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.

11. K. Kaniga, D. Trollinger, J. E. Galán, *J. Bacteriol.* **177**, 7078 (1995).
12. A. Deckhut *et al.*, *J. Immunol.* **151**, 2658 (1993); H. G. Ljunggren and K. Karre, *J. Exp. Med.* **162**, 1745 (1985).
13. RMA or RMA-S cells (C57BL/6J mouse lymphoma) were used as antigen presenting cells (APCs) after infection with *S. typhimurium* strains grown as described in (*12*, *15*). About 3×10^8 bacterial CFU were used to infect 10^7 APCs in a 3-ml volume of Dulbecco's modified Eagle's medium (DMEM) at 37°C for 2 hours. Control APCs were prepared by infection with $10^{7.5}$ plaque-forming units (PFU) of influenza A virus (strain A/PR/8/34) or incubation in 1 μM IVNP$_{366-374}$ synthetic peptide (ASNENMETM) for 2 hours. APCs subjected to the different procedures were washed, incubated for 1 hour in DMEM containing gentamicin at 100 μg/ml, and distributed in quadruplicate 100-μl cultures in 96-well plates with 2×10^5 APCs each. Influenza NP-specific T cell hybridoma 12.164 (2×10^5 cells) was added to the treated RMA or RMA-S cultures (*12*). Culture medium was collected after incubation for 44 hours at 37°C to determine the IL-2 concentration by a capture ELISA (Pharmingen).
14. SptP$^{\Delta 35-62}$-IVNP$_{366-374}$, which carries a mutation within the binding site of SicP, the SptP-specific chaperone [Y. Fu. and J. E. Galán, *J. Bacteriol.* **180**, 3393 (1998)], was constructed by introducing a 17–amino acid deletion in the SptP-IVNP$_{366-374}$ coding sequence of pSB762 by PCR. The resulting mutant protein is secreted from the bacteria at levels indistinguishable from those of wild type but it is not translocated into host cells (Y. Fu and J. E. Galán, unpublished results).
15. C. M. Collazo and J. E. Galán, *Mol. Microbiol.* **24**, 747 (1996).
16. C. M. Collazo, M. K. Zierler, J. E. Galán, *ibid.* **15**, 25 (1995).
17. M. Attaya *et al.*, *Nature* **355**, 647 (1992).
18. CTLs were quantitated as described in [J. Wunderlich and G. Shearer, in *Current Protocols in Immunology*, J. Coligan, A. Kruisbeek, D. Margulies, E. Shevach, W. Strober Eds. (Wiley, New York, (1991)]. Briefly, spleen cells from two or three mice were pooled and restimulated in vitro by coculture with irradiated syngeneic splenocytes infected with A/PR/8/34 influenza virus at a multiplicity of infection of 3 or treated with 1 μM synthetic peptide LCMVNP$_{118-126}$ (RPQASGVYM) for 5 days. EL-4 (*H-2^b*) and P815 (*H-2^d*) target cells were incubated with 1 μM IVNP$_{366-374}$ or LCMV NP$_{118-126}$ peptides and labeled with 0.15 mCi of sodium [^{51}Cr]chromate. Appropriate numbers of effector cells were combined with constant numbers of target cells in quadruplicate cultures to assess target lysis by ^{51}Cr release after cocultivation for 4 hours. Target lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum release was determined by Triton X-100 cell lysis. Spontaneous release was always <10% of maximum release. Data are presented as percent specific lysis, with the percent lysis of unsensitized target cells subtracted from the percent lysis of cognate peptide-sensitized target cells.
19. L. S. Klavinskis, A. Tishon, M. B. Oldstone, *J. Immunol.* **143**, 2013 (1989); J. L. Whitton *et al.*, *J. Virol.* **63**, 4303 (1989).
20. C57BL/6J or BALB/c mice were inoculated intragastrically with 1 to 2×10^8 CFU of appropriate live *S. typhimurium* strains grown as described in (*15*). Intragastric inoculation was repeated 4 and 6 weeks after the initial inoculation. Spleens were removed 2 weeks after the last bacterial inoculation to evaluate CTL activity in three mice from each group. To determine protective immunity, we inoculated groups of six to eight vaccinated mice intracerebrally with 10 median lethal doses (LD_{50}) of LCMV strain Armstrong. For control purposes, we inoculated mice intraperitoneally with $10^{7.5}$ PFU of influenza virus A/PR/8/34, 10^6 PFU of vaccinia influenza NP (*24*), or 10^4 PFU of LCMV strain Armstrong 4 weeks before assessment of immunity.
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.
22. J. Hackett, *Curr. Opin. Biotechnol.* **4**, 611 (1993); K. L Karem, J. N. Bowen, B. T. Rouse, *J. Gen. Virol.* **78**, 427 (1997).
23. W. Schmidt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3262 (1997); M. A. Ossevoort, M. C. Feltkamp, K. J. van Veen, C. J. Melief, W. M. Kast, *J. Immunother. Emphasis Tumor Immunol.* **18**, 86 (1995); J. Schlom *et al.*, *Breast Cancer Res. Treat.* **38**, 27 (1996).
24. Abbreviations for amino acid residues are as follows: 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, Y, Tyr.
25. G. L. Smith, J. Z. Levin, P. Palese, B. Moss, *Virology* **160**, 336 (1987).

