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Review Article

Signal transduction by FcεRI: Analysis of the early molecular events

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Abstract

We are analysing the initial molecular events stimulated by the high-affinity receptor for IgE, FcεRI. Earlier studies have shown that the first response when the receptor-bound IgE interacts with a multivalent antigen is a transphosphorylation of receptor tyrosines, induced by the approximation of two or more receptors by a constitutively associated src-family kinase (Lyn). The amount of weakly associated kinase regulates the intensity of the response. Several aspects are being analyzed: (i) the sites on Lyn and the receptor that account for the constitutive interaction; (ii) how the intrinsic affinity of a ligand for the receptor-bound IgE influences the responses; and (iii) the mechanism(s) by which low-affinity ligands can act as antagonists. In the latter studies, mast cell responses were followed by monitoring the phosphorylation of tyrosines on several proteins and secretion. At equivalent levels of receptor phosphorylation, a ligand with high affinity stimulated vigorous phosphorylation of downstream components, whereas a low-affinity ligand was unable to stimulate phosphorylation of the same components effectively. Cells stimulated with a mixture of high- and low-affinity ligands, under a protocol where simple displacement of one by the other was prevented, remarkably showed that excess low-affinity ligand inhibited the phosphorylation as well as degranulation by the high-affinity ligand. This antagonism results from a competition for the limiting amount of the constitutive initiating kinase. Related receptors that depend on recruitment of initiating kinases may be subject to similar regulatory mechanisms.

Key words: IgE receptor, mast cells, membrane receptors, tyrosine phosphorylation.

Overview

The IgE/mast cell system is the critical component by which the abnormal production of IgE in susceptible individuals elicits the allergic response. Therefore, a fuller understanding of this could potentially lead to new therapeutic approaches to this, the most prevalent of immunologic diseases. In order to have an experimental system that can be explored biochemically, it is critical to select one in which adequate amounts of materials are available. That is why many researchers have used a tumor line of mast cells for studies. Subsequent analyses, by ourselves and others, have confirmed that, with respect to certain generic aspects of the IgE/mast cell system we are exploring, the findings from the tumor cells selected for our work (RBL)[1,2] are readily applicable to normal human mast cells and basophils. However, it is clear that, for particular pathways, the details may vary according to both the species and developmental stage of the cells being investigated.

Many fundamental facts have been established about the interaction between IgE and mast cells and the mechanism by which antigen–IgE complexes stimulate the cells. The receptor has been molecularly defined with respect to the type, number and amino acid sequence of the subunits. Very recently, the first three-dimensional

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picture of the region of the receptor that binds IgE has been published.[3] Hopefully, the region on IgE that binds to the receptor will also be available at a sufficiently high resolution before too long. Such information could then be used to design specific inhibitors of the binding reaction. Some initial favorable clinical results with an anti-IgE antibody that inhibits binding of IgE without itself triggering anaphylactic or apparently other mast cell responses[4] suggest, therefore, that inhibiting the binding of IgE may be a practical way of obviating allergic responses.

The early stages of the mast cell response are other potentially useful targets for interfering with the allergic response and it is these events on which we have focused. In this short review, we summarize our current level of understanding of what is going on.

There is no evidence that the FcεRI receptor itself codes for an enzymatic activity, but it is now known that the receptor is associated with a tyrosine kinase and that phosphorylation of the receptor, on regions referred to as immunoreceptor tyrosine activation motifs (ITAM),[5] and of other targets is among the earliest consequences of activation of the receptor.

