Intracluster Restriction of Fc Receptor γ-Chain Tyrosine Phosphorylation Subverted by a Protein-tyrosine Phosphatase Inhibitor*

(Received for publication, December 26, 1995)

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This study shows that aggregation of U937 cell high affinity IgG Fc receptor (FcγRI) results in the transient tyrosine phosphorylation of FcγRI γ-chain but not the phosphorylation of γ-chains associated with nonaggregated IgA Fc receptors (FcαR) on the same cells. Thus, normally, tyrosine phosphorylation of γ-chains is limited to FcR in aggregates. In contrast, aggregation of FcγRI in the presence of vanadate induced the sustained tyrosine phosphorylation of FcγRI γ-chains and the rapid and extensive phosphorylation of nonaggregated FcαR γ-chains and low affinity IgG Fc receptors (FcαRII). This global phosphorylation of motifs on nonaggregated FcR was also detected upon aggregation of FcαR or FcγRII, which induced the phosphorylation of nonaggregated FcγRII γ-chains. Vanadate prevented dephosphorylation of proteins and increased kinase activity in stimulated cells. Evidence failed to support alternative explanations such as acquisition of phospho-tyrosine in stimulated cells. Evidence failed to support altered phosphorylation of proteins and increased kinase activity (15–17). Limitation to intracluster units is also evidenced by sustained binding of Lyn to FcγRII in isolated aggregates and phosphorylation of aggregate subunits in preference to an exogenously supplied substrate (14, 18). Both kinds of evidence suggest a spatial restriction of kinase activity to aggregates. It has been suggested that restriction is due to a requirement for aggregated receptors as sites for kinase activation (14, 19) and to a requirement that substrate be in the aggregated state (18). Another possibility is that kinase activity is under a positive control preventing activity in nonaggregated receptors. If this is the case, inhibition of the control might allow tyrosine phosphorylation of nonaggregated as well as aggregated FcR.

Evidence presented from an earlier report (20) and the present report is consistent with the second model. We used an assay system that allowed us to examine the effect on one FcR type of cross-linking another FcR type on the same cell. Western blots of precipitated FcR showed that normally there is no detectable kinase activity for nonaggregated FcR. However, in the presence of a phosphatase inhibitor, aggregation of one FcR type induced rapid and extensive phosphorylation of tyrosine motifs on noncross-linked FcR. The data in this report implicate phosphatases as necessary to prevent global FcR involvement and suggest that normal intracluster restriction of γ-chain phosphorylation may be due to this vanadate-sensitive mechanism.

MATERIALS AND METHODS

Cells, Antibodies, and Precipitants—U937 cells, subclone 10.6 (also called A12.13), were cultured in RPMI containing 10% fetal bovine serum and interferon-γ, as described previously (21), to increase expression of FcγRII (22), FcαR (6), and cell functions (6, 21). Antibodies and FcR ligands used for experiments included anti-FcγRI mAb 197 (Medarex, Annendale, NJ.), mAb HB63 (mIgG2a isotype control and high affinity FcγRI ligand), anti-FcγRII mAb 32.2, anti-FcR (22), FcRI (6), and cell functions (6, 21). Antibodies and FcR ligands used for experiments included anti-FcγRI mAb 197 (Medarex, Annendale, NJ.), mAb HB63 (mIgG2a isotype control and high affinity FcγRI ligand), anti-FcγRII mAb 32.2, anti-FcγRII mAb 66 and 97 (gifts from H. Kubagawa, University of Alabama at Birmingham), mAb P3 (mIgG1 isotype control), anti-FcγRII mAb and Fab IV.3 (Medarex), human IgG1 (high affinity FcγRI ligand), and the following Sepharose-conjugated antibodies: 32.2, human IgG (Sigma), A77, and IV.3. Anti-murine antibodies included F(ab’)2 sheep anti-mouse IgG F(ab’)2, preadsorbed against human IgG (Organon Technika, Durham, NC), goat anti-mouse γ-chain antibody (Pierce), FITC-conjugated F(ab’)2 goat anti-mouse IgG (CalTag, San Francisco, CA), and Sepharose-conjugated goat anti-mouse IgG (Organon Technika). Other precipitants included PY20-agarose (Transduction Labs, Lexington, KY), protein A-Sepharose (Sigma), and protein G-Sepharose (Genzyme, Cambridge, MA).
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Boston, MA). Primary immunoblot antibodies included rabbit anti-phosphotyrosine antibody (a gift from G. Lienhard, Dartmouth Medical School, Hanover, NH), horseradish peroxidase-conjugated PY20 (Transduction), rabbit anti-γ (a gift from J. P. Kinet, NIAID, National Institutes of Health), and rabbit anti-Syk (Upstate Biotechnologies, Inc., Lake Placid, NY). Secondary immunoblot antibodies included horseradish peroxidase-conjugated anti-rabbit IgG and anti-murine IgG (Bio-Rad).