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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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virtually the same time as that of FcεRI, but the phosphorylation of the kinase Erk2 was delayed. Higher and lower doses of the antigen correspondingly accelerated or slowed the kinetics of phosphorylation, but the order in which the proteins were modified was unaltered.

For methodological and other reasons, the absolute amounts of phosphotyrosine on different proteins are not directly comparable. Nevertheless, we estimated that the maximum amount of phosphotyrosines on the FcεRI, Syk, Nck, and Erk2 was about in the ratio of 3:1:0.2:0.4.

The IgE antibody to DNP (anti-DNP) bound the 2-nitrophenyl (2NP) moiety with an intrinsic affinity less than 0.001 of that for DNP (*9*). At comparable doses, the 2NP hapten conjugate induced less vigorous phosphorylation of the FcεRI than the DNP antigen, but this could be compensated for by the use of somewhat higher doses (Fig. 1B). Here the values shown correspond to the value at each time point divided by the maximum value achieved for that protein when stimulated as in Fig. 1A with the high-affinity ligand. Despite any ambiguity in comparing the extent of phosphorylation of different proteins directly (above), a comparison between the phosphorylation of the same components by the two stimuli is straightforward. The low-affinity ligand was progressively less effective in activating "downstream" components per unit of phosphorylation of the receptor (*11*). Thus, when the maximum phosphorylation of the receptor stimulated by the low-affinity ligand was more than twofold higher, the maximum phosphorylation of Syk was less than one-third of that achieved by the high-affinity ligand, and that for Erk2, whose activation is known to be "downstream" of Syk (*12*), was only one-tenth as much. These findings are consistent with a kinetic proofreading regime. [Our data place the activation of Nck temporally between Syk and Erk2 (Fig. 1A), but its location in one or another pathway is still unknown.]

Secretion of granular contents from the cells was also measured (Fig. 1C). The weakly bound 2NP ligand also stimulated release of hexosaminidase poorly, even at doses sufficient to stimulate phosphorylation of the FcεRI severalfold greater than that stimulated by the high-affinity DNP ligand. On the other hand, a conjugate made from the 2,4-dinitro-6-carboxyphenyl moiety (oDNCP), a hapten whose relative affinity for the anti-DNP IgE is 3% that of DNP (*9*), was almost as effective in stimulating degranulation as the DNP antigen in our initial experiments with cells loaded with bivalent anti-DNP IgE. However, we observed a clear difference when the cells were sensitized with a bispecific IgE antibody that is monovalent with respect to its DNP-binding site [(*10*) and below] (Fig. 1C).

