Kinetic Proofreading of Ligand-FcεRI Interactions May Persist beyond LAT Phosphorylation[1]

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Cells may discriminate among ligands with different dwell times for receptor binding through a mechanism called kinetic proofreading in which the formation of an activated receptor complex requires a progression of events that is aborted if the ligand dissociates before completion. This mechanism explains how, at equivalent levels of receptor occupancy, a rapidly dissociating ligand can be less effective than a more slowly dissociating analog at generating distal cellular responses. Simple mathematical models predict that kinetic proofreading is limited to the initial complex; once the signal passes to second messengers, the dwell time no longer regulates the signal. This suggests that an assay for kinetic proofreading might be used to determine which activation events occur within the initial signaling complex. In signaling through the high affinity IgE receptor FcεRI, the transmembrane adaptor called linker for activation of T cells (LAT) is thought to nucleate a distinct secondary complex. Experiments in which the concentrations of two ligands with different dwell times are adjusted to equalize the level of LAT phosphorylation in rat basophilic leukemia 2H3 cells show that Erk2 phosphorylation, intracellular Ca^{2+}, and degranulation exhibit kinetic proofreading downstream of LAT phosphorylation. These results suggest that ligand-bound FcεRI and LAT form a complex that is required for effective signal transmission. *The Journal of Immunology,* **2007, 178: 3530–3535.**

Kinetic proofreading of ligand receptor interactions has been invoked to explain why some ligands that bind cell surface receptors and trigger early activation events, such as receptor phosphorylation, fail to produce strong downstream signals (1, 2). In this mechanism, the formation of an active signaling complex requires a sequence of binding and phosphorylation events that are rapidly reversed if the ligand dissociates from the receptor. As a result, the effectiveness of rapidly dissociating ligands at inducing particular responses diminishes with the distance downstream. Two ligands that differ only in the rate at which they dissociate from the receptor may differ strongly in the level of cellular activation they induce, even when the concentrations of the two ligands are adjusted so that both give the same level of receptor occupancy.

Experiments of this type, probing signaling in the rat basophilic leukemia (RBL) cell line 2H3 (RBL-2H3) initiated by aggregation of the high affinity receptor for IgE (FcεRI), provide strong evidence for the kinetic proofreading model (3). A mathematical model of early signaling events in FcεRI-mediated signaling also provides a strong mechanistic basis for the kinetic proofreading in this system, which seems to arise from dual requirements for transphosphorylation by the two initiating kinases, Lyn and Syk (4). However, responses that apparently escape kinetic proofreading have been observed both in this system (5, 6) and in TCR signaling (7). A model explaining this escape has been proposed that involves the formation of a messenger that spreads the signal beyond the initial signaling complex containing the ligand and receptor (8, 9). In the case of *MCP-1*, a gene for which transcriptional activation appears to escape kinetic proofreading in RBL-2H3 cells, several experiments have been interpreted as supporting the hypothesis that calcium ions (Ca^{2+}) released from intracellular stores may serve as the messenger that allows escape (10). Transcriptional activation of other chemokines also appears to escape kinetic proofreading through similar mechanisms (6).

Given that many of the responses associated with cellular activation occur distal to the site of ligand receptor binding, it seems natural to ask whether other messengers might be found that would mark the end points of kinetic proofreading for some responses. An early event following the aggregation of FcεRI is phosphorylation of the transmembrane adaptor protein called linker for activation of T cells (LAT)[3] (11). This is thought to result from the movement of aggregated FcεRI complexes containing Syk into membrane lipid microdomains, where LAT preferentially localizes (12). On the one hand, it has been shown that disaggregation of the receptors leads to rapid dephosphorylation of LAT (13), which could suggest that downstream signaling events requiring phosphorylation of LAT should be subject to further kinetic proofreading. On the other hand, recent electron micrographs of membrane sheets prepared from RBL-2H3 cells following the aggregation of FcεRI revealed coclustered LAT in small islands that have little overlap with similar islands of FcεRI (14). Assuming that these islands are active in triggering downstream events, and that the micrographs also indicate the coclustering of LAT with downstream effectors such as the p85 subunit of PI3K and phospholipase C (PLC)-γ1, these images suggest that post-LAT responses may not be subject to further kinetic proofreading. A model that reconciles these two apparently contradictory observations is one in which Syk associated with the FcεRI complexes phosphorylates LAT

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[3] Abbreviations used in this paper: LAT, linker for activation of T cells; 2-NP, 2-nitrophenyl; PLC, phospholipase C; RBL, rat basophilic leukemia.