FcR Activation and the Respiratory Burst—U937 10.6 cells in supernatant (O2) assay medium were added to an equal volume of a second medium containing 10 μg/ml control antibodies or mAb 197, which cross-links FcγRI through Fc and Fab trivalent binding (23). Alternatively, cells were reacted with 5 μg/ml control or anti-Fcα antibody (24) for 20 min at 22 °C, centrifuged, resuspended in O2 assay medium, and added to an equal volume of a second medium containing 40 μg/ml anti-murine antibody. FcR aggregations were done at 37 °C. For assaying tyrosine phosphorylations or O2, the second medium was 10 mM luminol in phosphate-buffered saline (24). Orthovanadate (Na3VO4), buffered and at a concentration of 200 μM, was present during FcR aggregation except where indicated. To measure respiratory bursts, luminol-mediated chemiluminescence was monitored on a Pharmacia 1250 luminometer and is expressed in mV, as described previously (24).

Cellular Tyrosine Phosphoproteins—Cells reacted with antibodies were rapidly chilled, washed twice with cold phosphate-buffered saline, and boiled for 20 min in nonreducing SDS sample buffer. For reduction, boiling was continued for 3 min following the addition of 4% 2-mercaptoethanol. Proteins were separated by SDS-PAGE and analyzed by Western blot. phospho-γ in cellular proteins was distinguished from nonphosphorylated γ-chains through the migration pattern on nonreducing gels. Unreduced phosphorylated γ-chains migrate to a broad ~28-kDa position compared with unphosphorylated bands at ~22 kDa (6).

Immunoprecipitations and Western Blotting—Cells reacted and washed as above were solubilized at a concentration of 10^7/ml in cholate buffer (15 mM sodium cholate, 0.1% Nonidet P-40, 130 mM KCl, 200 μg/ml CaCl2, 200 μg/ml MgCl2, 10 mM NaF, 500 μg/ml Na3VO4 (pH 7.6), 5 mM sodium pyrophosphate, 5 mM NaH2PO4, 0.23 units/ml aprotinin, and 200 mg/ml benzamidine (both in 0.5% 2-mercaptoethanol). Proteins were separated by SDS-PAGE and transferred to polyester-supported nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were blocked and immunoblotted as described previously (6). Bands were visualized by the ECL procedure. Similar results were obtained using 0.5% Nonidet P-40, 0.5% digitonin as lysis buffer (6). For blotting, membranes were stripped twice at 50 °C for 30 min each with 2% SDS and 5% β-mercaptoethanol in 62 mM Tris, pH 6.8.