It is known that one molecule of IgE binds to one molecule of receptor and vice versa and that the IgE must be aggregated to initiate a response. The importance of aggregating the IgE has been clarified by showing that it is in fact the aggregation of the receptor to which the IgE is bound that is essential. Indeed, the smallest possible aggregates, dimers of receptors, have been shown to generate 'unit signals'.[6] Data from several laboratories allow the proposal of a specific molecular model for how the aggregation generates such signals. Such a model is illustrated in Fig. 1.[7,8] The evidence indicates that a small fraction of receptors are constitutively associated with weakly bound Lyn kinase. This kinase is unable to phosphorylate the receptor with which it is associated. However, when receptors are aggregated, if the receptors in the cluster are constitutively associated with Lyn kinase the latter can transphosphorylate the cytoplasmic domains of one or more receptors (and associated proteins) in the aggregate. This phosphorylation leads to the recruitment of additional (more tightly bound) Lyn kinase and other cellular proteins, which then propagate the biochemical cascade. The system is highly dynamic. That is, even stably clustered receptors appear to be actively phosphorylated and dephosphorylated. In the sections that follow, we describe in greater detail what information we have about these early events.

Interaction of kinase with receptor

Several groups have studied the interaction between FcεRI and Lyn kinase, using a variety of techniques. All of these studies have revealed a direct interaction between the kinase and the C-terminal cytoplasmic extension of the receptor's β-chain. No interaction has been detected between Lyn and the N-terminus of the β-chain (β_N) nor with the cytoplasmic domain of the γ-subunits (γ_C). In our recent studies, we have used Chinese Hamster Ovary (CHO) cells, which have small amounts of endogenous Lyn kinase and had been stably transfected with FcεRI. Providing the stimulus was sufficiently strong, we observed aggregation-induced phosphorylation of the transfected receptors. These cells were then retransfected with a variety of Lyn kinase constructs: (i) a catalytically inactive mutant; (ii) a construct consisting of the unique region of Lyn alone; and (iii) the wild-type kinase. Each of these types of transfectants yielded informative results.[9]

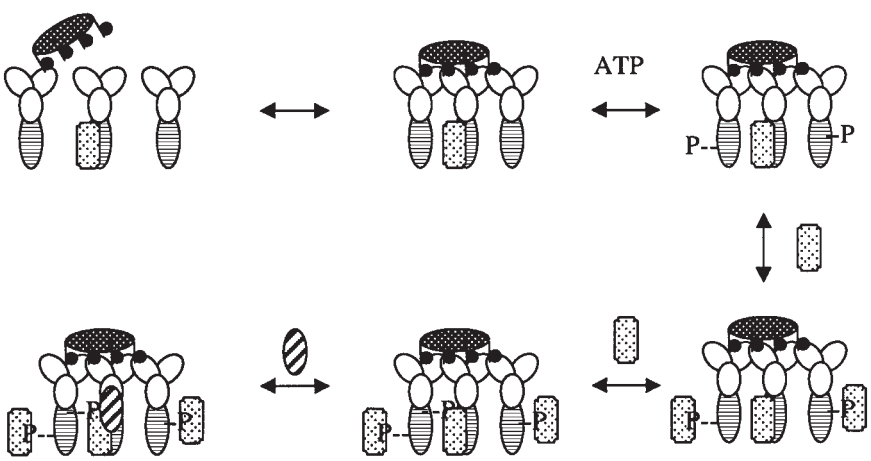

Fig. 1 Model for initial signal transduction events. Monomeric receptors (▤) become aggregated when the receptor-bound antigen-specific IgE binds with a multivalent antigen. Lyn kinase (▨), constitutively bound to a small fraction of the receptors, is then able to phosphorylate tyrosines on the newly approximated receptors. The phosphorylated receptors recruit additional lyn kinase, leading to further phosphorylation and recruitment of the next downstream component, syk kinase (▨).

When compared to the receptors in stimulated control cells, phosphorylation of the receptors in cells transfected with the catalytically inactive Lyn was markedly inhibited. This result indicates that the interaction between Lyn and the receptor is not dependent on an intact catalytic site on the kinase. It also suggests that the src-homology domain 2 (SH2) region of Lyn does not play a major role in regulating the level of phosphorylation of the receptor and may not necessarily be the basis of the recruitment to the phosphorylated receptors. In any event, the catalytically inactive Lyn was as effective as the unique domain alone in inhibiting the interaction between the receptor and the wild-type endogenous src-family kinase in the CHO cells. It appears likely that the principal interaction that the competition experiments are assessing is the constitutive interaction between Lyn and the receptor. Alternative constructs of Lyn could be used to probe further the nature of this interaction. However, such additional studies could only provide rather indirect evidence about which structures in Lyn are important. Rather than pursuing such intermediate results, what the field really needs is structural information at the atomic level of resolution. More detailed analyses must also control for the possibility that these interactions may be occurring in the context of specialized membrane domains (see later).