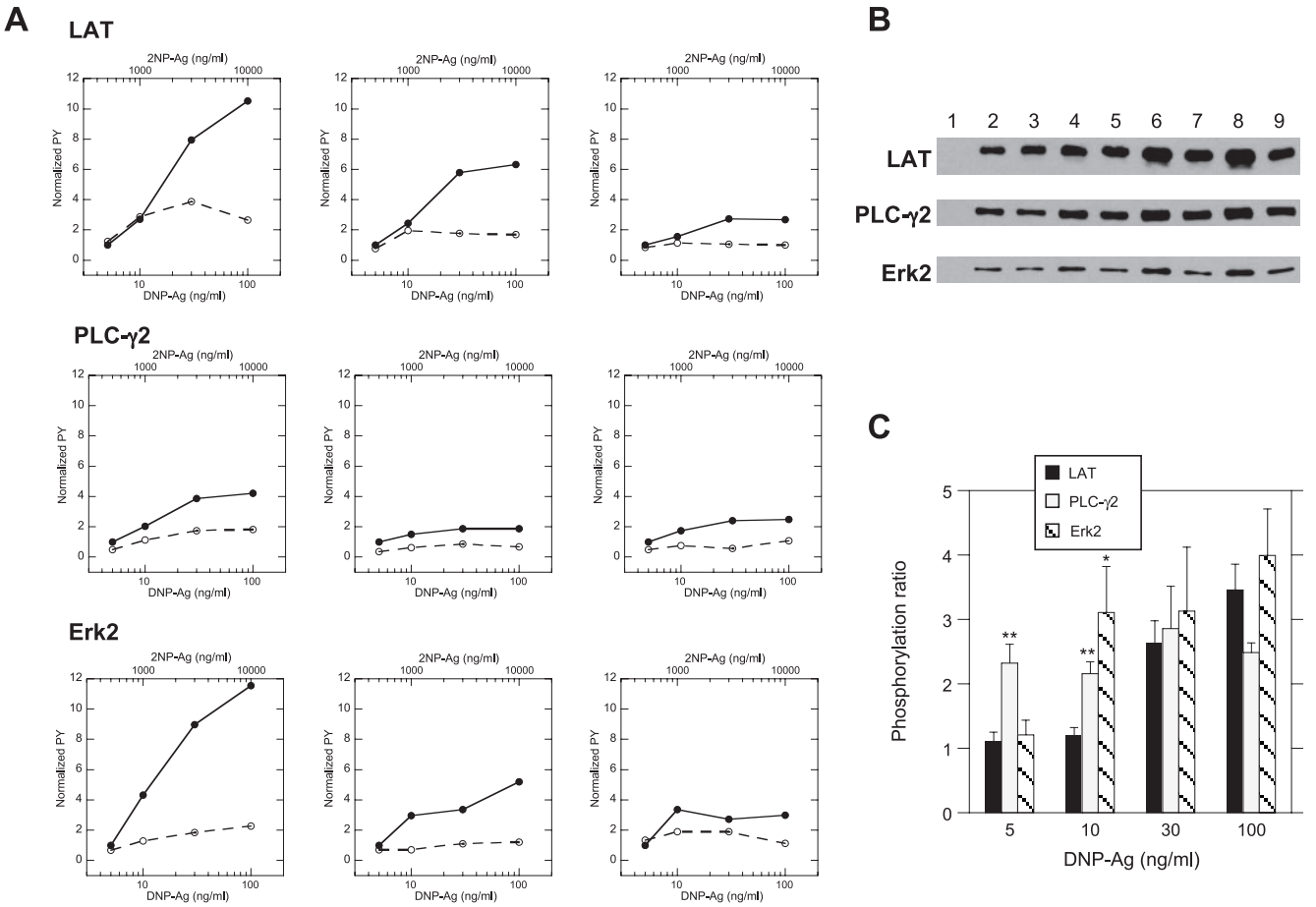

FIGURE 1. Relative levels of phosphorylation induced by DNP-Ag and a 100-fold higher concentration of 2NP-Ag for a range of ligand concentrations. *A*, Ligand dose-response curves for LAT, PLC-γ2, and Erk2 phosphorylation stimulated by DNP-Ag (solid lines, filled circles, concentration on lower *x*-axis) and a 100-fold higher concentration of 2NP-Ag (dashed lines, open circles, concentration on upper *x*-axis). Each column contains data from a single experiment. LAT and PLC-γ2 phosphorylation are detected at 3 min and Erk2 phosphorylation is detected at 5 min following stimulation. *B*, Representative Western blots used to generate the data plotted in *A*. *Lane 1*, control (resting), *lanes 2*, *4*, *6*, and *8*: DNP-Ag at 5, 10, 30, and 100 ng/ml, respectively; *lanes 3*, *5*, *7*, and *9*: 2NP-Ag at 500, 1000, 3000, and 10,000 ng/ml, respectively. *C*, Ratios of phosphorylation obtained for each dose combination tested. Values plotted are the average of the three experiments shown in *A* at each concentration (±SEM). The significance of the difference in phosphorylation at each dose combination was evaluated using a paired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

through a short-lived enzyme-substrate interaction that is too short to produce substantial coclustering of receptor and LAT but long enough to efficiently phosphorylate LAT. This is possible because the surface association of the Syk and LAT greatly enhances the rate of phosphorylation at the cell membrane (15, 16). Phosphorylated LAT clusters would then serve as messengers, allowing the escape from kinetic proofreading as described above. The model thus predicts that events downstream of LAT activation should not be subject to further kinetic proofreading of ligand receptor interactions provided that the duration of the catalytic interaction between Syk and LAT is much shorter than the lifetime of ligand receptor interactions, which is likely the case (17). This model is consistent with the observation that disaggregation of receptors upon the addition of a monovalent hapten results in rapid dephosphorylation of LAT (13) because activated Syk, which is required to sustain the phosphorylation of LAT, disappears rapidly following the breakup of receptor aggregates (18).