Assay for in Vitro Subunit Exchange—Cells were incubated with 197 or HB63 in activation medium, and solubilized at a concentration of 5 × 10^7/ml in 0.5% Nonidet P-40, 0.5% digitonin lysis buffer (6). Duplicate FcαR precipitations were performed on lysates from 1.5 × 10^8 cells on A77-conjugated beads. Also prepared were FcγRI- and FcγRII-depleted lysates from 2 × 10^8 cells. FcR were depleted through successive adsorptions by bead-conjugated antibodies: goat anti-murine for 3 h, goat anti-γ for 1 h, goat anti-murine for 1 h, A77 for 16 h, goat anti-murine for 1 h, hIgG for 1 h, and A77 for 1 h. Preadsorption was verified through anti-phosphotyrosine immunoblot of adsorbed proteins to detect FcR-associated phospho-γ. Duplicate FcαR precipitates were either kept on ice as controls or rotated with preadsorbed lysates from 4 × 10^8 cells for 1 h. Controls were precipitated using lysate aliquots representing 2 × 10^8 cells to assess the presence of free phospho-γ following FcR depletion. The control precipitations were performed on bead-conjugated PY20 or on anti-γ antibody-coated protein A-Sepharose for 1 h. All precipitates were washed three times with lysis buffer, and boiled in SDS-sample buffer. Precipitates were separated by SDS-PAGE and analyzed by Western blot.

Cytofluorographic Assay for Measuring FcR Aggregation/Internalization—Cells were incubated at 37 °C for 18 min with RPMI alone or containing 197 or HB63 (5 μg/ml). These cells were centrifuged, washed briefly, and incubated an additional 10 min with 5 μg/ml A77 or P3. Vanadate (200 μM) was present or absent throughout. All samples were washed three times with 0.1% bovine serum albumin in phosphate-buffered saline, stained with FITC-conjugated F(ab)2 goat anti-mouse IgG at 4 °C, and analyzed by cytofluorography as described previously (23, 25). Results are expressed as FITC-antibody binding sites/cell.

RESULTS

Transient Tyrosine Phosphorylations and the Effect of Vanadate—An early response by monocyte cells triggered through FcγRI is the tyrosine phosphorylation of several proteins including FcγRI-γ-chains (6, 7). Triggering also induces a transient respiratory burst that is tightly coupled to de novo receptor cross-linking (23). To determine whether induced tyrosine phosphorylations were also transient, we reacted U937 cells with anti-FcγRI mAb 197, which effectively cross-links because of trivalent binding. The time course of induced tyrosine phosphorylations was measured by Western blot. Under normal conditions (Fig. 1, right panel), tyrosine phosphorylations of pp72 (Fig. 1A), and γ-chains (Fig. 1B) were transient, peaking by ~3–5 min. Additional transiently phosphorylated proteins were detected with longer exposures. The lack of a sustained phosphorylation suggests that phosphatase activity is present in aggregated receptors. In the presence of vanadate, however, phosphorylations of 72-kDa proteins (Fig. 1A), γ-chains (Fig. 1B), and A77 for 16 h.

![Fig. 1. Signaling by FcγRI is normally transient.](http://www.jbc.org/figure)
Tyrosine phosphorylation of nonaggregated FcR γ-chains in activated cells. Some cells were reacted with 197 (+) (lanes 1 and 3) or HB 63 (−) (lanes 2 and 4) for 5 min in the absence of vanadate. Other cells were reacted with A77 (lane 5) or P3 (lane 6) followed by sheep anti-murine antibody (lanes 5 and 6) to cross-link (+) or not (−) FcαR. Washed cells were lysed with cholate buffer, and postnuclear supernatants were subjected to immunoprecipitation procedures. Nonaggregated FcαR (lanes 1 and 2), aggregated FcγRI (lanes 3 and 4), and nonaggregated FcγRI (lanes 5 and 6) were precipitated, and the nonreduced precipitates were separated by SDS-PAGE on 16% gels and analyzed by anti-phosphotyrosine Western blot. The bracket denotes nonphosphorylated γ, and bars denote phosphorylated γ.