Transfection with the wild-type Lyn enhances the sensitivity of the cells to an aggregating stimulus.[9] This is particularly notable with respect to stimulation with dimeric IgE. Thus, whereas the cells containing only the small amount of endogenous Lyn are unresponsive to dimers, those transfected with wild-type Lyn become responsive to such a stimulus. The responsiveness of such transfectants relative to the amount of Lyn they expressed was determined experimentally and the results have now been analysed quantitatively The analysis reveals that a single molecule of kinase per cluster is sufficient to initiate the response. This is entirely consistent with the molecular model.

The interpretation that it is the constitutive interaction that is affected is also consistent with findings reported by Wilson *et al*.[10] They have observed that a chimeric construct bearing the βc domain fails to become phosphorylated when transfected into RBL cells, but inhibits both baseline and aggregation-induced phosphorylation of the endogenous FcεRI. This result likely reflects competition by the transfected β-chain for limiting amounts of constitutively associated kinase. Thus, this experiment is, in effect, a mirror image to those we have described earlier.

Field *et al*. have provided persuasive evidence that phosphorylation of the clustered receptors occurs in specialized microdomains, so-called detergent-insoluble/glycolipid-enriched domains (DIG).[11,12] They have proposed that aggregation of the receptor promotes the association of the receptors with the DIG and that, because of the preferential partitioning of Lyn to those domains, phosphorylation of the receptors by Lyn ensues. Thus, in their proposal, it is the aggregation-induced coalescence of the special lipids surrounding the receptors with the lipid rafts enriched in Lyn kinase that initiates the response, rather than the transphosphorylation mechanism. Although we think that taken at its extreme the lipid domain coalescence model fails to explain a variety of experimental findings, it is relatively easy to think of how the two models could be complementary. We would propose that there is a specific constitutive interaction between Lyn and the unaggregated receptors and that Lyn can only phosphorylate immediately neighboring receptors, but not that with which it is associated. However, the contemporaneous coalescence of specialized lipids around the kinase and clustered receptor could be an accompanying or, possibly, even a contributing event.

Sites of tyrosine phosphorylation

The receptor with high affinity for IgE, FcεRI, belongs to a family of multichain immuno-recognition receptors (MIRR).[13] Like FcεRI, the cytoplasmic extensions of one or more of the subunits of each of these receptors contains one or more ITAM (see earlier), which contain a tyrosine residue in two canonical Tyr-X-X-Leu/Ile sequences[14] and which are substrates for src-family tyrosine kinases.

The molecular details of the early events triggered by FcεRI and by related receptors have been explored using genetically engineered chimeric proteins and mutational analysis. The findings have varied somewhat, depending on the system studied, and compared with what is observed with the intact receptor. Despite the power of protein engineering, the evidence that is derived from those approaches is largely circumstantial, particularly for mutants showing a loss of function. For example, substituting tyrosine for an alanine or phenylalanine not only substitutes a residue that can be phosphorylated for one that cannot, but also substitutes one that has very different hydropathicity and hydrogen-bonding properties. Significant conformational changes in the protein produced by such a substitution may contribute to, or even

account for, the functional alterations observed. In addition, genetic approaches can only indirectly give clues about the quantitative aspects of any modifications. In order to complement the mutational studies, we have examined the phosphorylation of the different subunits in the native FcεRI directly;[15] a kind of analysis that has not been previously reported for this or any of the other MIRR.

