(SV5) epitope [T. Hanke, P. Szawlowski, R. E. Randall, *J. Gen. Virol.* **73**, 653 (1992)] in pCDNA3 (Invitrogen). In vitro–translated, radiolabeled proteins were mixed with 2 μg of CaN (Sigma), 2 μg of CypA (Sigma) and 10 μM CsA (Sandoz) where appropriate, absorbed with protein A–Sepharose (Sigma), and immunoprecipitated with 2 μg of anti-HA (Boehringer) or 5 μg of anti-SV5 (Serotec). Complexes were analyzed by autoradiography or protein immunoblot analysis with polyclonal anti-CaN (Chemicon) or monoclonal anti-CaN(B) (Sigma).

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11. Vero cells were infected with wild-type BA71V ASFV or SV5-A238L recombinant ASFV (*12*) for 4 to 10 hours. BSC1 cells were infected with modified vaccinia ankara (MVA) expressing T7 RNA polymerase [G. Sutter, M. Ohlmann, V. Erfle, *FEBS Lett.* **371**, 9 (1995)] and transfected with pT7–SV5-A238L or pT7–SV5-IκB. Proteins were radiolabeled, and the lysed cell extracts were immunoprecipitated and analyzed as before (*9*).
12. Regions flanking the A238L ORF were cloned into KS vector (Stratagene), and the β-Gal gene downstream from the ASFV vp72 promoter [J. M. Rodriguez, F. Almazan, E. Vinuela, J. F. Rodriguez, *Virology* **188**, 67 (1992)] was cloned between them. In addition, SV5-tagged A238L was cloned downstream from the A238L promoter. Recombinant ASF viruses ΔA238L and SV5-A238L were isolated by using X-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside). Recombinant baculovirus expressing A238L (A238L–Bac) was constructed with use of the BAC TO BAC baculovirus expression system (Life Technologies). Anti-A238L was raised in rabbits by using bacterially expressed A238L purified by SDS-polyacrylamide gel–electrophoresis.
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17. Messenger RNA from RS-2 cells was converted to cDNA by using anchored oligo dT_{25}. PCR-amplified fragments corresponding to nucleotides 1309 to 1669 of the NFATc ORF (GenBank U08015) were cloned into pT7-Blue2 (Novagen), and the nucleotide sequence was determined.
18. The A238L ORF was cloned in pCDNA3. The reporter plasmids used were NFAT-luc, mutant NFAT-luc (mNFAT-luc), and AP-1-luc. Transfected RS-2 cells were treated after 16 hours with (where appropriate) 40 nM PMA (Sigma), 4 μM ionomycin (Sigma), and 1 μM CsA (Sandoz), and luciferase activity was assayed 24 hours later.
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Delivery of Epitopes by the *Salmonella* Type III Secretion System for Vaccine Development

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Avirulent strains of *Salmonella typhimurium* are being considered as antigen delivery vectors. During its intracellular stage in the host, *S. typhimurium* resides within a membrane-bound compartment and is not an efficient inducer of class I–restricted immune responses. Viral epitopes were successfully delivered to the host-cell cytosol by using the type III protein secretion system of *S. typhimurium*. This resulted in class I–restricted immune responses that protected vaccinated animals against lethal infection. This approach may allow the efficient use of *S. typhimurium* as an antigen delivery system to control infections by pathogens that require this type of immune response for protection.

The success of global vaccination programs requires efficacious vaccines that are stable and easy to administer (*1*). Viable carrier systems offer the greatest potential for innovative approaches to develop polyvalent vaccines. Efficient protection against infectious agents often requires the action of both humoral and cellular immune mechanisms. Therefore, an ideal polyvalent antigen delivery system should be capable of stimulating all desired effector cell populations of the immune system. Live replicating bacteria and viruses that stimulate complex immune responses have been rendered avirulent and endowed with the ability to express foreign proteins derived from pathogenic microorganisms (*2*). Avirulent strains of *Salmonella typhimurium* are being widely considered as delivery systems for heterologous antigens because of their ability to induce complex mucosal and systemic immune responses after oral administration (*3*). A characteristic feature of these bacteria is their ability to invade nonphagocytic cells such as those of the intestinal epithelium (*4*). After internalization, *S. typhimurium* remains confined to a membrane-bound compartment insulated from the cytosolic environment of the host cell (*5*). Localization within the "internalization" vacuole prevents delivery of expressed foreign antigens to the major class I antigen presentation pathway, thereby hampering the use of *Salmonella* vaccine carriers when this type of response is crucial for protection (for example, viral infections) (*6*). An attempt to circumvent this problem has been the use of *Salmonella* to deliver plasmid DNA to express antigens within the host-cell cytosol (*7*).

