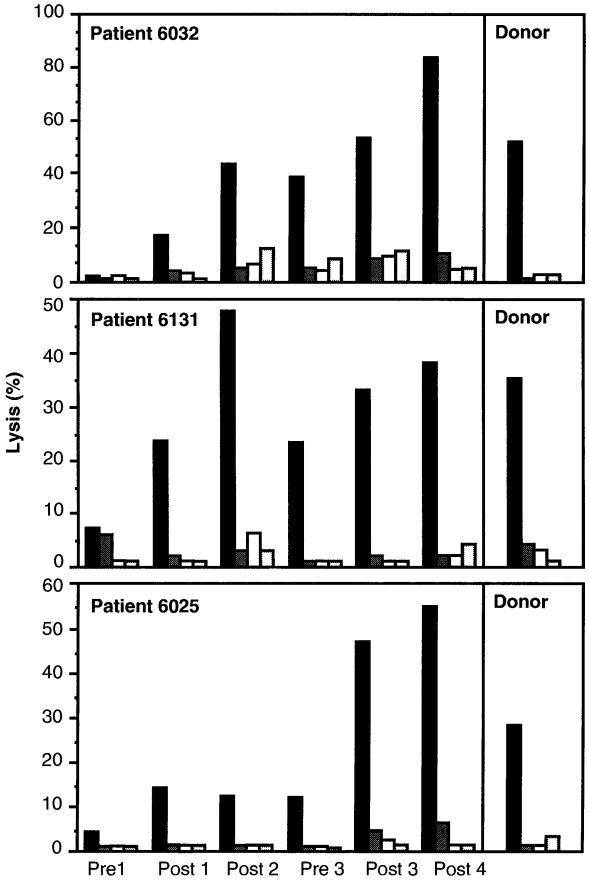

Fig. 1. Kinetics of reconstitution of CD8$^+$ class I MHC–restricted CMV-specific CTL responses in recipients of adoptive immunotherapy. CD8$^+$ CMV-specific CTL responses in BMT recipients and their respective bone marrow donors were evaluated in PBL by a CTL generation assay (*3*, *4*). Peripheral blood was obtained from the recipients immediately before initiating T cell infusions (pre 1), 2 days after each T cell infusion (post 1 to post 4), and just before the third infusion (pre 3). PBLs were separated by Ficoll-Hypaque density gradient centrifugation and stimulated at a responder-to-stimulator ratio of 20:1 with autologous fibroblasts infected 6 hours previously with CMV at an MOI of 5. Seven days later cultures were restimulated with CMV-infected fibroblasts and supplemented at restimulation with γ-irradiated PBL feeder cells. Two days after restimulation the media were supplemented with IL-2 (2 U/ml). Effector cells from these cultures were assayed in a 5-hour chromium release assay 7 days after restimulation for recognition of donor-derived MHC identical fibroblast target cells either infected with CMV for 24 hours (black bars) or mock-infected (dark gray bars) and allogeneic class I MHC–mismatched fibroblast target cells either infected with CMV (white bars) or mock-infected (light gray bars). Target cells were first treated with γ interferon (50 U/ml) for 2 days before infection with CMV to increase the sensitivity of the assay for the detection of CD8$^+$ CTLs (*15*). The data are shown at an effector-to-target ratio (E/T) of 20:1 for patient 6032 and the respective donor and at an E/T of 10:1 for patients 6131, 6025, and their respective donors.

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No changes in vital signs, oximetry, chest x-ray, or graft-versus-host disease status were observed with any of the 12 T cell infusions. This absence of toxicity made it possible to administer the cells on an outpatient basis.

The reconstitution of T cell immunity to CMV in treated hosts was evaluated by assaying peripheral blood lymphocytes (PBLs) obtained before initiating T cell therapy and 48 hours after infusion for CMV-specific $CD8^+$ CTL and $CD4^+$ helper T cell (T_H) responses. Consistent with the profound immunodeficiency observed early after allogeneic BMT, CMV-specific $CD8^+$ CTL and $CD4^+$ T_H responses were not detected in any of the three patients before adoptive therapy (Figs. 1 and 2). Forty-eight hours after the first T cell infusion, weak $CD8^+$ CMV-specific class I MHC–restricted CTL responses were detected in each of the three patients, and these CTL responses were augmented with subsequent infusions of CTL clones (Fig. 1). Persistent cytolytic responses were detected in PBLs obtained 1 week after the second infusion (just before the third infusion), suggesting that the increasing responses detected after each cell dose reflected both persistent and recently infused CTL clones. The magnitude of lytic activity detected in all recipients after the third cell dose was equivalent to or greater than that observed in the immunocompetent bone marrow donors (Fig. 1). Importantly, we did not detect recovery of CMV-specific $CD4^+$ T_H responses in recipients of $CD8^+$ CTL clones during the 4 weeks of therapy (Fig. 2). Previous analysis of the temporal kinetics of endogenous reconstitution of CMV-specific T cell responses after transplant in 56 allogeneic BMT recipients