Phosphorylation of the β-subunit

Both canonical tyrosines present in the ITAM of the β-subunit were phosphorylated, although not to the same degree. The several-fold greater phosphorylation of Tyr228 than of Tyr214 was observed both *in vivo* and *in vitro* and to a similar degree. *In vivo*, the level of phosphorylation is the result of a dynamic process of phosphorylation and dephosphorylation. It may be supposed that *in vivo* the different levels of phosphorylation result from a different susceptibility of the two tyrosines to a phosphatase. However, this would not explain why a similar differential was observed *in vitro* in the presence of the inhibitor vanadate, where all tyrosine phosphatase activity is eliminated. Alternatively, the differential may reflect a different accessibility of the two tyrosines to the receptor-bound kinase(s). If so, then the difference must be more or less absolute for some of the aggregated receptors, because the phosphorylation *in vitro* reached a plateau value at similar rates for both tyrosines. We have no additional data to support other explanations.

Recently, various downstream signalling molecules were shown to interact with a diphosphorylated β ITAM peptide.[16] It is possible that alternative phosphorylations of the β ITAM tyrosines lead to differential interactions with such distal components (see, for example, Ortega *et al*.[17]). The results on the β-subunit resemble those found with the Ig-α chain of the B cell antigen receptor, where only the canonical tyrosines in the ITAM were phosphorylated and to different extents.[18] The level of phosphorylation in Ig-α was minimally affected when one of the canonical tyrosines was mutated to phenylalanine.

Our data have shown that the non-canonical Tyr224 (which is conserved in the human, mouse and rat β ITAM) can also become phosphorylated. This tyrosine is at position – 4 from the C-terminal canonical tyrosine. Among the members of the MIRR family, only murine CD3-γ has a non-canonical tyrosine within the ITAM, at position – 3 from the C-terminal canonical tyrosine. In neither instance is the tyrosine surrounded by a sequence that is typically found in substrates for src-family kinases.

Phosphorylation of the γ-subunit

The phosphorylation of the two canonical tyrosines in the ITAM of the γ-subunit was more equivalent than in the case of the β-subunit. *In vivo*, the relative phosphorylation of Tyr47 (N-terminal peptide) was always larger than Tyr58 (C-terminal peptide) and the opposite was true *in vitro*, but in both cases the ratio was reasonably close to one. The extent of phosphorylation of the γ tyrosines, relative to β Tyr218, was two- to three-fold larger *in vivo* than *in vitro*. If one takes into account that about 90% of the radioactivity in the N-terminal γ peptide *in vivo* is phosphotyrosine, the average of the ratios *in vivo* and *in vitro* of phosphorylation of the two ITAM tyrosines in γ is roughly one.

Several reports have suggested that it is the phosphorylated γ-subunit that interacts with Syk kinase. Thus Syk, as well as its isolated SH2 domains, preferentially binds to the isolated γ- rather than the β-subunit.[19] It has also been shown that peptides containing the γ ITAM bind to and activate Syk kinase.[19] Syk and the related ZAP-70 tyrosine kinases contain two consecutive SH2 domains, whose integrity is required for binding to ITAM and activation.[20] Furthermore, Syk and the related Zap-70 used by other MIRR family members interact only with bisphosphorylated ITAM. Our observations that the phosphorylation of the two tyrosines in the ITAM of γ are approximately equivalent is consistent with the mechanistic model these data imply.

Dephosphorylation of the receptor

Although the Lyn kinase associated with the IgE receptor from resting cells displays tyrosine kinase activity, little or no phosphorylation of the receptor itself is observed unless the receptors are aggregated.[21] These observations and those made on the effect of inhibitors of phosphatases imply that protein tyrosine phosphatase(s) (PTP) are continuously modulating the resting system. The studies with inhibitors and other experimental approaches[22] have shown that the PTP also continuously act on aggregated receptors. Finally, when individual receptors dissociate from the aggregate, for example, by addition of monomeric hapten after stimulation by multivalent antigen, rapid dephosphorylation of the receptor and of other cellular proteins is observed.

