Altered Patterns of Tyrosine Phosphorylation and Syk Activation for Sterically Restricted Cyclic Dimers of IgE-FcϵRI[†]

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ABSTRACT: Previous studies in our laboratory established that the symmetrical bivalent ligand, N,N′-bis-[[ϵ-(2,4-dinitrophenyl)amino]caproyl]-L-tyrosyl]-L-cystine ((DCT)$_2$-cys), stably cross-links anti-2,4-dinitrophenyl-immunoglobulin E (IgE) bound to high affinity receptors FcϵRI on the surface of RBL-2H3 cells, forming mostly cyclic dimers containing two IgE-FcϵRI and two (DCT)$_2$-cys (Posner et al. (1995) *J. Immunol. 155*, 3601−3609). These cyclic dimers do not trigger Ca^{2+} or degranulation responses under a variety of conditions. However, we find that the linearly cross-linked IgE-FcϵRI formed at higher concentrations of (DCT)$_2$-cys do trigger degranulation in the presence of cytochalasin D, an inhibitor of actin polymerization. We further investigated stimulation by (DCT)$_2$-cys of the earliest known events in the functional response, i.e., tyrosine phosphorylation of the β and γ subunits of FcϵRI. At the higher (DCT)$_2$-cys concentrations corresponding to linear dimers and maximal degranulation, tyrosine phosphorylation of both β and γ are observed. At lower (DCT)$_2$-cys concentrations where cross-linking is maximal and cyclic dimers are overwhelmingly dominant, only γ tyrosine phosphorylation is observed. Cytochalasin D does not affect these phosphorylation patterns, but instead appears to enhance coupling to downstream signaling events. Phosphorylation of Syk occurs at the higher (DCT)$_2$-cys concentrations in parallel with β phosphorylation but does not occur in its absence at the lower (DCT)$_2$-cys concentrations. These results suggest that cyclic dimers of IgE-FcϵRI are sterically restricted such that they stimulate tyrosine phosphorylation of γ but not β, and this is not sufficient for Syk binding and/or activation.

In mast cells, including the RBL-2H3 mucosal mast cell line, immunoglobulin E (IgE)[1] bound to high affinity receptors, FcϵRI, mediates cellular activation which culminates in the release of preformed and newly synthesized mediators of inflammatory and allergic responses (Beaven & Metzger, 1993; Metcalfe et al., 1992). Aggregation of FcϵRI is essential for initiating the signaling process which includes FcϵRI immobilization (Menon et al., 1986a,b), tyrosine phosphorylation of FcϵRI subunits (Paolini & Kinet, 1991) and other substrates (Li et al., 1992; Benhamou et al., 1992), Ca^{2+} mobilization (Beaven et al., 1984; Millard et al., 1989), protein kinase C activation (White et al., 1988; Ozawa et al., 1993), and phospholipase A_2 activation (Garcia-Gil & Siraganian, 1986; Hirasawa et al., 1995). Phosphorylation of the FcϵRI-β and -γ subunits is among the earliest biochemical events to follow FcϵRI aggregation (Paolini & Kinet, 1991). The src family tyrosine kinase, Lyn, is sufficient for aggregation-dependent β/γ phosphorylation (Scharenberg et al., 1995) and is likely responsible for this step. Phosphorylation of the tandem tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) sequence of FcϵRI-γ is required for binding and activation of the tyrosine kinase Syk (Jouvin et al., 1994; Wilson et al., 1995), and this leads to tyrosine phosphorylation of a number of other proteins, including phospholipase Cγ (Park et al., 1991; Li et al., 1992).

Although considerable progress has been made, the mechanism by which aggregation of IgE-FcϵRI initiates the tyrosine phosphorylation cascade is incompletely understood. Pribluda et al. (1994) provided evidence for a transphosphorylation mechanism by which Lyn associated with one receptor in a cross-linked aggregate phosphorylates the ITAM tyrosine residues of an adjacent receptor. One unresolved question is whether the structural orientation of cross-linked FcϵRI complexes influences their capacity to initiate the first steps in this process. Previous studies with dimer-forming anti-FcϵRI mAb suggested the possibility that steric restriction of the cross-linked receptor complexes can influence signaling (Ortega et al., 1988). Our studies with the symmetrical bivalent ligand (DCT)$_2$-cys showed that cyclic dimers of IgE-FcϵRI formed with this ligand do not trigger Ca^{2+} mobilization or cellular degranulation, even when these complexes are extended into larger aggregates by several different dimer-forming anti-IgE mAb (Posner et al., 1995 and unpublished results). In contrast, small amounts of linear (noncyclic) aggregates form at concentrations of (DCT)$_2$-cys higher than those optimal for cross-linking and cyclic dimer formation, and these linear aggregates can synergize with the anti-IgE mAb to stimulate significant Ca^{2+} and degranulation responses (Posner et al., 1995).