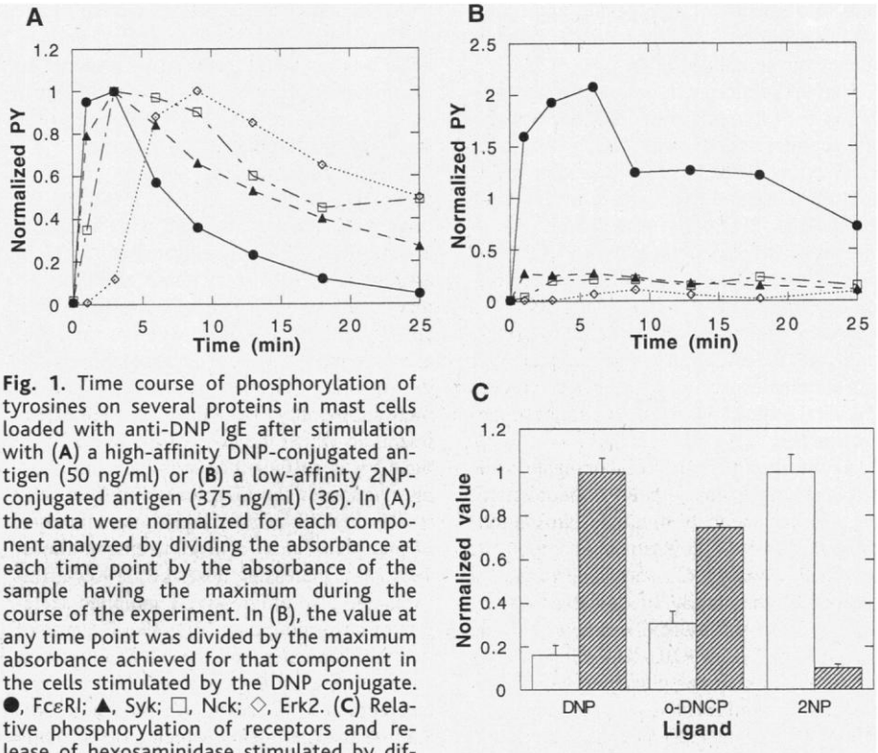


Fig. 1. Time course of phosphorylation of tyrosines on several proteins in mast cells loaded with anti-DNP IgE after stimulation with (**A**) a high-affinity DNP-conjugated antigen (50 ng/ml) or (**B**) a low-affinity 2NP-conjugated antigen (375 ng/ml) (*36*). In (A), the data were normalized for each component analyzed by dividing the absorbance at each time point by the absorbance of the sample having the maximum during the course of the experiment. In (B), the value at any time point was divided by the maximum absorbance achieved for that component in the cells stimulated by the DNP conjugate. ●, FcεRI; ▲, Syk; □, Nck; ◇, Erk2. (**C**) Relative phosphorylation of receptors and release of hexosaminidase stimulated by different antigens. Release was measured on adherent cells as described (*24*, *37*, *38*). The absorbance related to the phosphotyrosine on receptors in the sample of cells stimulated with the 2NP-conjugated antigen was designated as "1.00," and the values for the cells stimulated with the alternate ligands were related to this value (open bars). The same procedure was used to compare the release of hexosaminidase (hatched bars), except that the data were normalized to the release stimulated by the DNP ligand. The net release in the duplicate samples stimulated with the DNP antigen was 26.07 ± 1.7%. The error bars show the range of duplicate samples.

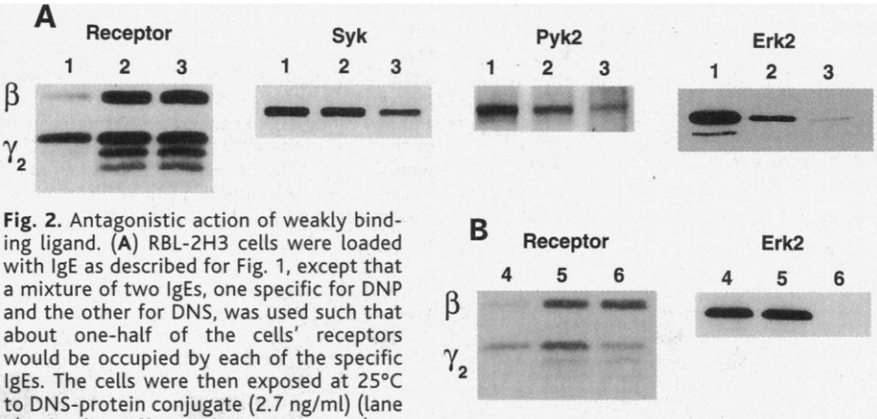


Fig. 2. Antagonistic action of weakly binding ligand. (**A**) RBL-2H3 cells were loaded with IgE as described for Fig. 1, except that a mixture of two IgEs, one specific for DNP and the other for DNS, was used such that about one-half of the cells' receptors would be occupied by each of the specific IgEs. The cells were then exposed at 25°C to DNS-protein conjugate (2.7 ng/ml) (lane 1), the low-affinity 2NP conjugate (100 ng/ml) (lane 3), or both simultaneously (lane 2). Portions were removed for immunoprecipitation of solubilized proteins at the times of maximum phosphorylation of each component and then separated by gel electrophoresis and assayed for phosphotyrosine. The far left panel shows phosphorylation of the β and γ chains in the receptors from 10^6 cell equivalents. Because the immunoprecipitations of the receptors were through their bound IgE with anti-IgE, the precipitates contained receptors bound to both the DNP- and DNS-specific IgE. The other panels show immunoprecipitations with anti-Syk (10^6 cell equivalents), anti-Pyk2 (5×10^6 cell equivalents), and anti-Erk2 (4×10^6 cell equivalents). (**B**) The experiment is like that shown in (A), except that separate portions of the cells were sensitized alternatively with anti-DNS and anti-DNP IgE and then mixed after first washing the cells well to remove unbound IgE. The cells were then stimulated with the DNS-protein conjugate alone (lane 4), the low-affinity 2NP conjugate alone (lane 6), or both simultaneously (lane 5). Only the phosphorylation of the receptor and of Erk2 is shown.

Here, the maximum size of the aggregates that are generated can be no greater than the valence of the antigen (*13*).

Variant peptides can antagonize the stimulation of T lymphocytes induced by wild-type peptides under nondisplacing conditions (*3*). We examined whether a similar effect would be observed in responses mediated by FcεRI. RBL-2H3 cells were loaded with a mixture of two monoclonal mouse IgEs, one specific for DNP and the other for the non–cross-reacting dansyl moiety (DNS) (*9*). The cells were then exposed to the DNS-protein conjugate, the low-affinity 2NP conjugate, or both simultaneously, and immunoprecipitates of several proteins were assayed for phosphotyrosine (Fig. 2A).

At the dose of the DNS conjugate used, the phosphorylation of FcεRI, although modest, was sufficient to stimulate phosphorylation of the three downstream components examined: Syk, Pyk2, and Erk2. Pyk2, a member of the family of "focal adhesion" kinases, is phosphorylated subsequent to the activation of Syk in RBL-2H3 cells (*14*). As before, the FcεRI was phosphorylated when the cells were stimulated with only the low-affinity 2NP ligand (*15*), but phosphorylation of the downstream components was diminished. When the cells were stimulated with the mixture of non–cross-reacting ligands, the phosphotyrosine in the total receptor subunits was undiminished and phosphorylation of Syk and Pyk2 was minimally reduced, but phosphorylation of Erk2 was substantially decreased (*16*).