1B), and other proteins (Fig. 1A) accumulated, reaching a maximum by 18 min. This indicates that vanadate blocked normal dephosphorylation. Incubation with vanadate alone did not increase tyrosine phosphorylations (lane −24). The continued accumulation of phosphoproteins (Fig. 1A) suggests sustained activation of kinases that phosphorylate FcR tyrosines. A shorter exposure of the left panel revealed a decrease in total cellular unphosphorylated γ concomitant with an increase in phosphorylated γ-chains. This clearly demonstrated a shift to the phosphorylated form of a significant portion of total cellular γ.

Triggering under the same conditions resulted in a transient burst of O2 production (Fig. 1C). Respiratory burst kinetics were similar to the tyrosine phosphorylation response in the absence of vanadate.

Absence of Bystander Involvement during FcR Cross-linking—According to reports, FcγRI aggregation does not result in the phosphorylation of nonaggregated (bystander) FcαR- associated γ-chains (15–17). To determine whether nonaggregated FcR in monocytes becomes phosphorylated, we used an assay system in which FcR of one class were aggregated and nonaggregated FcR of another class were examined for phosphorylation of their associated γ-chains. We aggregated FcγRI in the absence of vanadate for an optimal time (5 min) (Fig. 1) and examined aggregated FcγRI and nonaggregated FcγRγ by immunoprecipitating the receptors from lysates of the cells. As shown in Fig. 2, nonaggregated FcαR contained only a trace of phosphotyrosine compared with aggregated FcγRI. In the converse experiment, FcαR were aggregated with little effect on FcγRI γ (Fig. 2). Longer incubations did not increase phosphorylation of nonaggregated receptors (not shown). These results indicate that nonaggregated bystanders were not significantly targeted by aggregation-activated kinases.

Tyrosine phosphorylation of bystander FcR in the presence of Vanadate—Because FcγRI triggering in the presence of vanadate resulted in extensive phosphorylation of cellular γ-chains (Fig. 1), we examined the possibility that this may have included the phosphorylation of nonaggregated FcR. We cross-linked FcγRI and examined FcγRγ in receptor immunoprecipitates. As shown by Western blot (Fig. 3A), γ-chains co-precipitating with nonaggregated FcαR were extensively phosphorylated. Blotting with anti-γ antibodies confirmed this and demonstrated similar intensities of phospho-γ bands in nonaggregated FcαR and aggregated FcγRI (Fig. 3A). In the same experiment, we cross-linked FcαR and examined nonaggregated FcγRI γ in receptor immunoprecipitates (Fig. 3B). As shown (Fig. 3B), nonaggregated FcγRI γ was phosphorylated in anti-FcαR-activated but not in nonactivated cells. Recoveries of receptors in precipitates in all cases were assessed by anti-γ chain blots.

Cross-linking of FcαR was also executed in the presence of hlgG1 to block a potential Fc interaction of anti-FcαR with FcγRI. The results show that γ-chains in the hlgG1-FcγRI complexes had become phosphorylated (Fig. 4, A and B, lane 1). Furthermore, the possibility of anti-murine antibody cross-linking and stimulating via bound hlgG1 was also eliminated by an oxidase assay in which cells preincubated with hlgG1 or not and incubated with the same set of antibodies were found to be activated only through IgA receptors. Values from the oxidase assay (in mV) were 4451 ± 228 for A77-hlgG1-coated cells, 414 ± 752 for A77-coated cells, and 19 ± 9 and 9 ± 2 for P3-hlgG1 and P3-reacted cells, respectively. All received second antibody. These results eliminated Fc bridging as the source of nonaggregated involvement.