These results suggested that the cyclic dimers of (DCT)$_2$-cys and IgE-FcϵRI have structural constraints that affect their functional competence in critical signaling events. We have

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[1] Abbreviations: IgE, immunoglobulin E; DNP, 2,4-dinitrophenyl; DCT, [ϵ-[(DNP)amino]caproyl]-L-tyrosine; (DCT)$_2$-cys, *N,N*′-bis[DCT]-L-cystine; ITAM, immunoreceptor tyrosine-based activation motif.

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examined this question in the present study, and we find that these cyclic dimers have a unique pattern of FcεRI tyrosine phosphorylation that correlates with no Syk activation or downstream signaling. The results indicate that structural restrictions within FcεRI aggregates can influence the earliest tyrosine phosphorylation events.

MATERIALS AND METHODS

Reagents. The mAb anti-2,4-dinitrophenyl (DNP) IgE used in these studies (Liu et al., 1980) was purified as previously described (Subramanian et al., 1996). The monovalent ligand, [ε-[(DNP)amino]caproyl]-L-tyrosine (DCT), was obtained from Biosearch, Inc. (San Rafael, CA), and the bivalent ligand $(DCT)_2$-cys was synthesized and characterized in this laboratory as described previously (Kane et al., 1986; Subramanian et al., 1996). BSA, conjugated with an average of 15 DNP groups (DNP-BSA), was prepared as previously described (Eisen et al., 1959). Cytochalasin D was purchased from Sigma Chemical Co (St. Louis, MO).

β-Hexosaminidase Release Assay. RBL-2H3 cells (Barsumian et al., 1981) sensitized with anti-DNP IgE were incubated at 37 °C overnight within a 48 well plate at a density of 2.5×10^5 cells/well. These adherent cell monolayers were washed twice with warm BSS (20 mM Hepes, pH 7.4, 135 mM NaCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5.6 mM glucose) and incubated with or without 3 μM cytochalasin D for 15 min at room temperature. These solutions were replaced by 250 μL of prewarmed BSS with or without cytochalasin D and containing the appropriate ligands, followed by incubation with the cells for 1 h at 37 °C. A 50 μL aliquot of each supernatant was then assayed for β-hexosaminidase content by a colorimetric enzyme assay (Schwartz et al., 1979). The aliquot was incubated with 200 μL of 1 mM enzyme substrate (*p*-nitrophenyl *N*-acetyl-β-D-glucosaminide) in 0.05 M citrate buffer (pH 4.5) for 1 h. After quenching by addition of 500 μL of 0.1 M sodium carbonate (pH 9), β-hexosaminidase concentration was determined by measuring enzyme reaction product absorbing at 400 nm and comparing to total product associated with cells lysed in 1% TX-100.

Preparation of Cell Lysates. RBL-2H3 cells sensitized with anti-DNP IgE were replated overnight in six well plates (4×10^6 cells/well) and washed twice with BSS. Then prewarmed BSS (2 mL) containing appropriate ligands was added, and cells were incubated for 5 min at 37 °C as indicated in the figure legends. This solution was quickly replaced with 500 μL of cold lysis buffer (Field et al., 1995) containing 0.2% Triton X-100, and 2 μM DCT was added to accelerate ligand dissociation (Posner et al., 1991) for more efficient solubilization of the cross-linked IgE-FcεRI (Mao et al., 1992). After 40 min at 4 °C, wells were gently scraped with a pipette tip, and lysates were removed and centrifuged at 13000*g* for 5 min. Either supernatants were used for immunoprecipitations, or 100 μL was mixed with 100 μL of a 1× SDS/reducing sample buffer (Chang et al., 1995) and boiled immediately for 5 min for whole cell lysate blot analysis.