To test whether the inhibitory effect of the low-affinity ligand was mediated by a released soluble factor, separate portions of cells were loaded with anti-DNS IgE and anti-DNP IgE alternatively and, after being washed to remove unbound IgE, were mixed. They were then stimulated under the three conditions as in Fig. 2A (Fig. 2B). The phosphorylation of the receptor proceeded as before, as did the phosphorylation of Erk2 by the DNS conjugate or the lack thereof by the 2NP conjugate. However, phosphorylation of Erk2 was no longer reduced in response to the mixture of ligands. Thus, the low-affinity ligand is only inhibitory when bound to the same cells as the high-affinity ligand.

The time dependence of the inhibitory effect was explored (Fig. 3A). Again, the cells were doubly sensitized with anti-DNP and anti-DNS IgE and exposed to the high-affinity DNS conjugate either alone or admixed with an excess of the low-affinity 2NP conjugate. The inhibition was not transient, and the temporally more distal components of the signaling cascade (Pyk2 and Erk2) were more profoundly affected. In six such experiments in which the dose of the low-affinity ligand was sufficient to stimulate the phosphorylation of the receptor severalfold relative to the high-affinity ligand, the inhibition of phosphorylation of Erk2 averaged almost 70% (*17*). Increasing doses of the low-affinity ligand also progressively inhibited secretion of hexosaminidase (Fig. 3B). Because of the ineffectiveness of the 2NP antigen in stimulating even the phosphorylation of the receptors at 37°C (see above), this and three additional experiments, which gave virtually identical results, were conducted at 25°C. As a control, release from cells loaded only with anti-DNS IgE was not inhibited by the added 2NP ligand, ruling out the trivial explanation of a "toxic" effect of 2NP.

In this system, it has been proposed that the initiating event is an aggregation-induced transphosphorylation of the receptor by the small amount of lyn constitutively associated with the FcεRI (*18*) and that the amount of weakly associated kinase can limit the intensity of the response (*19*). We therefore hypothesized that clusters of receptors induced by an excess of low-affinity ligand could sequester the kinase, so that the smaller number of clusters associated with the high-affinity ligand would be deprived of lyn and, therefore, of the means to initiate the signaling cascade. If so, then in an experiment such as that shown in Fig. 2A, phosphorylation of those receptors specifically aggregated by the high-affinity ligand would be expected to be reduced when isolated from the cells stimulated with the mixture of high- and low-affinity ligands, even though the receptor population as a whole showed a higher amount of phosphorylation. We had technical difficulty in efficiently immunoprecipitating the high-affinity DNS ligand, so for these experiments we used preformed oligomers (dimers) of rat IgE as a surrogate high-affinity ligand (*20*).

Cells were first partially loaded with mouse anti-DNP IgE and then stimulated with rat IgE dimers alone, the low-affinity 2NP conjugate alone, or a mixture of both (Fig. 4). The dimer-clustered receptors stimulated phosphorylation of Erk2, whereas, as before, the low-affinity 2NP ligand was not only deficient in stimulating phosphorylation

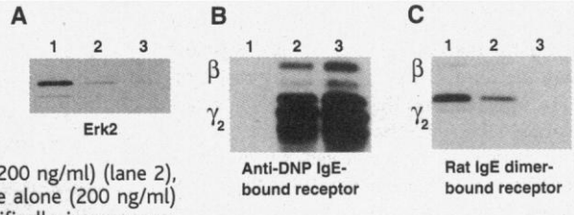
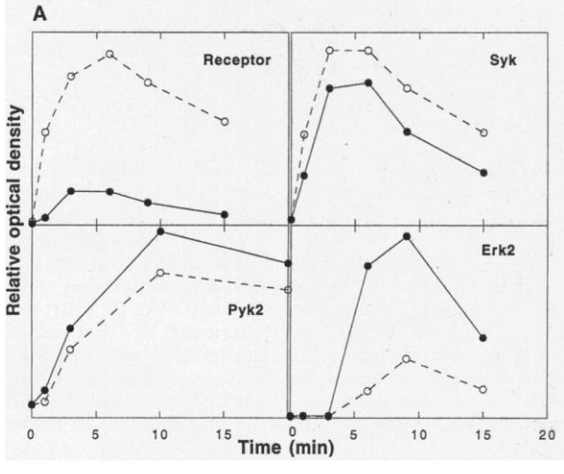


Fig. 4. Molecular mechanism of antagonism. Cells were first partially loaded with mouse anti-DNP IgE. At time zero, they were then reacted with rat IgE dimer alone (100 ng/ml) (lane 1), a mixture of dimer (100 ng/ml) and low-affinity 2NP conjugate (200 ng/ml) (lane 2), or the low-affinity 2NP conjugate alone (200 ng/ml) (lane 3). Cell extracts were specifically immunoprecipitated with (**A**) anti-Erk2, (**B**) anti-mouse IgE, or (**C**) anti-rat IgE, and the precipitated material was analyzed on gels for protein-bound phosphotyrosine as before.