In this paper we report a set of experiments designed to test the hypothesis that activated LAT could function as a messenger linking activated receptor complexes and later cellular responses. Following Torigoe et al. (3) we measure the responses of RBL-2H3 cells to differing doses of slowly and rapidly dissociating ligands. We find that by setting the concentration of the rapidly dissociating ligand ~100-fold higher than that of the slowly dissociating ligand, we are able to elicit similar levels of LAT phosphorylation. If the LAT messenger hypothesis is correct, there should be no difference in responses downstream of LAT phosphorylation elicited by the two ligands. We find instead that nearly all of the measured responses — phosphorylation of the intracellular proteins PLC-γ2 and Erk2, changes intracellular Ca^{2+} levels, and degranulation — are substantially smaller for stimulation by the rapidly dissociating ligand, demonstrating that kinetic proofreading of ligand receptor interactions continues to have functional consequences downstream of LAT activation.

Materials and Methods

Reagents, Abs, and cells

Indo-1-acetoxymethyl ester was obtained from Invitrogen Life Technologies. Reagents for ECL were obtained from Pierce. Goat anti-mouse IgE was affinity purified by a mouse IgE-conjugated Sepharose column. Rabbit anti-Syk serum was a gift from Dr. U. Blank (Institut National de la Santé et de la Recherche Médicale, Paris, France). The IgG fraction of rabbit anti-LAT serum was purified by protein A-Sepharose (Amersham Biosciences) column. Rabbit anti-PLC-γ2 and rabbit anti-Erk2 Abs were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-phosphotyrosine Ab 4G10 was obtained from Upstate Biotechnology. Mouse monoclonal anti-DNP IgE (19) was affinity purified from ascitic

fluid by using a column of Sepharose conjugated with trinitrophenyl lysine. $(DNP\text{-}cap)_{4.6}$-Fab and $(2NP\text{-}cap)_{5.3}$-Fab (where 2NP is 2-nitrophenyl) were prepared as described previously (20). RBL-2H3 cells were maintained as described previously (21). The cDNA of N-terminal truncated LAT was a gift of Dr. L. Samelson (National Cancer Institute, Bethesda, MD). Rabbit anti-LAT sera were generated against truncated LAT-GST by Sigma-Aldrich.