Immunoprecipitations. Lysates (500 μL) were precleared by incubating either with 50 μL of anti-mouse IgG1 (heavy chain specific) conjugated to agarose beads (Sigma Chemical Co.) or with 50 μL of protein A−agarose beads (Pierce Chemical Co., Rockford, IL) for 30 min at 4 °C while gently rotating. After sedimenting the beads at 13000*g* for 2 min, the supernatants were gently rotated at 4 °C either with 50 μL of anti-mouse Ig (heavy and light chain specific)−agarose (Sigma Chemical Co.) for 1.5 h or with 2 μL of rabbit anti-rat Syk antiserum (a gift from Dr. J.-P. Kinet, Harvard Medical School) for 1 h, followed by 50 μL of protein A beads. These beads were washed twice with ice cold lysis buffer without Triton X-100. Proteins were eluted from beads by boiling in 100 μL of SDS/nonreducing sample buffer.

Protein Blotting. Samples were separated on SDS gels (12% acrylamide) and then transferred to Immobilon poly(vinylidene difluoride) (Millipore Corp., Bedford, MA) with a semidry transfer apparatus (Integrated Separation Systems, Hyde Park, MA). Membranes were blocked with 4% (w/v) BSA and probed with 1:2500 horseradish peroxidase-conjugated recombinant anti-phosphotyrosine antibody, RC20 (Transduction Laboratories, Lexington, KY). Blots were developed with ECL chemiluminescence and Hyperfilm-ECL (Amersham Corp., Arlington Heights, IL).

RESULTS AND DISCUSSION

Figure 1 compares $(DCT)_2$-cys concentration dependence curves for cellular degranulation and predicted equilibrium distributions of cross-linked IgE-FcεRI aggregate states. The distributions shown in Figure 1A are based on the theory of Dembo and Goldstein (1978) in conjunction with our experimental binding measurements. The shapes and positions of maxima for these curves as determined by the binding parameters were discussed in detail previously (Posner et al., 1995; Holowka & Baird, 1996). A central feature is that maximal cross-linking of IgE-FcεRI occurs at the total $(DCT)_2$-cys concentration (C_{Tmax}) given by

$$C_{Tmax} = (2K_1)^{-1} + [IgE]_{Total} \quad (1)$$

where $K_1 = 2 \times 10^9$ M^{-1} is the established equilibrium constant for $(DCT)_2$-cys binding monovalently to IgE and $[IgE]_{Total}$ is the total concentration of IgE present in a given sample. For the experiments presented here, $[IgE]_{Total}$ is ~0.7 nM such that $C_{Tmax} = \sim 1$ nM. Because cyclic dimers containing two IgE-FcεRI and two $(DCT)_2$-cys have been found to be highly stable (Posner et al., 1995), the following are predicted from this analysis: formation of trimer and larger cross-linked IgE-Fc aggregates is effectively prevented; cyclic dimers are the highly predominant aggregate species when cross-linking is maximal; linear dimers are present in relatively low amounts, and these are maximal at $(DCT)_2$-cys concentrations significantly higher (and lower) than the concentration corresponding to maximal cross-linking and cyclic dimers (Figure 1A). The predicted width of the cyclic dimer peak and the positions of the maxima for the linear dimers are less certain than the maximum corresponding to C_{Tmax} because of their dependence on the correlated parameters K_2, the linear cross-linking constant, and J_2, the dimer cyclization constant. Our binding experiments have not yet established these values, but the values used in Figure 1A lie within a reasonable range (Posner et al., 1995; see Figure 1 legend). The general features of Figure 1A do not depend critically on the exact values of K_2 and J_2.

Figure 1B shows the degranulation response of RBL-2H3 cells to $(DCT)_2$-cys in the presence and absence of cytocha-