In the same experiment, FcγRI were cross-linked (Fig. 4, A and B, lane 5), causing the extensive phosphorylation of FcαR. Anti-γ immunoblots of aggregated (lane 6) compared with nonaggregated FcγRI (lane 1) show similar intensities of phospho-γ bands, suggesting that similar numbers of chains in nonaggregated ligand-occupied FcγRI were phosphorylated. Noticeable decreases in unphosphorylated γ and increases for phospho-γ within individual samples (lanes 1 and 6) imply an efficient shift in state. Similar mobility patterns for phospho-γ in each case are consistent with equivalent site modifications by kinases. These results demonstrate the efficient phosphorylation of tyrosines on nonaggregated FcR γ-chains.

To examine phosphorylation kinetics, we measured the onset
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**Fig. 4.** Ligand-blocked nonaggregated FcγRI are efficiently phosphorylated. A, immunoprecipitation of FcγRI after FcαR cross-linking in the presence of hlgG1. Cells were preincubated for 20 min with 10 μg/ml hlgG1 and A77 (lanes 1 and 2) and then incubated for 18 min with sheep anti-murine antibody (lanes 1 and 2) in the presence of 200 μM vanadate. FcγRI and FcαR were precipitated from lysates via 32.2 (lane 1) and anti-murine antibody (lane 2), respectively. B, efficient transphosphorylation of FcγRI. Cells were incubated as above with 197 (+) or HB63 (−). FcγRI were precipitated via anti-murine antibody (lanes 5 and 6). Nonreduced precipitates were analyzed by sequential anti-phosphotyrosine and anti-γ Western blot. Band intensities for phospho-γ (lanes 1 and 5) indicate similar efficiencies of phosphorylation. FcαR precipitates (lanes 3 and 4) are shown for comparison.

and maximal phosphorylation times for γ-chains of aggregated FcγRI and nonaggregated FcαR. As shown by anti-phosphotyrosine and anti-γ Western blot (Fig. 5), phosphorylation of FcαRγ was detectable by 6 min and plateaued by 18 min (Fig. 5). FcγRIγ phosphorylation was detectable by 3 min and plateaued between 12 and 18 min. Similar maximal intensities were observed, and anti-γ blots show similar amounts of FcR in precipitates. These results show the rapid and prolonged phosphorylation of aggregated and nonaggregated FcR γ-chains.

Phospho-γ is Not Acquired through Subunit Exchange—To determine whether nonaggregate phosphorylation could be an artifact of immunoprecipitation in which phospho-γ exchanged for unphosphorylated γ, or vice versa, immunoprecipitates of nonaggregated FcαR (containing phospho-γ-chains) were incubated for the usual time with an unstimulated lysate pre-cleared of endogenous FcR α-chains. Exchange was judged by comparing the original with lysate-incubated precipitates. As shown in Fig. 6A, these two were identical, indicating that FcαR did not exchange its associated phospho-γ during immunoprecipitation. In the converse experiment (Fig. 6B), unphosphorylated FcαR in precipitates were incubated in lysates of FcγRI-stimulated cells. The lysates had been pre-cleared of FcγRI and FcαR (Fig. 6B, lower panel) but contained free phospho-γ chains (lane 7). Exchange was again judged by comparing original with lysate-incubated precipitates. The results show that unphosphorylated γ in FcγRI precipitates was not exchanged for phospho-γ. Collectively, the results show that phospho-γ was not acquired through subunit exchange in vitro.

To determine whether phospho-γ exchange in vivo explains the appearance of phospho-γ in nonaggregated FcR, we triggered FcγRII and precipitated from the cell lysate nonaggregated FcγRII. FcγRII lack γ-chains but are phosphorylated in cytoplasmic domain motifs upon cross-linking (10). Fig. 7A shows that nonaggregated FcγRII in FcγRII-activated but not in nonactivated cells were phosphorylated on tyrosines. Similarly, upon triggering through FcγRII, γ-chains for FcγRI and FcαR became phosphorylated (Fig. 7B). These data indicate that FcR lacking exchangeable γ-chains are phosphorylatable bystanders and, with cross-linking, are able to induce γ-chain phosphorylation. This suggests that direct kinase activity rather than...
subunit exchange in vivo explains bystander γ-chain phosphorylation.