Cell lysates and immunoprecipitation

RBL-2H3 cells were sensitized with anti-DNP IgE overnight, harvested with trypsin-EDTA, and washed with buffer A (150 mM NaCl, 5 mM KCl, 5.4 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM PIPES (pH 7.2) containing 0.1% BSA) and resuspended in buffer A at 6.2×10^6 cells/ml. Cells were activated with varying concentration of DNP- or 2NP-conjugated Fab as described above at 37°C. Reactions were stopped by mixing two volumes of cell suspension and one volume of ice-cold 3× solubilization buffer giving a final concentration of 0.1% Triton X-100, 50 mM Tris, 50 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM iodoacetate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml each aprotinin, leupeptin, and pepstatin A (pH 7.6), and incubated for 15 min at 4°C. After 10 min of centrifugation, target proteins were immunoprecipitated with appropriate Abs using protein A-Sepharose beads (Amersham Biosciences).

Phosphotyrosine assay

Immunoprecipitated proteins were extracted with hot SDS sample buffer and electrophoresed on polyacrylamide gels. Phosphotyrosine was detected by Western blotting using 4G10 under conditions that favored accurate quantification as described previously (22). Specifically, the linear range of detection was determined by scanning a blot from a gel loaded with increasing amounts of immunoprecipitated Syk. Appropriate exposures were chosen for scanning that fell within the linear range of Ab staining.

Measurement of intracellular calcium

Cells were loaded with indo-1-acetoxymethyl ester (1 μM) in buffer A containing 250 μM sulfinpyrazone for 15 min at 37°C. Cells were washed twice and resuspended at 2×10^6 cells/ml in the same buffer. The relative concentration of intracellular calcium ions was measured ratiometrically in a PTI QM-2000-2 spectrofluorometer (Photon Technology International) equipped with a magnetic stirrer and temperature control. The ratio of 400–490 nm fluorescence intensity was plotted (excitation 340 nm) as a relative measure of intracellular calcium ion concentration.

Hexosaminidase assay

The activity of hexosaminidase was measured as described (23), using *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide as a substrate.

Results

Phosphorylation of LAT, PLC-γ2, and Erk2

Phosphorylation of LAT tyrosines in RBL-2H3 cells is dependent upon the cross-linking of FcεRI and Syk tyrosine kinase activity (24). Previously, it was determined that a concentration increase of 10–100-fold was required to equalize the level of receptor phosphorylation induced by the rapidly dissociating (lower affinity) 2NP-conjugated Ags (2NP-Ag) in comparison with the more slowly dissociating (higher affinity) DNP-conjugated Ags (DNP-Ag) (3). A 375 ng/ml dose of 2NP-conjugated Ag was sufficient to give roughly double the level of receptor phosphorylation as a 50 ng/ml dose of DNP-conjugated Ag of approximately the same valence. Under these conditions, downstream responses were progressively attenuated for the 2NP-Ag in comparison to the DNP-Ag. Syk phosphorylation was attenuated ~4-fold.

Fig. 1 shows that it is possible to equalize the levels of LAT phosphorylation induced by DNP-Ag and 2NP-Ag over a limited range of Ag concentrations. We find that when the ratio of 2NP-Ag to DNP-Ag concentration is 100:1, the level of LAT phosphorylation is equalized when the DNP-Ag concentration is between 5 and 10 ng/ml (Fig. 1*C*). Above this range, the level of phosphorylation induced by 2NP-Ag reaches a maximum, whereas the level induced by DNP-Ag continues to rise. Therefore, we can only test