Contact of *S. typhimurium* with host cells results in activation of a specialized protein secretion system (type III) that is encoded in a pathogenicity island at centisome 63 of its chromosome (*4*). This protein secretion system delivers a set of bacterial effector proteins into the host-cell cytosol, which leads to stimulation of signal transduction pathways that result in a variety of responses such as actin cytoskeleton reorganization and activation of transcription factors (*4*). In an effort to improve the ability of *Salmonella* to elicit class I–restricted immune responses to those epitopes, we investigated the potential of this system to deliver heterologous epitopes into the host-cell cytosol. To this end, we chose SptP, a *S. typhimurium* effector protein that is delivered into the host cell through the centisome 63 type III secretion system but is not required for efficient bacterial entry into nonphagocytic cells (*8*). We constructed a chimeric form of SptP that carries a class–I restricted epitope consisting of residues 366 to 374 from the influenza virus nucleoprotein ($IVNP_{366-374}$) found to be immunodominant in mice of the *H-2*^{b} haplotype (*9*). The epitope was introduced at a permissive site of SptP (*10*) located between the two predicted independent domains of this protein (Fig. 1) (*8*). The chimeric SptP-$IVNP_{366-374}$ protein was secreted into the culture supernatant of both wild-type *S. typhimurium* and the isogenic avirulent *aroA sptP* mutant strain SB824 at concentrations indistinguishable from those of wild-type SptP (Fig. 1). Both strains efficiently delivered SptP-$IVNP_{366-374}$ into the cytosol of infected cultured epithelial cells (Fig. 1). In contrast, and as expected, the isogenic *S. typhimurium sipD* mutant strain SB221 did

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not translocate the chimeric protein into the host-cell cytosol, although it could secrete SptP-IVNP$_{366-374}$ to the infection medium (Fig. 1). SipD is essential for type III protein translocation into host cells, although it is completely dispensable for protein secretion (*8, 11*). Similar results were obtained when a different epitope derived from the lymphocytic choriomeningitis virus nucleoprotein (LCMVNP$_{118-126}$) was introduced at the same site of SptP (Fig. 1). Thus, SptP can serve as a molecular courier to deliver foreign peptides of immunological interest to the cytoplasm of target cells.

We then examined the ability of wild-type, *aroA*, and *aroA sptP S. typhimurium* expressing the chimeric protein SptP-IVNP$_{366-374}$ to deliver the influenza nucleoprotein (NP) epitope to a class I–restricted antigen presenting pathway. Murine RMA thymoma cells (*H-2^b*) were infected with the different *S. typhimurium* strains and the ability of the infected cells to present the influenza NP epitope to the class I–restricted T cell hybridoma 12.164 was assessed by measuring its interleukin-2 (IL-2) secretory response (*12, 13*) (Fig. 2). The RMA thymoma cells infected with *S. typhimurium* strains expressing SptP-IVNP$_{366-374}$ were efficiently recognized by the epitope-specific T cell hybridoma. Antigen presentation was strictly dependent on the cytosolic delivery of the epitope by the *S. typhimurium* type III system, as RMA cells infected with a *sipD*$^-$ mutant strain, which can secrete SptP-IVNP$_{366-374}$ but cannot deliver it into the cell cytosol (Fig. 1), did not stimulate the T cell hybridoma (Fig. 2). *Salmonella typhimurium* strains expressing a deletion mutant of SptP-IVNP$_{366-374}$ (SptP$^{\Delta 35-62}$-IVNP$_{366-374}$) lacking the binding site for its specific chaperone, and therefore efficiently secreted but not translocated into the host cell (*14*), did not present antigen in infected RMA cells (Fig. 2). Similarly, the COOH-terminal half of the influenza virus NP fused to InvJ (InvJ-IVNP$_{335-498}$), a protein substrate of the type III system that is secreted but it is not translocated into host cells (*15, 16*), also did not present antigen in infected RMA cells (Fig. 2). *Salmonella typhimurium* is therefore capable of delivering foreign epitopes to the class I antigen presenting pathway with proteins translocated by the type III secretion system.