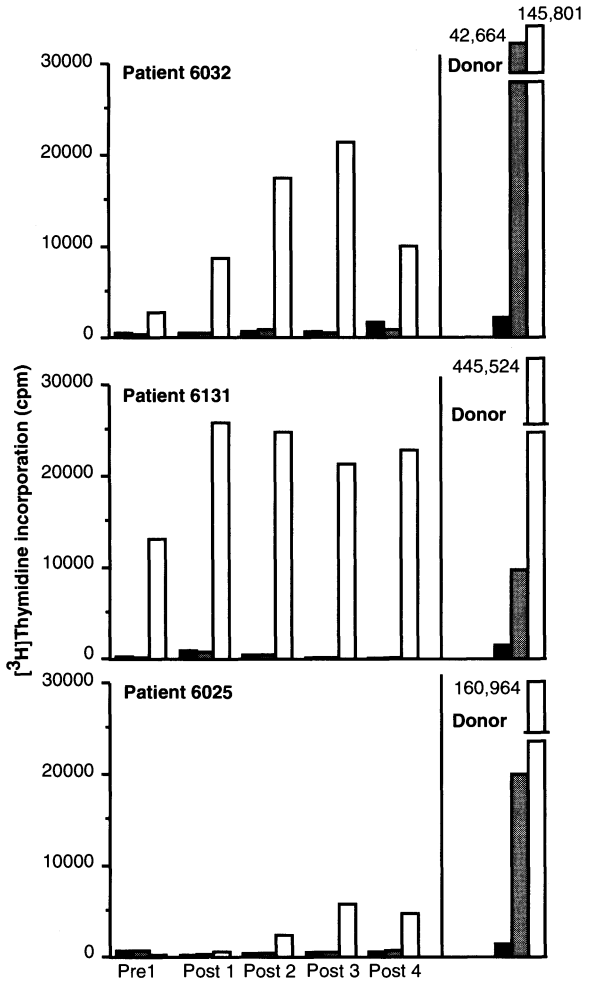

Fig. 2. Kinetics of reconstitution of $CD4^+$ CMV-specific T_H responses in recipients of adoptive immunotherapy. $CD4^+$ CMV-specific T_H responses of PBLs obtained from BMT recipients and their CMV-seropositive bone marrow donors were evaluated concurrently with $CD8^+$ CTL responses. Triplicate cultures of 2×10^5 PBLs per well were plated in 96-well round bottom plates with a control antigen prepared by glycine extraction of mock-infected fibroblasts (black bars), a CMV antigen preparation prepared by glycine extraction of CMV-infected fibroblasts (gray bars) (*4*), and with phytohemagglutinin (10 μg/ml) (white bars). Wells were pulsed with 2 μCi of [^{3}H]thymidine for the final 18 hours of a 96-hour incubation. The results are expressed as the mean counts per minute of triplicate wells.