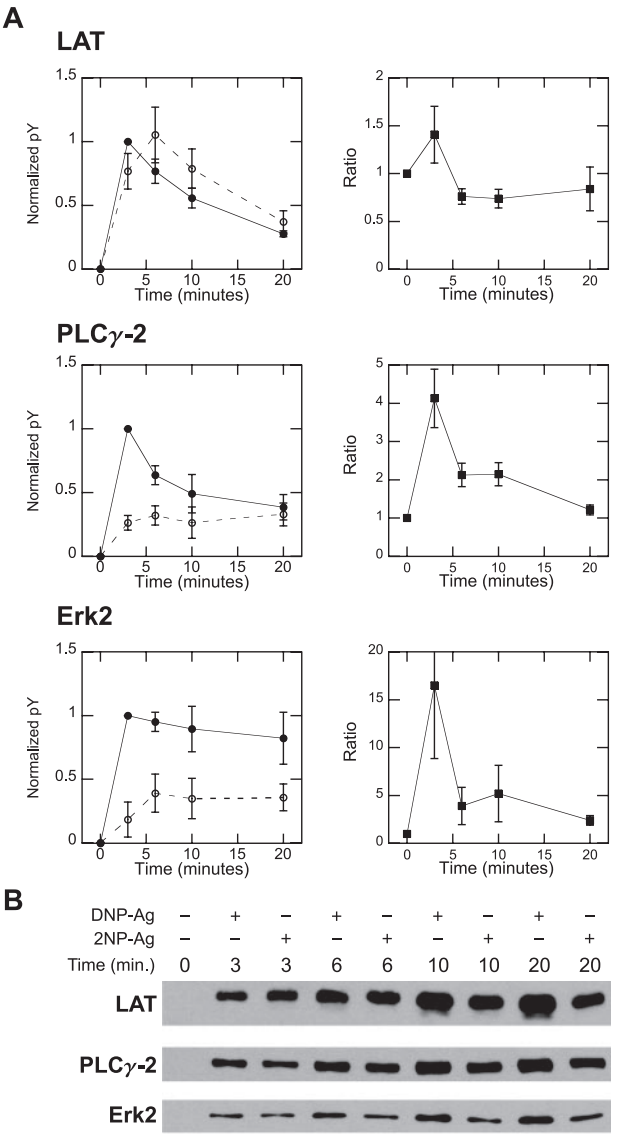

FIGURE 2. Kinetics of LAT, PLC-γ2, and Erk2 phosphorylation following stimulation of DNP-Ag (solid lines, filled circles) and 2NP-Ag (dashed lines, open circles) at 10 ng/ml and 1000 ng/ml concentrations, respectively. *A*, *Left column*, relative phosphorylation levels normalized at 3 min after DNP-Ag stimulation; *right column*, ratio of phosphorylation levels induced by DNP-Ag and 2NP-Ag. Average values of three separate experiments are shown (±SEM). Differences between the curves for PLC-γ2 and Erk2 were found to be significant using Student's *t* test for paired data at the 3-, 6-, and 10-min time points ($p < 0.05$ at each time point, tested separately). *B*, Representative Western blots used to obtain the data shown in *A*.

for kinetic proofreading downstream of LAT at the lower combinations shown in Fig. 1.

We chose to measure the phosphorylation of PLC-γ2 and Erk2 as indicators of IgE-mediated responses downstream of LAT (25). Although PLC-γ1 has been linked more directly with LAT than PLC-γ2, we found that the available Abs for PLC-γ1 were not sufficiently sensitive for the comparison of the responses induced by the two ligands we studied here. As shown in Fig. 1, PLC-γ2 phosphorylation is attenuated by a factor of ~2 at both the 5/500 and the 10/1000 ng/ml DNP-Ag/2NP-Ag dose combinations. Erk2 phosphorylation is attenuated by about a factor of three at the 10/1000 dose combination, but not at the 5/500 combination. This apparent lack of kinetic proofreading of Erk2 phosphorylation at

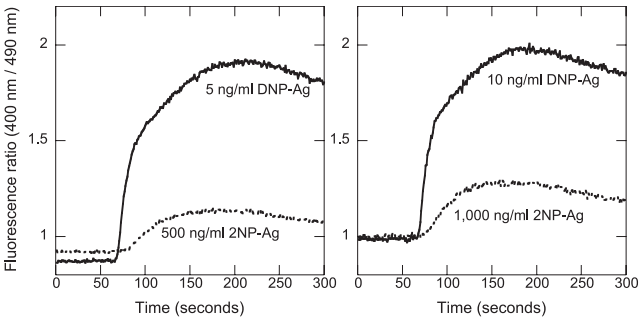

FIGURE 3. Intracellular Ca^{2+} mobilization by DNP-Ag and 2NP-Ag. Ligands were added to a suspension of RBL-2H3 cells (2×10^6 cells/ml) at $t = 60$ s and the response was monitored for 4 min at 37°C. Curves shown are representative of three separate experiments.