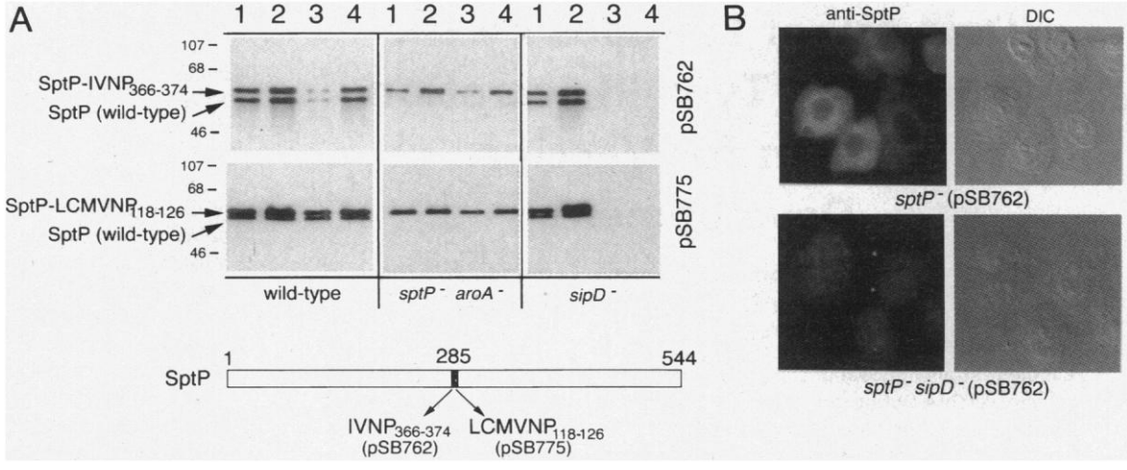

Fig. 1. Translocation of SptP chimeric proteins into cultured cells. (**A**) Henle-407 cells were infected with different *S. typhimurium* strains carrying the indicated plasmids and the presence of SptP fusion proteins in the different fractions examined as described in (*15*). Lane 1, whole cell lysate of non-cell–associated bacteria; lane 2, bacteria-free infection medium; lane 3, Triton X-100 insoluble fraction containing internalized bacteria; lane 4, Triton X-100 soluble Henle-407 cell lysate containing translocated proteins. SptP and fusion derivatives were detected by protein immunoblotting with a monoclonal antibody to SptP. The relevant phenotype of the infecting strains is indicated at the bottom of each panel. (**B**) Henle-407 cells were infected with wild-type *S. typhimurium* or the isogenic translocation defective, hypersecreting *sipD* mutant strain carrying the plasmid pSB762, which encodes SptP-IVNP$_{366-374}$. Infected cells were then stained with a monoclonal antibody to SptP as described in (*15*). Micrographs of the SptP stained cells and the corresponding DIC images are shown. Similar results were obtained with the same strains carrying pSB775.