Bystander Phosphorylation Is Not Due to FcR Co-aggregation—We investigated the possibility that vanadate may have induced co-aggregation of nonaggregated with aggregated FcR, making nontargeted Fcγ available to aggregate-docked kinases. Aggregation was assessed by measuring internalization of receptors. Following aggregation and a predetermined interval for internalization of FcγRI, the cells were fluorescently labeled to quantitate FcγRI and FcαR remaining on the surface. The results (Fig. 8) show that 197-FcγRI aggregates were effectively internalized (>60%) without a concomitant reduction in surface FcαR. As similar results were obtained in the presence and absence of vanadate (Fig. 8), the data do not support a vanadate-induced co-aggregation.

Other indirect evidence suggests a lack of co-aggregation. As shown in Figs. 3, 4, and 5, nonaggregated FcγR were deficient in tyrosine phosphoproteins that co-precipitate with aggregated FcγR. In several experiments, the co-precipitating panel consisted of 32-, 52-, 66-, and 72-kDa (Syk kinase) bands. Discrete co-precipitations are consistent with a lack of co-aggregation of FcγR types.

Tyrosine Phosphorylation of Nonaggregated γ by Treatment of CD11s with Pervanadate—Treatment of nonactivated cells with vanadate prereacted with H2O2 to produce peroxidase induced the tyrosine phosphorylation of γ. This did not occur upon treatment with vanadate or H2O2 alone. These data (Fig. 9) show that γ-chains can become phosphorylated in the absence of any FcγR cross-linking. Based on this, it appears that the kinases that interacted with nonaggregated FcγR were negatively regulated by tyrosine phosphatases.

DISCUSSION

Transient and Cluster-restricted Tyrosine Phosphorylation of Aggregated FcγR—Evidence is presented showing that under normal conditions FcγRI-induced tyrosine phosphorylations are transient. Similar observations have been reported by Duchemin et al. (7) and by Swieter et al. (26) for aggregated FcγRI in rat basophilic leukemia cells. In our experiments, peaks of phosphorylation occurred by 3–5 min. Importantly, even at the peak of this activity, phosphorylation of γ-chains triggered by FcγRI aggregation occurred on subunits of the aggregated receptors but was absent from noncross-linked FcαR on the same cells. Similarly, cross-linking of FcαR did not cause phosphorylation of γ-chains on FcγRI. This absence of nonaggregated FcαR involvement indicates that tyrosine phosphorylation in monocytes is normally restricted to FcγR in aggregates or clusters. As previously mentioned, this lack of bystander involvement is normal for nonaggregated FcγRI γ-chains in suboptimally FcγRI-triggered basophils (15–17).

Restriction of Kinase Activity to Clusters Subverted by Vanadate—Interestingly, we found that aggregation of FcγRI in the presence of vanadate resulted in tyrosine phosphorylations not only of γ-chains associated with aggregated FcγRI but also of γ-chains associated with nonaggregated FcαR and of the cytoplasmic domain of nonaggregated FcγRI. Phosphorylation of nonaggregated FcαR began shortly after the onset of phos-

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The activation of tyrosine kinases is a critical step in many cellular processes. FcR, for example, can activate tyrosine kinases, but the mechanism is complex. It is well known that clustering of FcR is essential for kinase activation, but aggregation of FcR can also contribute to kinase activation.

In the presence of vanadate, there was an inhibition of protein tyrosine phosphatase activity, and also an increased or sustained tyrosine kinase activity. This was demonstrated in the observation that FcR aggregation-dependent increase in cellular phosphotyrosine occurred over a longer time than the normal peak activity would have predicted. This transient FcR-triggered tyrosine phosphorylations would have declined after the first 3-5 min of stimulation. Phosphorylation in the presence of vanadate was greater during this initial period and it continued to accumulate for 12-18 min. Thus, vanadate prevented dephosphorylation of γ-chains, and it appears to have either prevented deactivation (and promoted release) of kinases in aggregates or activated kinases preassociated with nonaggregated FcR.