We recently investigated some of the characteristics of the PTP responsible for these phenomena. We first developed an *in vitro* assay to test the PTP activity in total cell lysate towards receptors that had been phosphorylated in response to

aggregation.[23] This assay has also been used to localize candidate PTP to subcellular fractions and to compare the susceptibility of aggregated and disaggregated receptors. Finally, we have examined the kinetics of dephosphorylation for FcεRI and other cellular proteins *in vivo,* in order to gain insights about the underlying regulation.

In our assays, the isolated receptors had 'no' phosphatase associated with them. Swieter *et al.* have reported such an association,[24] but our results are not necessarily in conflict. First, Swieter *et al.* isolated their receptors by procedures designed to minimize the dissociation of weakly interacting phosphatase and indeed found that the activity they measured was relatively easily dissociable. In contrast, we deliberately used procedures that would tend to dissociate weakly interacting phosphatases, in part because it is difficult to distinguish between contaminants, spurious associations and physiologic ones. In addition, we deliberately wanted to avoid assaying 'trace' amounts of activity. Our assays were conducted at a lower temperature (30°C compared with 37°C) and generally for much shorter times than those employed by Swieter *et al*. Indeed, the activity we are measuring appears in some instances 100-fold greater than can be estimated from their paper (see also later).

The studies using cell fractions have suggested that there are cytosolic phosphatases that in principle may participate in the dephosphorylation of receptors. However, much of the activity that we have observed is localized in the membrane fraction and permeabilized cells, which have lost many of their cytosolic proteins and are as efficient in dephosphorylating receptors as intact cells. Therefore, it appears that membrane-bound phosphatases are importantly involved. This finding is, of course, consistent with a receptor-associated phosphatase, such as described by Swieter *et al*.[24] *In vivo*, a membrane-bound phosphatase could be vastly more effective than *in vitro* assays may reveal, because of proximity and other effects.

That the relevant phosphatase is likely to be membrane bound raises the question of whether it may be CD45. Although CD45 has been found to be necessary for activating a T lymphocyte cell line through the FcεRI with which it had been transfected,[25] the evidence that CD45 is required for the initial IgE-mediated activation of mast cells is contradictory.[26–28] There is also another consideration. We have already referred to the experiments of Field *et al.*, which suggest an important, possibly critical, role for specialized membrane domains.[11,12] One could propose a model in which such domains are deficient in phosphatases. This paucity, coupled with an enrichment in kinase(s), could promote the phosphorylation of the aggregated receptors that become associated with these regions. Indeed, CD45 is thought to be excluded from these specialized domains[29] and there are experimental data that suggest that this exclusion may be involved in regulating the activation of the kinase Lck in T lymphocytes.[30]

We have tested the possibility that in RBL cells, the specialized membrane regions may be deficient in PTP necessary to dephosphorylate FcεRI, but found no evidence for such a deficiency.[23] We aggregated receptors under conditions shown to promote the association of the receptors with such domains and then blocked continued kinase action with ethylenediaminetetraacetic acid (EDTA). There was, nevertheless, prompt dephosphorylation of the receptors. These observations parallel our previous findings on the dynamic phosphorylation/dephosphorylation to which smaller aggregates of the receptor are subject.[22]

We have also examined this latter aspect quantitatively *in vitro,* by comparing the susceptibility of aggregated versus disaggregated receptors to dephosphorylation by PTP. The results have shown that aggregation did not protect the phosphorylated tyrosines from hydrolysis. These results lead us to a simple kinetic scheme consistent with our proposed molecular model (Fig. 2).[23]

Earlier data has shown that k_{-22} is substantial and effectively overwhelms k_{22}. The new data has indicated that k_{-12} approximately equals k_{-22}. Possibly, by promoting interactions with the cytoskeleton, phosphorylation stabilizes the aggregated state of receptors, thereby enhancing the ratio k_f/k_r by decreasing k_r. It follows that the concentration of the aggregated phosphorylated species, which appears to be the critical component that initiates the cascade of events, will be independently determined by the ratio k_f/k_r for both the unphosphorylated and the phosphorylated receptors and by k_{12}/k_{-12}. Thus, the cell maintains a constant brake on this system through the constitutive action of phosphatases and aggregation moves the system, principally by enhancing the effectiveness of kinases.