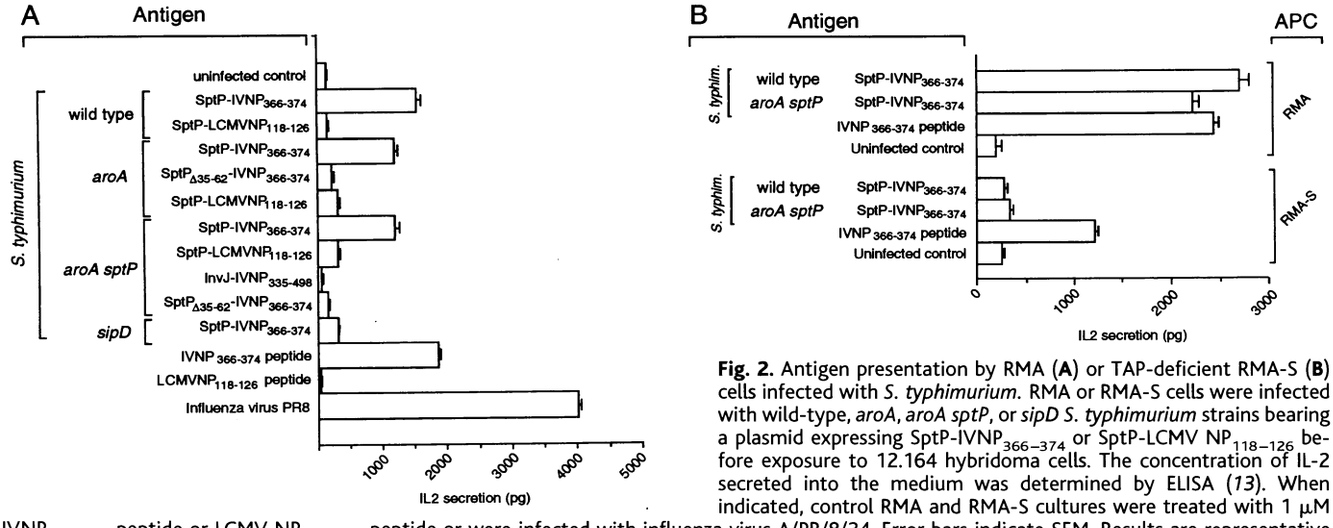

Fig. 2. Antigen presentation by RMA (**A**) or TAP-deficient RMA-S (**B**) cells infected with *S. typhimurium*. RMA or RMA-S cells were infected with wild-type, *aroA*, *aroA sptP*, or *sipD S. typhimurium* strains bearing a plasmid expressing SptP-IVNP$_{366-374}$ or SptP-LCMV NP$_{118-126}$ before exposure to 12.164 hybridoma cells. The concentration of IL-2 secreted into the medium was determined by ELISA (*13*). When indicated, control RMA and RMA-S cultures were treated with 1 μM IVNP$_{366-374}$ peptide or LCMV NP$_{118-126}$ peptide or were infected with influenza virus A/PR/8/34. Error bars indicate SEM. Results are representative of three independent experiments.

Peptides delivered to the cytosol by *S. typhimurium* required a functional peptide transporter system (TAP) for their transfer to the endoplasmic reticulum and loading by class I molecules, because TAP2-defective RMA-S mutant cells (*17*) infected with *S. typhimurium* SptP-IVNP$_{366-374}$ were markedly impaired in their capacity to stimulate the T cell hybridoma compared with wild-type RMA cells (Fig. 2). This result provides independent evidence that peptides displayed at the RMA cell surface by class I molecules occupied a cytosolic compartment before they were transferred into the endoplasmic reticulum.

We then examined the potential of avirulent *S. typhimurium* strains expressing SptP-IVNP$_{366-374}$ to induce cytotoxic T lymphocytes (CTLs) in vivo. C57BL/6J mice orally inoculated with an avirulent *S. typhimurium sptP aroA* mutant strain expressing SptP-IVNP$_{366-374}$ developed CTL precursors that lysed target cells infected with influenza virus or loaded with the synthetic peptide NP$_{366-374}$ (Fig. 3A) (*18*). In contrast, and consistent with the in vitro antigen presentation results, mice inoculated intragastrically with *Salmonella* expressing either SptP-LCMVNP$_{118-126}$ or InvJ-IVNP$_{335-498}$ failed to stimulate CTLs directed to influenza virus epitopes (Fig. 3A). Thus, cytoplasmic targeting of the type III system hybrid substrates dictates the outcome of the class I–restricted response to a foreign epitope: pronounced CTL responses were induced by the *S. typhimurium* mutant strain expressing SptP-IVNP$_{366-374}$ but not by the strain expressing the nontranslocated InvJ-IVNP$_{335-498}$ chimeric protein.

To assess the ability of the type III–mediated *S. typhimurium* antigen delivery system to induce protective class I–restricted CTLs, we chose murine lymphocytic choriomeningitis virus (LCMV) infection because CTLs play a dominant role in protection against this viral disease (*19*). In this model, intracerebral inoculation with LCMV results in lethal choriomeningitis, which can be prevented by a single clonal population of LCMV-specific CTLs (*19*). BALB/c mice immunized intragastrically with the *aroA sptP S. typhimurium* mutant strain expressing SptP-LCMVNP$_{118-126}$ were completely protected against lethal intracerebral challenge with a virulent strain of LCMV (*20*). In contrast, mice immunized intragastrically with *S. typhimurium aroA* mutant strain expressing an irrelevant epitope (SptP-IVNP$_{366-374}$) succumbed to the same challenge (Fig. 3B). Consistent with the hypothesis that protection was mediated by an H-2–restricted immune response, the same *S. typhimurium aroA* mutant strain expressing SptP-LCMVNP$_{118-126}$ did not protect C57BL/6J mice against an identical infection with LCMV (*21*). Furthermore, protection required cytosolic delivery of the epitope by the type III secretion system, as vaccination with a *S. typhimurium sipD*$^-$ strain defective in translocation but not in secretion of SptP-LCMVNP$_{118-126}$ did not protect mice against LCMV infection (Fig. 3B). Induction of protective immunity in mice by *Salmonella* vaccination was correlated with the presence of LCMV-specific CTLs although quantities of splenic CTL precursor were lower in mice vaccinated with *Salmonella* than in mice infected with a sublethal dose of LCMV (Fig. 3C).