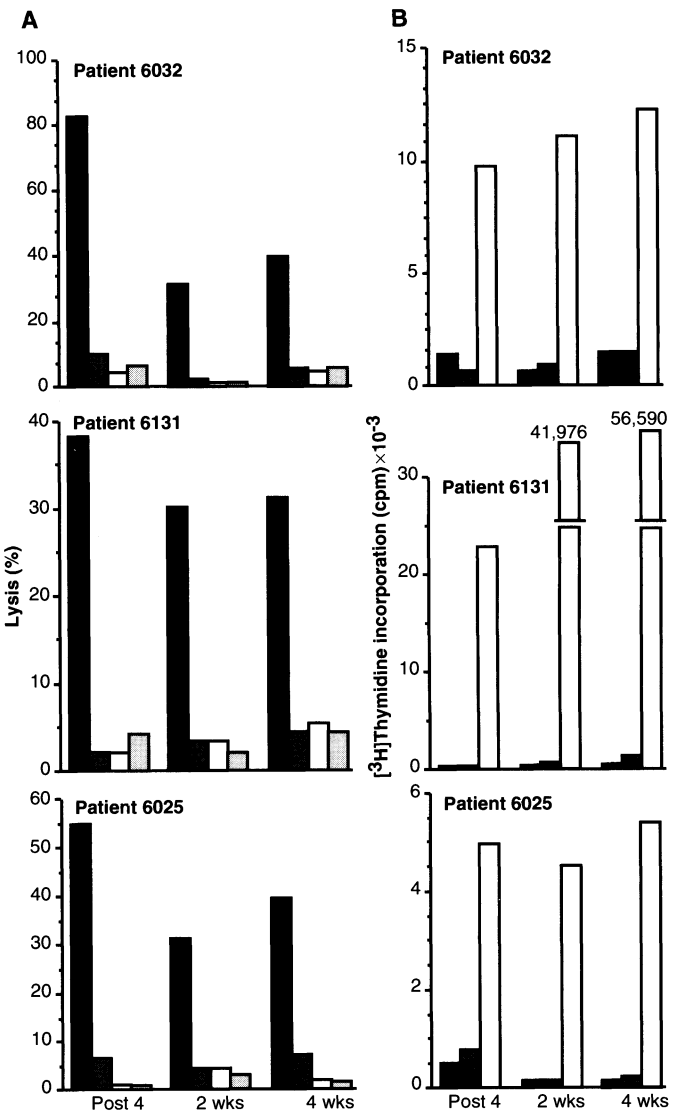

Fig. 3. Evaluation of recipients of adoptive immunotherapy following completion of T cell infusions for persistence of $CD8^+$ CMV-specific CTL responses and recovery of $CD4^+$ CMV-specific T_H responses (**A**) $CD8^+$ CMV-specific CTL responses of PBLs obtained from recipients 2 and 4 weeks after completion of the four T cell infusions were evaluated by using the CTL generation assay described in Fig. 1. Target cells include donor-derived fibroblasts either infected with CMV (black bars), or mock-infected (dark gray bars), and allogeneic MHC–mismatched fibroblasts either infected with CMV (white bars) or mock-infected (light gray bars). Data are shown at an E/T of 20:1 for patient 6032 and at an E/T of 10:1 for patients 6131 and 6025. (**B**) $CD4^+$ CMV-specific T_H responses of PBLs obtained from recipients 2 and 4 weeks after completion of the four T cell infusions were evaluated by using the assay described in Fig. 2. Responses were measured to control antigen (black bars), CMV antigen (gray bars), and PHA (white bars).

Table 1. $CD8^+$ CMV-specific CTL clones used in immunotherapy recognize CMV-infected cells without the requirement of viral gene expression. $CD8^+$ CMV-specific T cell clones were isolated from donors with minor modifications (*16*) of described methods (*3*). T cell clones were evaluated in a 5-hour chromium release assay for recognition of autologous and MHC-mismatched CMV-infected fibroblast target cells in which endogenous viral gene expression following viral entry was blocked with actinomycin D (*3*). Target cells were either infected with CMV at a multiplicity of infection (MOI) of 5 for 24 hours (CMV), infected with CMV at an MOI of 5 for 4 hours in the presence of actinomycin D (20 μg/ml) (CMV/ActD), or mock-infected (Mock) and labeled with 200 μCi of ^{51}Cr. The data are expressed as the percent of lysis of each target at effector-to-target ratios of 1.25:1/5:1 and show the cytolytic reactivity for 5 of 40 clones administered to patient 6032, 5 of 7 clones administered to 6131, and 5 of 5 clones administered to 6025.

Donor clone	Target cell (% lysis)					
	Autologous			MHC-mismatched		
	CMV	CMV/ ActD	Mock	CMV	CMV/ ActD	Mock
Patient 6032						
2F4	41/54	40/50	0/1	0/0	0/0	1/1
42C4	44/57	41/55	0/2	0/2	0/1	0/0
31E1	34/46	31/44	1/1	1/2	0/1	0/0
16D9	38/50	38/47	0/1	1/1	0/0	0/0
1D4	43/56	39/52	1/2	2/1	1/0	0/1
Patient 6131						
27E1	51/59	40/47	1/2	2/4	4/3	4/4
50A12	41/67	31/44	0/2	3/2	0/0	0/2
20H1	48/49	32/38	1/2	0/2	0/0	0/1
16H6	52/61	39/52	2/3	1/2	0/1	0/1
6E5	45/62	42/48	2/3	2/4	0/1	1/4
Patient 6025						
58G9	30/37	26/39	1/0	1/1	0/0	0/0
16D7	28/38	28/36	0/1	0/1	0/0	0/0
52D5	36/45	37/40	0/0	0/0	0/0	0/2
18H9	25/31	32/34	0/0	1/1	0/0	0/0
19C7	26/35	20/31	0/0	0/0	0/0	0/0

showed that recovery of CMV-specific $CD4^+$ T_H responses before or concurrently is obligatory for the generation of detectable $CD8^+$ CTL responses to CMV (*4*). Thus, the adoptive transfer of $CD8^+$ CMV-specific CTL clones is effective in providing reconstitution of CMV-specific CTL responses in immunodeficient hosts.