Quantitative analysis

Competition for limited kinase

Under certain conditions of stimulation, phosphorylation of the receptors reaches a plateau well before aggregation

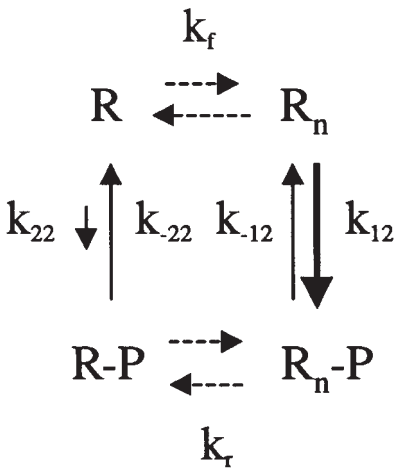

Fig. 2 Kinetic scheme for phosphorylation of receptors after aggregation. R, FcεRI with bound monomeric IgE. The binding of multivalent antigen to the IgE leads to the formation of aggregated receptors, R_n, with a forward rate constant of k_f and a dissociation rate constant of k_r. The size of the arrows depicting the phosphorylation and dephosphorylation of the aggregated and monomeric receptors reflects the experimental finding that phosphorylation is governed by the fact that $k_{12} > k_{22}$ and not because k_{-12} differs from k_{-22}. P, phosphotyrosine.

of additional receptors has ceased.[31] We have eliminated several obvious explanations, such as that phosphatase is activated (earlier), and have hypothesized that aggregated receptors are competing for limited amounts of the initiating src-family kinase.

To test this hypothesis, a proportion of receptors was stably aggregated by dimers of rat IgE (weak stimulus) prior to the aggregation by antigen of other receptors on the same cells bearing anti-dinitrophenyl (anti-DNP) mouse IgE (strong stimulus). In some instances, the latter aggregates were dissociated a few minutes later by adding monovalent hapten. The receptors were individually analysed by immunoprecipitating them with species-specific anti-IgE.[32]

We observed that receptors aggregated by a strong stimulus became phosphorylated at the same time as those receptors that were stably aggregated by dimeric IgE were becoming dephosphorylated. Therefore, initially, the dimers become phosphorylated by virtue of their constitutively associated Lyn kinase. By the addition of antigen, a competing reaction was promoted and when these new aggregates were in excess, they monopolized the limited Lyn to which the FcεRI have access.

The addition of hapten disaggregated the competing species and their rapid dephosphorylation restored the pool of Lyn to its original size, so that the stable dimers regained access to Lyn and were rephosphorylated. The rephosphorylation of the pre-existing oligomers is consistent with our previous results that demonstrated that at least modest-sized aggregates of FcεRI are subject to repeated cycles of phosphorylation, dephosphorylation and rephosphorylation over considerable periods of time.[22]

Effects of affinity

Ligands binding to cell-surface receptors typically stimulate elaborate biochemical cascades. If the initiating interaction(s) must be preserved during the subsequent steps, then ligands with lower affinity, which generally means those forming complexes with shorter lifetimes, will be less likely to stimulate responses that go to completion. This process has been dubbed 'kinetic proofreading'[33] and has been creatively applied to signalling by the antigen receptors of T cells and possibly to account for the action of variant peptides that can act as partial agonists or antagonists.[34]

We have examined the kinetic proofreading formulation in the context of FcεRI and explored whether ligands of differing affinity can act as mutual antagonists under conditions where simple displacement cannot occur.[35] We loaded cells with IgE and monitored responses to high- or low-affinity ligands. On addition of the high-affinity DNP-antigen, tyrosines on both the β- and γ-subunits of FcεRI were phosphorylated with comparable kinetics. The phosphorylation of the kinase Syk and the adaptor protein Nck reached a maximum shortly afterwards and the phosphorylation of the extracellular signal-regulated (ERK)2 kinase even later. 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.