We show here that delivery of epitopes through the *S. typhimurium* type III secretion system results in efficient stimulation of class I–restricted protective antiviral immune responses. Use of this system will expand the efficient use of *S. typhimurium* as a carrier of heterologous antigens to vaccinate against infections in which this type of response is crucial for protection (*22*). In addition, avirulent *S. typhimurium* expressing tumor-specific antigens may allow use of this system for treatment of neoplastic diseases by induction of cancer cell–specific class I–restricted CTLs (*23*).

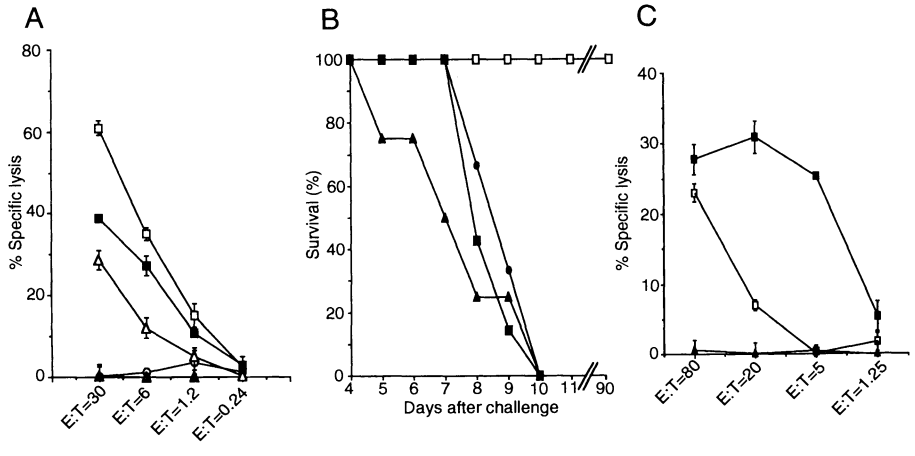

Fig. 3. (**A**) Lysis of IVNP$_{366-374}$ peptide-sensitized EL-4 target cells by restimulated splenocytes from mice orally immunized with the *aroA sptP S. typhimurium* strain SB824 expressing SptP-IVNP$_{366-374}$, an irrelevant peptide (SptP-LCMVNP$_{118-126}$), or InvJ-IVNP$_{335-498}$ (*18, 20*). Control mice were vaccinated intraperitoneally with live influenza virus A/PR/8/34 or recombinant vaccinia virus expressing influenza nucleoprotein (vaccinia IVNP)(*25*). Effector-to-target cell ratios (E:T) are indicated. Error bars indicate SEM. Results are representative of three independent experiments. Open squares, SptP-IVNP$_{366-374}$; open circles, SptP-LCMVNP$_{118-126}$; closed squares, vaccinia IVNP; open triangles: influenza virus strain A/PR/8/34; closed triangles, InvJ-IVNP$_{335-498}$. (B and C) Immune response of mice immunized with avirulent *S. typhimurium* expressing SptP-LCMVNP$_{118-126}$. (**B**) Survival of vaccinated mice after LCMV intracerebral challenge inoculation (*20*). Groups of BALB/c mice were alternatively orally vaccinated with the avirulent *aroA sptP S. typhimurium* strain SB824 expressing SptP-LCMVNP$_{118-126}$ or SptP fused to an irrelevant epitope (SptP-IVNP$_{366-374}$), an avirulent *S. typhimurium sipD*$^-$ strain, defective in translocation but not in secretion of SptP-LCMVNP$_{118-126}$, or mock vaccinated with broth. Open squares, *aroA* (SptP-LCMVNP$_{118-126}$) (n = 8); closed squares, *aroA* (SptP-IVNP$_{366-374}$) (n = 7); closed triangles, *sipD* (SptP-LCMVNP$_{118-126}$) (n = 4); closed circles, mock control (n = 6). (**C**) Percent specific lysis by in vitro restimulated splenocytes from mice inoculated with *aroA sptP S. typhimurium* expressing SptP-LCMVNP$_{118-126}$ or SptP fused to an irrelevant peptide (SptP-IVNP$_{366-374}$). Control mice were vaccinated by intraperitoneal inoculation with live LCMV. Error bars indicate SEM. Results are representative of three independent experiments. Open squares, aroA (SptP-LCMVNP$_{118-126}$); closed triangles, aroA (SptP-IVNP$_{366-374}$); closed squares, LCMV.