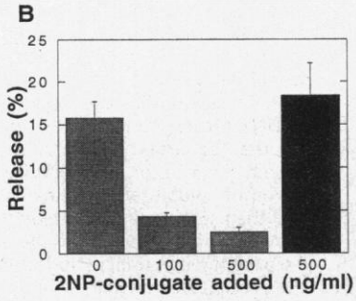


Fig. 3. Inhibition of cellular response by low-affinity antigen. (**A**) Time dependence of the inhibitory effect. Cells were loaded with a mixture of anti-DNP and anti-DNS IgE and stimulated with DNS conjugate (2.7 ng/ml) (●) or DNS conjugate (2.7 ng/ml) plus 2NP conjugate (100 ng/ml) (○). The indicated proteins were analyzed for phosphotyrosine. The dimensions of the ordinate scales for the four panels have been roughly normalized. (**B**) Inhibition of hexosaminidase release by low-affinity ligand. The experiment was performed at 25°C. Cells were loaded either with both anti-DNS and anti-DNP (grey bars) or with only anti-DNS (black bar). Samples were reacted for 30 min with DNS conjugate (2.7 ng/ml), in addition to 2NP conjugate (0, 100, or 500 ng/ml). The bars indicate the absolute percentage of release after subtracting the percentage of hexosaminidase released spontaneously (2.13 ± 0.11%). The error bars show the range for the duplicate incubations studied. Three additional experiments gave virtually identical results.

of Erk2 by itself but also inhibited the action of the high-affinity stimulant. Under these conditions, the receptors clustered by the low-affinity antigen were phosphorylated (Fig. 4B). However, the phosphotyrosine on the receptors that were clustered by the high-affinity ligand was reduced by about two-thirds (Fig. 4C).

In the previous experiments, we deliberately used a protocol in which the receptors were clustered in two distinct pools—one for each ligand. When we instead used IgE of a single specificity in combination with limited amounts of low- and high-affinity ligands, the receptor clusters would contain both high- and low-affinity ligands. Depending on a number of variables, addition of low-affinity ligand either inhibited or augmented downstream signaling stimulated by high-affinity ligand. However, when we used the bispecific antibody, where the receptors binding the low- or high-affinity ligand cannot co-cluster, the low-affinity ligand reproducibly inhibited signaling from receptors binding the high-affinity ligand, as in the protocols with two distinct ligands (*21*).

The essential feature of systems subject to kinetic proofreading is that the cascade of events that follows interaction with a ligand persists only as long as the initiating interaction is maintained (*22*). We have determined that the FcεRI-initiated cascade of cellular responses behaves like a system subject to kinetic proofreading. The molecular explanation for which we have obtained direct evidence relates to findings on how receptors that are not themselves kinases may need to compete for the limited extrinsic kinase that initiates the transphosphorylation of approximated receptors (*19*). In such a system, weakly clustered receptors act like the "dog in the manger" (*23*): Like the dog, they impede access to a necessity (the kinase) in spite of their inability to use it productively. The pathophysiological sequelae produced by such activators are likely to differ from those capable of stimulating the full cellular response. As already noted (*22*), it is the lifetime of individual receptors in a cluster that determines their likelihood of initiating a "complete" cascade. Therefore, the ratio of late to early signals stimulated by a receptor, such as an FcγR, that uses a similar signaling cascade as FcεRI but whose interaction with its cognate Ig is much weaker might be different (lower) than that stimulated by FcεRI (all other things taken equal), with corresponding differences in the cellular response. Some relevant although limited data on this matter [Table I in (*24*)] show no such tendency, but a kinetic analysis or an analysis of later events (for example, gene transcription) might uncover such a trend.

In principle, the molecular mechanism we propose can relate to other receptors that require recruitment of an extrinsic component in limited supply and that are subject to a kinetic proofreading regimen. In the immune system, the family of multichain immune recognition receptors and the cytokine receptors are obvious candidates.