The anti-DNP IgE binds the 2-nitrophenyl (2NP) moiety with a relative intrinsic affinity less than 0.001 of that for DNP. 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 using somewhat higher doses. The striking finding was that, per unit phosphorylation of the receptor, the low-affinity ligand was progressively less effective in activating 'downstream' components. Thus, even when the phosphorylation of the receptor stimulated by 2NP was more than two-fold higher, the maximum phosphorylation of Syk was less than one-third of that achieved by the high-affinity ligand and for ERK2, only one-tenth as much. The

weakly bound 2NP ligand also stimulated release of hexosaminidase very poorly. These findings are consistent with a kinetic proofreading regimen.

Variant peptides can antagonize the stimulation of T lymphocytes induced by wild-type peptides under non-displacing conditions. We have examined whether a similar effect is observed in responses mediated by FcεRI.[35] Cells were loaded with a mixture of two monoclonal mouse IgE, one having a high affinity for DNP and the other having a high affinity for the non-cross-reacting dansyl moiety (DNS). The cells were then exposed either to the DNS-protein conjugate, to the low-affinity 2NP conjugate or to both simultaneously and immunoprecipitates of several proteins were assayed for phosphotyrosine.

In response to the dose of the DNS conjugate used, the phosphorylation of FcεRI was modest but 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.[36] When the cells were stimulated with only the low-affinity 2NP ligand, the receptors were phosphorylated but phosphorylation of the downstream components was progressively diminished. If the cells were stimulated with the mixture of non-cross-reacting ligands, the phosphotyrosine in the total receptor subunits was undiminished, phosphorylation of Syk and Pyk2 was minimally reduced, but phosphorylation of ERK2 was substantially decreased. Secretion of hexosaminidase was also measured. Increasing doses of the low-affinity ligand progressively inhibited release. We eliminated the possibility that the 2NP was simply 'toxic' and showed that in order to manifest its inhibitory capacity, the low-affinity ligand had to be bound to the same cells as the high-affinity ligand.

Further experiments have shown that the weakly clustered receptors act like the 'dog in the manger': despite their inability to use Lyn productively, they impede the access of the more stable clusters to the kinase. In principle, the molecular mechanism we have uncovered 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.

Closing remarks

Considerable headway is being made in defining, in molecular terms, the early events associated with the binding of IgE to the high-affinity receptor and the consequences of multivalent antigen binding to the cell-bound IgE. The molecular events are highly analogous to the initiation of cellular responses by many other cell-surface receptors that use clustering of the receptors as a fundamental mechanism. However, much remains to be uncovered. At the structural level we are only at the beginning, both with respect to the receptor and to downstream components. Although for the latter we have by now quite a few X-ray structures, there is increasing evidence that signalling is not occurring, either free in solution or even simply through sequential interactions between isolated components. Rather, there is mounting evidence that signalling occurs in the context of multicomponent macromolecular complexes. The fact that, in the case of signalling pathways, these 'protein machines' may be metastable structures evolving over time, vastly complicates their analysis.

We are only beginning to discern the true complexity of the systems we are exploring. Consider that a dimer of FcεRI, which contains 12 canonical and two non-canonical tyrosines in the ITAM that may or may not be phosphorylated, has potentially 2^{14} (> 17 000) unique states and Syk kinase, with six phosphorylatable tyrosines,[37] has 64 potential states. Determining which of these are truly functionally significant is a major undertaking. Although computers can provide a suitable substitute for our obviously inadequate personal memory banks to store the details of the elaborate pathways, they will have to be provided with new software so that the massive amount of new data can be usefully integrated. One way is illustrated by the work of Bhalla and Iyengar, who have shown how data on individual pathways that are known to be linked can be analysed.[38] They have come up with some striking examples of how such networked pathways may lead to a variety of 'emergent' properties; that is, properties that were not predictable from behavior of the individual pathways. This is the exciting prospect before us as we move into the brave new 'era of pathway quantitation'.[39]

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