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tween SptP and residues 118 to 126 of the LCMV nucleoprotein. Both plasmids are derived from the low-copy plasmid pWSK-II [R. F. Wang and S. Kushner, *Gene* **100**, 195 (1991)]. Plasmid pSB776, which encodes a fusion between the first 179 amino acids of InvJ and residues 335 to 498 of the influenza virus nucleoprotein, was constructed by polymerase chain reaction (PCR). This plasmid is derived from the low-copy plasmid pYA292 [J. E. Galán, K. Nakayama, R. Curtiss III, *Gene* **94**, 29 (1990)]. Strain SB824 was constructed by introducing the *sptP*::*kan* mutant allele from strain SB237 (*8*) into the Δ*aroA* strain SL3261 [S. K. Hoiseth and B. A. Stocker, *Nature* **291**, 238 (1981)] by P22HT*int* transduction.

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21. A group of five C57BL/6J mice received live *aroA sptP S. typhimurium* mutant strain expressing SptP-LCMVNP$_{118-126}$ intragastrically three times as described in (*19*). None of the mice in this group survived intracerebral inoculation with 10 LD_{50} of LCMV strain Armstrong. In contrast, another group of six C57BL/6J mice that had been injected with 10^4 PFU of LCMV strain Armstrong 4 weeks before intracerebral inoculation with LCMV, survived.
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An Unusual Mechanism for Ligand Antagonism

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The ratio of late to early events stimulated by the mast cell receptor for immunoglobulin E (IgE) correlated with the affinity of a ligand for the receptor-bound IgE. Because excess receptors clustered by a weakly binding ligand could hoard a critical initiating kinase, they prevented the outnumbered clusters engendered by the high-affinity ligands from launching the more complete cascade. A similar mechanism could explain the antagonistic action of some peptides on the activation of T cells.

Binding of ligand to a cell surface receptor often stimulates an elaborate biochemical cascade. If one of the initiating interactions must be preserved during the course of subsequent time-dependent, energy-consuming steps, the fidelity of the response can be considerably greater than would be predicted simply from the free energy released by the initial interaction with ligand. That is, ligands with lower affinity—which generally means those forming complexes with shorter lifetimes—would be less likely to stimulate responses that went to completion, a process that in related multistep systems has been dubbed "kinetic proofreading" (*1*). This formulation has been applied to explain the discriminatory prowess of the antigen receptors of T cells (*2*) and possibly to account for the action of variant peptides that can act as partial agonists or antagonists (*3*). Some experimental evidence supports this formulation (*4*, *5*), but specific molecular explanations have not yet been described.

The clonotypic antigen receptor on T cells is one of a family of receptors called "multichain immune recognition receptors" that share numerous structural and functional attributes (*6*). The high-affinity receptor for the Fc portion of immunoglobulin E (IgE), FcεRI, is a member of this family. We examined the kinetic proofreading formulation in the context of FcεRI and explored whether ligands of differing affinity could act as mutual antagonists under conditions at which simple displacement could not occur.

We loaded the FcεRIs on rat mucosal-type mast cells (line RBL-2H3) (*7*) with a monoclonal IgE specific for the 2,4-dinitrophenyl (DNP) hapten (*8*). The IgE's affinity for several nitrophenyl hapten analogs relative to DNP was ascertained (*9*), selected multivalent hapten-protein conjugates were prepared (*10*), and the cellular responses to high- or low-affinity ligands were monitored. The phosphorylation of tyrosines on the receptor and of several proteins in response to aggregation of FcεRI was quantitated (Fig. 1, A and B). The high-affinity DNP antigen stimulated phosphorylation of tyrosines on the β and γ subunits of FcεRI with comparable kinetics, and the data shown for the receptor represent the combined values for the two types of subunits (Fig. 1A). The phosphorylation of the kinase Syk and the adaptor protein Nck reached a maximum at

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