References and Notes

1. J. J. Hopfield, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4135 (1974); J. Ninio, *Biochimie* **57**, 587 (1975); M. Yarus, *Trends. Biochem. Sci.* **17**, 171 (1992); *ibid.*, p. 130; S. M. Burgess and C. Guthrie, *ibid.* **18**, 381 (1993).
2. T. W. McKeithan, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5042 (1995).
3. A. Sette *et al.*, *Annu. Rev. Immunol.* **12**, 413 (1994).
4. L. Racioppi, F. Ronchese, L. A. Matis, R. N. Germain, *J. Exp. Med.* **177**, 1047 (1993); B. D. Evavold, J. Sloan-Lancaster, P. M. Allen, *Immunol. Today* **14**, 602 (1993); J. Sloan-Lancaster, A. S. Shaw, J. B. Rothbard, P. M. Allen, *Cell* **79**, 913 (1994); L. Racioppi and R. N. Germain, *Chem. Immunol.* **60**, 79 (1995); J. D. Rabinowitz, C. Beeson, D. S. Lyons, M. M. Davis, H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1401 (1996); J. Sloan-Lancaster and P. M. Allen, *Annu. Rev. Immunol.* **14**, 1 (1996); J. D. Rabinowitz *et al.*, *Immunity* **5**, 125 (1996); C. Wulfing *et al.*, *J. Exp. Med.* **185**, 1815 (1997).
5. J. Madrenas *et al.*, *Science* **267**, 515 (1995).
6. A. D. Keegan and W. E. Paul, *Immunol. Today* **13**, 63 (1992).
7. E. L. Barsumian, A. McGivney, L. K. Basciano, R. P. Siraganian, *Cell. Immunol.* **90**, 131 (1985).
8. F. T. Liu *et al.*, *J. Immunol.* **124**, 2728 (1980).
9. Sepharose beads coupled with trinitrophenyl-lysine (0.01-ml packed volume) were mixed with varying concentrations of hapten in buffer {1.8 mM Ca^{2+}, 1 mM Mg^{2+}, and gelatin [or bovine serum albumin (BSA)] (1 mg/ml) in 119 mM NaCl, 5 mM KCl, and 25 mM Pipes (pH 7.2)}. To each duplicate tube, we added 0.5 μg of Fab fragments prepared (*25*) from the anti-DNP IgE (*8*) and radioiodinated. The final volume was 0.522 ml. After 10 min at ≈25°C (sufficient time for binding to reach equilibrium), the tubes were centrifuged, and 0.25 ml of the supernatant and the remaining supernatant plus pellet were counted. For the indicated concentrations, the percentage of inhibition (in parentheses), the concentration required to inhibit activity by 50% (IC_{50}) (*26*) as assessed from a logarithmic plot of the data, and the relative affinity (aff.) based on it, were as follows: 2,4-dinitrophenyl-εNH_2 caproate (DNP): 0.1 μM (34.3 ± 0.6), 0.25 μM (51.2 ± 4.6), 0.5 μM (70.2 ± 1.9); IC_{50}, 0.21; aff., 1.0; 2,4-dinitro-6-carboxyphenyl-εNH_2 caproate (oDNCP): 5 μM (48.7 ± 0.9), 10 μM (61.1 ± 6.6), 25 μM (82.7 ± 6.3); IC_{50}, 5.7; aff., 0.037; and 2-nitrophenyl-εNH_2 caproate (2NP): 200 μM (34.3 ± 4.2), 350 μM (51.0 ± 2.7), 500 μM (65.6 ± 1.7); IC_{50}, 324; aff., 0.0006. Differences in binding between 25° and 37°C for any of the ligands were too small to be reproducible, suggesting that the temperature dependence of binding was similar for the different ligands.
10. Antigens were prepared either from BSA or from bovine IgG Fab fragments purified by absorption with protein G Sepharose and by gel filtration on Sephadex G-200. The proteins were conjugated directly or with the εNH_2-caproate (cap) derivatives of the haptens (*27*). Protein concentrations were measured with a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) (*28*), and the protein-bound hapten was estimated spectrophotometrically (*29*). The antigens were estimated to be (DNS_{69})-BSA, $(DNP\text{-}cap)_{3.5}$-Fab, $(oDNCP\text{-}cap)_{4.3}$-Fab, and $(2NP\text{-}cap)_{4.5}$-Fab. Anti-DNS IgE, a monoclonal mouse antibody, has an affinity of $5.9 \times 10^8\ M^{-1}$ for DNS lysine (*30*). By fusing the anti-DNP and anti-DNS hybridomas, a "quadroma" was constructed [K. Subramanian, thesis, Cornell University, Ithaca, NY (1995)], and bispecific antibodies were purified by successive affinity chromatography on Sepharose conjugated with TNP or DNS.
11. Complete time courses of phosphorylation such as those shown in Fig. 1, A and B, were examined for five different doses of the high-affinity ligand and for three different doses of the low-affinity ligand. At all times and doses, the low-affinity ligand was progressively inefficient in stimulating the phosphorylation of later components. For example, a dose of the low-affinity ligand that stimulated up to a 16-fold higher amount of phosphorylation of the receptor compared with the high-affinity ligand stimulated at maximum only about half as much phosphorylation of the next downstream component, Syk.
12. N. Hirasawa, A. Scharenberg, H. Yamamura, M. A. Beaven, J.-P. Kinet, *J. Biol. Chem.* **270**, 10960 (1995).
13. We have provided experimental data (*31*) and a rationale (*19*) for why smaller aggregates are disadvantaged when clustered receptors must compete for limited amounts of the initiating kinase. It follows that the smaller clusters are also more sensitive to the intrinsic affinity of the ligand that induced them. Our findings on the effect of cluster size are consistent with observations on the effect of antigenic valency in triggering cells sensitized with antibodies of different affinities (*32*).
14. H. Okazaki, J. Zhang, M. M. Hamawy, R. P. Siraganian, *J. Biol. Chem.* **272**, 32443 (1997).
15. Figures 2 and 4 suggest possible qualitative differences in the phosphorylation of the γ subunits stimulated by the high- and low-affinity ligands, but we observed no consistent pattern in numerous experiments. Also, quantitative analysis indicated no difference in the ratio of phosphotyrosine on the β and γ subunits of receptors aggregated by the ligands of differing affinity. A difference in the pattern of phosphotyrosine associated with the ζ chain when T cell receptors are stimulated alternatively by agonist and antagonist peptides has been reported [for example, (*5*)]. Possibly this difference is related to the three-fold greater multiplicity of phosphorylation sites in the ζ chain compared with the γ chain.
16. Unlike the phosphorylation of the receptor and Syk, phosphorylation of Erk2 decreased at higher doses of antigen at which the phosphorylation of the receptor and Syk was still increasing (*21*). Thus, the inhibition of the phosphorylation of Erk2 with the mixture of antigens might have resulted from the high total dose of antigen. The results of an experiment such as that shown in Fig. 2 at doses of antigen well below those at which the decline in the phosphorylation of Erk2 by the high-affinity antigen is observed demonstrated that the inhibition by the low-affinity antigen is not simply due to an excessively high dose of antigen.
17. With sufficient low-affinity ligand to enhance the maximal phosphorylation of the receptor about four-fold compared with the phosphorylation produced by the high-affinity ligand alone, the maximal phosphorylation of Erk 2 was inhibited by 83% (Fig. 3A). In five other experiments for which similar calculations were made and in which the receptor phosphorylation was enhanced twofold to fivefold by the addition of the low-affinity ligand, the inhibition varied between 55 and 82%, with an average of 66% for the six experiments.
18. T. Yamashita, S.-Y. Mao, H. Metzger, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11251 (1994); V. S. Pribluda, C. Pribluda, H. Metzger, *ibid.*, p. 11246.
19. C. Torigoe, B. Goldstein, C. Wofsy, H. Metzger, *ibid.* **94**, 1372 (1997); C. Wofsy, C. Torigoe, U. M. Kent, H. Metzger, B. Goldstein, *J. Immunol.* **159**, 5985 (1997).
20. D. M. Segal, J. D. Taurog, H. Metzger, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2993 (1977).
21. C. Torigoe, J. K. Inman, H. Metzger, unpublished results.
22. When we speak of maintaining the initiating interaction, we refer not to the persistence of a cluster (a somewhat ambiguous concept in a dynamic system) but to the lifetime of individual receptors within the cluster [H. Metzger, *Mol. Immunol.* **19**, 1071 (1982)]. That lifetime is directly related to k_{-1} for the binding reaction, which (rather than k_{+1}) largely accounts for the differences in affinity for antigen-antibody or receptor-ligand interactions [L. A. Day, J. M. Sturtevant, S. J. Singer, *Ann. N.Y. Acad. Sci.* **103**, 611 (1963)].
23. Aesop, in *Folklore and Fables*, C. W. Eliot, Ed. (Grolier Enterprises, Danbury, CT, 1985), p. 27.
24. G. Alber, U. M. Kent, H. Metzger, *J. Immunol.* **149**, 2428 (1992).