The ability of adoptively transferred T cells to persist in vivo is important for optimal efficacy in murine therapy models and is likely to be essential in human hosts who remain immunocompromised (*8*). The persistence of transferred CTL clones was evaluated in these three recipients by assaying PBLs for CMV-specific $CD8^+$ CTL and $CD4^+$ T_H responses after completion of the four infusions. After 2 weeks, all three patients exhibited a modest decline in CMV-specific CTL responses, consistent with either limited survival of some transferred T cells or migration from the vascular compartment to tissue sites (Fig. 3A). On repeat analysis 4 weeks after therapy, CTL responses remained detectable in the peripheral blood despite no recovery of CMV-specific $CD4^+$ T_H responses in two of the patients (6032 and 6025) and the development of a very weak $CD4^+$ T_H response in the other (6131) (Fig. 3, A and B). Thus, adoptively transferred $CD8^+$ CTL immunity persists for at least 1 month. Evaluation of CTL persistence for longer time periods has been complicated in these patients by the recovery of CMV-specific $CD4^+$ T_H responses, making it possible that endogenous $CD8^+$ CTLs are also being generated. Healthy CMV-seropositive individuals have a CTL response to CMV that is heterogeneous at the clonal level with respect to T cell receptor (TCR) β gene rearrangements (*9*), when examined by Southern (DNA) blot analysis (*10*). Preliminary studies on CMV-specific CTL clones that were isolated from patient 6131 within 1 week of T cell infusions indicated that the TCR β gene rearrangements present in such clones were identical to those of the CTL clones that had been previously infused into the patient.

The adoptive transfer of Ag-specific T cells is effective therapy for viruses and tumors in animal models (*11*). Our data show that large numbers of clonally derived T cells with defined antigen specificity can be generated and adoptively transferred without toxicity to selectively reconstitute immune responses in humans. None of these three BMT patients who had immunity reconstituted by adoptive transfer developed CMV viremia or pneumonia, consistent with previous evidence that the recovery of endogenous $CD8^+$ CTL responses confers protection from CMV disease (*4*). However, larger numbers of patients will need to be treated to permit definitive evaluation of the prophylactic antiviral effects of adoptive T cell transfer. The successful reconstitution of CMV-specific immunity in immunodeficient BMT patients by adoptive immunotherapy suggests that the administration of autologous expanded CMV-specific CTLs may diminish the incidence and severity of CMV disease in solid organ transplant recipients and patients with AIDS (*1*, *12*) and has implications for the investigation of antigen-specific T cell clones as therapeutic reagents for other human diseases. Human immunodeficiency virus (HIV)-specific $CD8^+$ CTLs can be detected during the asymptomatic stage of HIV infection (*13*); thus the isolation, expansion, and subsequent infusion of HIV-specific CTL clones at the later symptomatic stages of HIV infection when CTL responses are deficient might provide a therapeutic antiviral effect. Moreover, the potential for generating T cell clones in response to defined antigens expressed by human tumors for use in tumor therapy has been illustrated by the recent identification in human melanoma of genes encoding antigens recognized by MHC-restricted tumor-specific T cell clones (*14*).

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16. For isolation of $CD3^+$, $CD8^+$, $CD4^-$ CMV-specific T cell clones, autologous fibroblasts were infected with AD169 strain CMV for 6 hours and then cultured for 7 days with autologous PBLs in RPMI supplemented with 25 mM Hepes, 11% human CMV-seronegative AB serum, 4 mM L-glutamine, and 25 μM 2-mercaptoethanol. Cultures were restimulated with autologous CMV-infected fibroblasts and γ-irradiated (3300 rads) PBLs as feeder cells and supplemented 2 days later with 2 to 5 U/ml of interleukin-2 (IL-2). Seven days after restimulation, $CD8^+$ T cells were enriched and plated in 96-well round bottom wells at 0.3 to 0.8 cell/well with autologous γ-irradiated feeder cells, autologous CMV-infected fibroblasts, and 50 U/ml of IL-2. Wells positive for growth were evident in 10 to 14 days. Clones that had class I MHC–restricted CMV-specific cytolytic reactivity were

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-

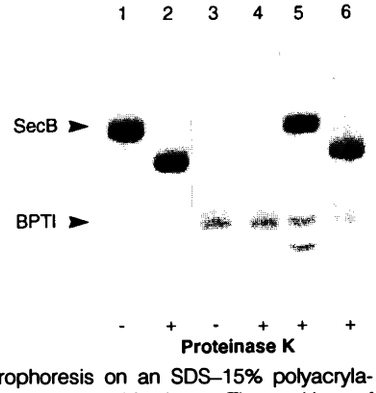

Fig. 1. Proteolysis as an assay of ligand binding. Incubation mixtures (0.4 ml, 10 mM Hepes, pH 7.6) contain purified proteins as follows: lanes 1 and 2, SecB (4 μg) purified as described (*14*); lanes 3 and 4, R·BPTI (1 μg); lane 5, SecB (4 μg) and R·BPTI (1 μg); and lane 6, SecB (4 μg) and native BPTI (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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