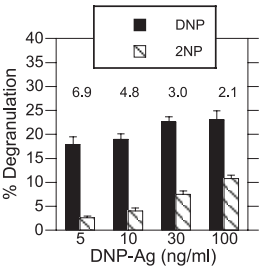

FIGURE 4. Hexosaminidase release induced by DNP-Ag and 2NP-Ag ligands. The *x*-axis concentrations are shown for DNP-Ag. Numbers above the bars are the ratios of the release stimulated by DNP-Ag to the release stimulated by a 100-fold higher concentration of 2NP-Ag. Adherent RBL-2H3 cells in 96-well plates were activated for 30 min at 37°C. Spontaneous release was subtracted from each data point. Data shown are the average of three experiments (±SEM). The observed differences are significant for all dose combinations ($p < 0.01$; Student's *t* test on paired data).

the 5/500 dose combination stands in contrast to subsequent experiments we performed that show strong proofreading of both intracellular calcium and degranulation responses (see next section) and may be the result of the small sample size. Two of the three experiments presented in the bottom row of Fig. 1*A* do show the attenuation of Erk2 phosphorylation at the 5/500 dose combination.

Comparisons done at a fixed time after stimulation are not sufficient to provide conclusive evidence of kinetic proofreading because of the possibility that the response to one ligand may lag the response to the other ligand. At early times, a simple lag in the response could be misinterpreted as evidence of kinetic proofreading. To address this possibility, we measured the phosphorylation kinetics of the three proteins using a 10/1000 dose combination as shown in Fig. 2. Over the 20 min following ligand addition the ratio of induced LAT phosphorylation remained fairly constant, whereas the levels of PLC-γ2 and Erk2 phosphorylation are significantly depressed. The levels of PLC-γ2 phosphorylation appear to converge at later times, whereas the levels of Erk2 phosphorylation remain ~3-fold lower for stimulation with 2NP-Ag.

Intracellular Ca^{2+} and degranulation

We also tested other cellular responses downstream of LAT phosphorylation for kinetic proofreading. As shown in Fig. 3, levels of intracellular calcium mobilization following the addition of an Ag are substantially lower for the 2NP-Ag in comparison with DNP-Ag at both dose combinations measured. In contrast to the results for Erk2 phosphorylation, we find strong evidence of kinetic proofreading of the intracellular calcium response at the lower dose combination (5/500 ng/ml DNP-Ag/2NP-Ag) as well as at the higher dose combination (10/1000 ng/ml DNP-Ag/2NP-Ag).

The degranulation response also exhibits a high degree of kinetic proofreading at both ligand dose combinations as shown in Fig. 4. At the 5/500 and 10/1000 ng/ml dose combinations, degranulation exhibits the highest degree of kinetic proofreading of all of the responses studied. Because the products of degranulation accumulate outside of the cell, the difference in the measured degranulation represents an integral of the difference over time and is thus a better indicator of the overall difference in the responses than the ratio at a single time point. For example, the phosphorylation kinetics of PLC-γ2 shown in Fig. 2 exhibit clear differences that would not be reflected by the phosphorylation ratio at the 20-min time point. It is noteworthy that even at the highest 2NP-Ag dose studied (10,000 ng/ml) the level of degranulation is lower than that for the lowest dose of DNP-Ag studied (5 ng/ml). This demonstrates that even very high doses of 2NP-Ag ligand cannot overcome the signaling defect arising from the short half-life of the ligand receptor bond.

Discussion

The observation that FcεRI and LAT form distinct and largely nonoverlapping clusters in the plasma membrane of mast cells (14) suggested to us that activated LAT might serve as a messenger that allows escape from kinetic proofreading. A model consistent with the available data is that a transient interaction between FcεRI complexes containing the activated kinase Syk could phosphorylate LAT, leading to the formation of distinct LAT clusters in which subsequent activation events would no longer be sensitive to the lifetime of ligand receptor bonds. According to this model, equalizing the levels of LAT activation for two ligands differing in the dwell time of their interaction with receptors would produce equal responses downstream of LAT activation. In the experiments we have reported here, we were able to equalize total levels of LAT phosphorylation at two dose combinations of DNP- and 2NP-conjugated Ags, but we found the lower-affinity 2NP-Ag elicited weaker downstream responses. All four responses we tested indicated continued sensitivity to the duration of the ligand receptor bond, i.e., kinetic proofreading, at ligand doses that equalized the level of LAT phosphorylation. The only response that did not clearly exhibit kinetic proofreading was Erk2 phosphorylation at the 5/500 ng/ml DNP-Ag/2NP-Ag dose combination, although a modest level of proofreading occurred in two of three experiments. Other responses to this dose combination exhibited strong kinetic proofreading, and it is possible that measurement of the kinetics at this combination would reveal proofreading at other time points, as was the case for PLC-γ2 phosphorylation (see Fig. 2) at the 10/1000 ng/ml dose combination.