25. S. Haba and A. Nisonoff, *J. Immunol. Methods* **138**, 15 (1991).
26. Y. Cheng and W. H. Prusoff, *Biochem. Pharmacol.* **22**, 3099 (1973).
27. K. E. Stein, C. Kanellopoulos-Langevin, D. I. Cohen, J. K. Inman, *J. Immunol. Methods* **37**, 83 (1980).
28. P. K. Smith *et al.*, *Anal. Biochem.* **150**, 76 (1985).
29. R. F. Ashman and H. Metzger, *J. Biol. Chem.* **244**, 3405 (1969); C. H. Schneider, S. Lazary, W. Wirz, A. L. de Weck, *Immunochemistry* **11**, 447 (1974).
30. V. T. Oi *et al.*, *Nature* **307**, 136 (1984).
31. B. M. Vonakis, H. Chen, H. Haleem-Smith, H. Metzger, *J. Biol. Chem.* **272**, 24072 (1997).
32. A. M. Collins, D. Thelian, M. Basil, *Int. Arch. Allergy Immunol.* **107**, 547 (1995); A. M. Collins, M. Basil, K. Nguyen, D. Thelian, *Clin. Exp. Allergy* **26**, 964 (1996).
33. C. Wofsy, U. M. Kent, S.-Y. Mao, H. Metzger, B. Goldstein, *J. Biol. Chem.* **270**, 20264 (1995).
34. E. Woldemussie, K. Maeyama, M. A. Beaven, *J. Immunol.* **137**, 1674 (1986).
35. V. S. Pribluda, C. Pribluda, H. Metzger, *J. Biol. Chem.* **272**, 11185 (1997).
36. RBL-2H3 cells were incubated with anti-DNP IgE, and, after washing, 6.2×10^6 cells/ml were incubated at 25°C in a buffer (*9*) containing Fab fragments (50 ng/ml) conjugated with DNP- or 2NP-caproate (*10*). Reactions were stopped by transferring cells directly into solubilization buffer at 0°C. All the methods used to analyze specific proteins for phosphotyrosine have been described (*33*). Antibodies not previously cited were anti-PLC γ1 from Upstate Biotechnology (Lake Placid, NY), a mixture of five monoclonal antibodies that cross reacts with Nck [D. Park and S. G. Rhee, *Mol. Cell. Biol.* **12**, 5816 (1992)]; goat polyclonal anti-Erk2 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal IgG1 anti-Pyk2 (Transduction Laboratories, Lexington, KY); biotin-conjugated mouse monoclonal IgG2b anti-phosphotyrosine, 4G10 (Upstate Biotechnology); and horseradish peroxidase–conjugated avidin (Sigma). The cell equivalents analyzed were adjusted so that the samples would give approximately similar exposures on the autophotographs.
37. U. M. Kent *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3087 (1994).
38. Cells reacted with bispecific anti-DNP/anti-DNS IgE (0.3 μg/ml) (*10*) (a fivefold to sixfold excess of IgE per receptor) for ≈16 hours were washed and reacted with the alternative ligands at 28°C. After 30 min, release of hexosaminidase was measured. Cells in separate wells were stimulated identically and analyzed for phosphotyrosine after 3 min. The doses of ligand (adjusted to induce ample phosphorylation of the receptors) were 10, 100, and 2500 ng/ml for the DNP-, oDNCP-, and 2NP-protein conjugates, respectively. Separate experiments confirmed that higher doses of the oDNCP and 2NP conjugates gave no additional release. The experiments were conducted at a lower than optimal temperature (*34*) because of the unusual difference in the temperature response of the cells to the two ligands: Whereas the DNP conjugate stimulated increased phosphorylation with increasing temperature between 0° and 37°C, the 2NP conjugate yielded decreased phosphorylation above 15°C, although the ratio of modification in γ compared with β was always around 2:1 (*35*).
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Fidelity of T Cell Activation Through Multistep T Cell Receptor ζ Phosphorylation