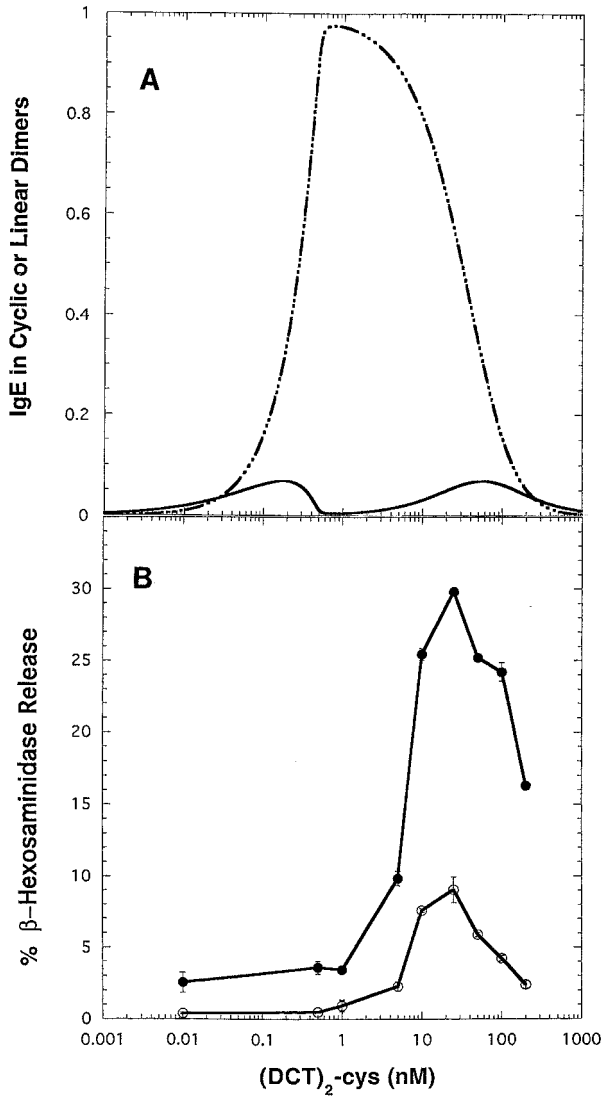

FIGURE 1: Comparison of degranulation responses and equilibrium distributions of cyclic and linear dimers of IgE-FcεRI at varying concentrations of $(DCT)_2$-cys. (A) Equilibrium distributions of cyclic (−···−) and linear (−) cross-linked complexes (fraction of receptor-bound IgE) predicted by Dembo−Goldstein theory and binding data (Posner et al., 1995); $K_1 = 2 \times 10^9$ M^{-1}, $K_{2,3D} = 2 \times 10^{10}$ M^{-1} ($K_{2,2D} = 2 \times 10^{-10}$ cm^{-2}), $J_2 = 2000$. (B) Degranulation of RBL-2H3 cells sensitized with anti-DNP IgE in response to $(DCT)_2$-cys in the presence (●) and absence (○) of 3 μM cytochalasin D. Spontaneous release of β-hexosaminidase in the presence and absence of cytochalasin D was <10% and was subtracted from the stimulated release.

lasin D, an inhibitor of actin polymerization. As previously shown, $(DCT)_2$-cys in the absence of cytochalasin D or a synergizing anti-IgE mAb causes very little degranulation (Kane et al., 1986; Posner et al., 1995); in this experiment a small peak (maximum <10% release) occurs in the range of 10−100 nM. In contrast, cells treated with cytochalasin D respond well to $(DCT)_2$-cys (maximum ∼30% release) in the range of 10−100 nM. This same concentration range of $(DCT)_2$-cys was shown previously to synergize maximally with the independently binding anti-IgE mAb, B1E3 (Posner et al., 1995). In other samples of the representative experiment shown in Figure 1B, an optimal dose of DNP-BSA (500 ng/mL) caused β-hexosaminidase release of 83% in the presence of cytochalasin D and 60% in its absence, whereas cytochalasin D without antigen caused <5% degranulation (data not shown). That cytochalasin D potentiates the rate and/or extent of degranulation responses to multivalent antigens was demonstrated previously (Seagrave & Oliver, 1990; Narasimhan et al., 1990), with evidence that at least part of this effect is due to enhancement of the terminal steps in FcεRI-mediated exocytosis (Narasimhan et al., 1990).