These results are difficult to reconcile with the idea that phosphorylated LAT plays the role of a messenger coupling activation of the receptor complex to downstream activation events at physically separate locations. The observation of continued kinetic proofreading of ligand receptor interactions suggests the alternative view that an activated receptor and LAT remain colocalized within a complex for a time comparable to the duration of the ligand receptor bond. It is possible that the additional proofreading occurs subsequent to LAT phosphorylation but before the formation of stable LAT clusters, but this possibility also requires a relatively long-lived FcεRI-LAT complex. Although there is no evidence for a direct interaction between FcεRI and LAT, fluorescence microscopy indicates that the TCR and LAT colocalize

transiently following receptor stimulation (26). It is also possible that protein-lipid interactions mediate the interaction between FcεRI and LAT. It is known, for example, that LAT is found predominantly in lipid raft portions of the plasma membrane, whereas upon aggregation FcεRI receptors shift from the non-raft domains to raft domains (27). Another possibility is that a series of weak protein-protein interactions mediate formation of transient LAT-FcεRI complexes, analogous to the role SLP-76 plays in stabilizing the recruitment of PLC-γ to LAT (28).

Several factors complicate the interpretation of the experiments we have performed here and require further investigation. First, there is the possibility that the two ligands induce different patterns of LAT phosphorylation, leading to differential activation of downstream events. For example, it has been observed previously that DNP- and 2NP-conjugated Ags induce different relative phosphorylation levels of the γ_2 and β subunits of FcεRI even when the total levels of receptor phosphorylation are comparable (20). LAT contains multiple tyrosines that become phosphorylated upon Ag stimulation, and these are coupled differentially to downstream effectors such as PLC-γ, Sos, and PI-3K (29). Of these tyrosines, four are essential for LAT-dependent signaling mediated by FcεRI (30). Recent evidence also indicates that phosphorylation of LAT tyrosines may play a negative role in mast cell activation (31) that could further amplify the effects of differences in phosphorylation patterns. These observations suggest the need for future experiments using site-specific anti-phosphotyrosine Abs to resolve any differences that may be occurring in the pattern of LAT phosphorylation.

A second confounder is the possibility that the two ligands differentially activate signaling pathways that run in parallel to FcεRI-Syk-LAT activation (32). Our results showing that LAT phosphorylation levels off at >1,000 ng/ml 2NP-Ag (Fig. 1*A*) while both intracellular calcium (data not shown) and degranulation (Fig. 4) continue to increase demonstrate that LAT phosphorylation is not the sole determinant of downstream response levels. One candidate for an alternative pathway is Fyn-mediated activation of Gab2 (33), which appears to play a major role in the degranulation response. A second candidate is activation of the non-T cell activation linker (NTAL), which is also likely to make a significant contribution to all of the downstream responses examined here (25). Recent experiments using inhibitors of actin polymerization also suggest that DNP- and 2NP-conjugated Ags induce different levels of coupling between FcεRI and the cytoskeleton (20), which could lead to differential activation of a variety of responses, receptor internalization and degradation being the most obvious.

In conclusion, the experiments described here represent an initial attempt to shed light on the molecular interactions that function during a signaling response by using the lifetime of ligand receptor interactions as a probe. The fact that downstream responses remain sensitive to this lifetime even when the level of LAT phosphorylation is equalized suggests that either FcεRI and LAT colocalize for a substantial period of time (on the order of seconds) or that the kinetic proofreading of early events leads to differential coupling of the initial complex to downstream pathways, possibly through the induction of different phosphorylation patterns or differential coupling to the cytoskeleton. Distinguishing between these two possibilities will require additional experiments, both of the biochemical type reported here and those involving in vivo imaging techniques such as FRET. It is also clear that to understand how cell signaling systems convert a difference in ligand receptor lifetime into a complex pattern of differential responses will require the development of detailed mathematical models. We are in the process of extending our current model of the FcεRI-Lyn-Syk pathway (2) to include the large number of adaptor and effector molecules that couple to the ligand receptor complex. As we have discussed elsewhere (34), the multivalent nature of these interactions produces a combinatorial complexity that poses a major barrier to quantitative modeling. With the recent development of a number of computational tools to address these challenges (35), we are optimistic that new insights will be forthcoming in the near future.

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Disclosures

The authors have no financial conflict of interest.

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