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The T cell receptor (TCR) αβ heterodimer interacts with its ligands with high specificity, but surprisingly low affinity. The role of the ζ component of the murine TCR in contributing to the fidelity of antigen recognition was examined. With sequence-specific phosphotyrosine antibodies, it was found that ζ undergoes a series of ordered phosphorylation events upon TCR engagement. Completion of phosphorylation steps is dependent on the nature of the TCR ligand. Thus, the phosphorylation steps establish thresholds for T cell activation. This study documents the sophisticated molecular events that follow the engagement of a low-affinity receptor.

The αβ TCR is part of a large protein complex composed of the CD3 γ, δ, ε, and ζ chains. These chains contain signaling motifs called ITAMs (immune receptor tyrosine-based activation motifs) with the consensus sequence $YXX(L/I)X_{6-8}YXX(L/I)$ (*1, 2*). Upon phosphorylation, this motif is sufficient to transduce signals from the TCR (*3*). The γ, δ, and ε chains each contain one ITAM, and ζ contains three ITAMs. The multiple ITAMs are thought to amplify signals from the TCR; however, it is not immediately clear why such a complicated receptor system arose solely for the purpose of signal amplification. Another function of the TCR complex could be to qualitatively evaluate ligands of the αβ TCR. To examine this possibility, we studied the effect of different physiologic TCR ligands on phosphorylation of individual tyrosines of the TCR ζ chain. Although ζ is not absolutely required for T cell development (*4, 5*), ζ is critical for the selection of the TCR repertoire and for the prevention of autoimmunity (*6*). With six potential phosphorylation sites, herein referred to as A1, A2, B1, B2, C1, and C2 (Fig. 1A), ζ could yield more than 60 different phospho-species and amplify initial signals. Thus, molecularly, ζ is well suited for processing information received by the αβ TCR. The mechanism of signal initiation through phosphorylation of ζ, however, has not been ascertained. Discreet phosphoforms of ζ exist in resting and in activated T cells, with apparent molecular sizes of 21 and 23 kD, respectively. Specific phospho-species giving rise to these discreet forms have not been identified because of the complexity of the molecule (*7–9*). The ratio of p21 and p23 can be altered after stimulation of T cells with suboptimal ligands, further suggesting a discriminatory role of ζ phosphorylation in T cell activation (*10, 11*).

We first wished to identify the molecular composition of p21. We raised six antisera, each specific for one of the six phosphotyrosines in ζ (*12*). We demonstrated their specificity by using phosphorylated and unphosphorylated ζ proteins, as well as ζ proteins with substitutions of individual tyrosines to phenylalanine, where the tyrosine of interest cannot be phosphorylated (Fig. 1B) (*13*). The antibodies were used to examine the phosphorylation status of TCR ζ in the resting Th1 clone 3.L2, which is specific for Hb(64–76)/I-E^k (Fig. 2, leftmost lane of each panel) (*14*). Two of the six specific phospho-ITAM sera (against the B1 and C2 sites) recognized p21 in resting T cells (Fig. 2, D and G) (*15*). A2 was recognized variably (Fig. 2C) (*16*). This pattern was observed in at least seven independent experiments for each antisera. The B1 and C2 phosphotyrosines were located within the same ζ homodimer, because immunoprecipitation with anti-pC2 and subsequent protein immunoblotting with anti-pB1 revealed p21 (*17*). Thus, in resting T cells, the p21 form of ζ has two prominent phosphotyrosines, B1 and C2.

We also examined the pattern of ζ phosphorylation observed in resting mature T cells directly isolated from mice. We made use of TCR transgenic mice harboring the Hb(64–76)/I-E^k–specific TCR 2.102 (*14*) bred onto a RAG-1 deficient background (*18*). Spleens from such mice are greatly enriched in resting, mature $CD4^+$ T cells. Lysates from freshly isolated spleen cells were studied for their ζ phosphorylation (*19*). Phosphorylation of the p21 form of ζ, consisting of prominent B1 and weak C2 phosphorylation, was found (*17*), thus extending our findings to resting ex vivo T cells.

We next stimulated the T cell clone 3.L2 with antigen presenting cells (APCs) pulsed with the antigenic peptide Hb(64–76) and examined the phosphorylation of ζ (*15*). Such stimulation fully activates the 3.L2 T cell and causes cell proliferation (*14*). The phosphorylation of p21 rapidly increased, and a 23-kD phospho-form of ζ appeared (Fig. 2A). The increase in p21 phosphorylation was due

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