To further support this conclusion, evidence is presented that argues against alternative explanations. We show that γ-chain phosphorylation did not occur as a result of (i) anti-FcR bridging of nontargeted γ-chains in overtly aggregated FcR. It was extensive, as demonstrated by a dramatic shift of total cellular and FcR-associated γ-chains from the nonphosphorylated to the phosphorylated state. Collectively, the results show an activation-dependent phosphorylation of nonaggregated FcR γ-chains and motifs under conditions that inhibit phosphatase activity.

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This conclusion is further supported by the observation that pervanadate treatment of cells resulted in the phosphorylation of γ-chains without any FcR cross-linking. Pervanadate is a potent inhibitor of phosphotyrosine dephosphorylation. It increases tyrosine phosphorylations in a number of cell types (Roberts et al., 2001), including T lymphocytes (Roberts et al., 2002). The effect of pervanadate in this study suggests that relevant kinase activation can occur independently of aggregation and, conversely, that γ-chains need not be aggregated to be substrates. Since γ-chains do not require the aggregated state for phosphorylation, this state is unlikely to dictate restriction of kinase activity to clusters.

Thus, it is clear from results in this report that kinase activity for γ-chains is not obligatorily limited to clusters of FcR, although clustering is the normal mechanism for kinase activation. It is also clear that aggregation of γ-chains is not a physical requirement for kinase interaction with their tyrosines. Pribluda et al. (1992) have described clustered FcγRIγ motifs as the normal and necessary state of substrate for activated kinases to phosphorylate in trans their nearest neighbor FcγRI γ-chains. The same group has also shown FcγRI dimers to be a sufficient size to satisfy the requirements for transphosphorylation (33). Therefore, our observations are not inconsistent with the model of Pribluda et al., since nonaggregated FcγRI and FcαRI may exist as dimers prepared to transphosphorylate paired chains but needing something more for kinase activation.

Our central conclusion is that there is a vanadate-sensitive mechanism that prevents kinase activation and the tyrosine phosphorylation of the nonaggregated FcR component chains. Normal intracluster restriction of γ-chain phosphorylation may be due to this mechanism, and phosphatases as regulatory molecules are implicated in the process.

Vanadate subverted the normal cellular mechanism, but it is not clear by what mechanism. One difficulty in interpreting molecular events is that we do not know in sufficient detail how kinases that phosphorylate FcγRI γ-chains become activated, with what proportion of receptors they are associated, and what other regulatory molecules are present. One interpretation of our data is that vanadate blocked the deactivation (dephosphorylation) of kinases in aggregates and caused their release. However, in view of the effect of pervanadate, it is more likely that kinases are sufficiently phosphorylated with, or recruited to, nonaggregated FcR and phosphorylate receptors once activated. Yamashita et al. (1991) estimate that ~25% of resting FcγRI are associated with Lyn kinase in rat basophilic leukemia cells. Wang et al. (1992) identified Lyn and Hck associated with resting FcγRI. Kent et al. (1993) found phosphorylation activity in FcγRI aggregates and Swiet et al. (1992) found it in monomers. In our experiments, FcR-mediated oxygen radical production may have converted some vanadate to pervanadate or, alternatively, stimulation may have caused channeling of orthovandate intracellularly. Either way, it would appear that phosphatases that were phosphorylated were inhibited that were functionally associated with kinases in nonaggregated FcR. Thus, a reasonable hypothesis is that nonaggregated FcγRI phosphorylation is normally negatively regulated by phosphotyrosine phosphatases and that aggregation induces FcγRI phosphorylation by transiently inactivating the phosphatases.

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J. Biol. Chem. 1996, 271:11099-11105.
doi: 10.1074/jbc.271.19.11099

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