Comparison of Figures 1A and 1B shows consistency with previous results (Posner et al., 1995) that the response to $(DCT)_2$-cys is minimal at concentrations corresponding to maximal cross-linking and maximal formation of cyclic dimers (∼1 nM). Instead, this response is near-maximal at a higher concentration of $(DCT)_2$-cys (∼25 nM) where linear dimers become significant. The earlier studies (Posner et al., 1995) also showed very little response at lower concentrations of $(DCT)_2$-cys including concentrations at which a second peak of linear chains is predicted (Figure 1A). As discussed in detail previously (Posner et al., 1995; Holowka & Baird, 1996), equilibrium theory predicts the number of linear cross-links at each of these two maxima to be the same; however, other features, such as dynamic interchanges between paired IgE-FcεRI, can be quite different in the two concentration regimes. For example, at the low concentration maximum there is essentially no free $(DCT)_2$-cys in solution, and cross-links are likely to break and re-form between the same pair of IgE-FcεRI. In contrast, there is excess $(DCT)_2$-cys in solution at the high concentration regime such that cross-link breakage more likely results in filling of the vacated IgE combining sites with $(DCT)_2$-cys from solution, and consequently linear dimers would tend to form continuously between new pairs of IgE-FcεRI. The lifetime for proximity of a given pair of IgE-FcεRI in linear dimers in this high concentration regime is expected to be directly related to the inverse of the dissociation rate constant (i.e., ∼200 s; Subramanian et al., 1996) and substantially less than the proximity lifetime for the low concentration linear dimers because of continual re-cross-linking of the same pairs. Thus, our observation that cellular degranulation does not occur in the low concentration regime is consistent with the possibility that these persistently cross-linked IgE-FcεRI linear dimers undergo a transition to a desensitized state.

To investigate the basis for cyclic dimer deficiency in stimulating cellular responses, we examined the earliest steps of signal transduction. In particular, we compared the tyrosine phosphorylation of the FcεRI-β and -γ subunits at 1.2 nM $(DCT)_2$-cys corresponding to maximal cyclic dimers and at 25 nM $(DCT)_2$-cys where linear dimers of IgE-FcεRI are significant. As shown in Figure 2, tyrosine phosphorylation of both FcεRI-β and -γ subunits are stimulated by DNP-BSA (Ag) and by 25 nM $(DCT)_2$-cys (D2). The amount of phosphorylation observed is greater for DNP-BSA than for $(DCT)_2$-cys, consistent with relative amounts of degranulation stimulated (see above). Several distinct γ_2 bands are phosphorylated after stimulation with DNP-BSA, as observed previously with this ligand (Paolini & Kinet, 1993). The striking qualitative difference with 1.2 nM $(DCT)_2$-cys is that no stimulated tyrosine phosphorylation of β is detected, although γ phosphorylation is similar to that observed for 25 nM $(DCT)_2$-cys. Tyrosine phosphorylation of FcεRI β and -γ is maximal after 5 min with $(DCT)_2$-cys (Figure 2), but a similar pattern of differential phosphorylation is seen between 1 and 10 min of stimulation (data not shown). Consistent with the degranulation results (Posner et al., 1995), no phosphorylation of β or γ was detected at a lower concentration of $(DCT)_2$-cys (0.25 nM)

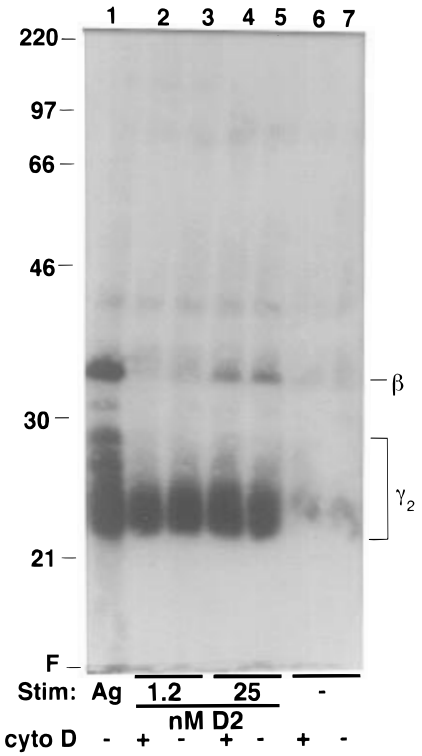

FIGURE 2: Differential tyrosine phosphorylation of FcεRI-β and -γ subunits in response to selected concentrations of $(DCT)_2$-cys. RBL-2H3 cells sensitized with anti-DNP IgE were stimulated for 5 min at 37 °C with indicated ligands in the presence (lanes 2, 4, 6) or absence (lanes 1, 3, 5, 7) of 3 μM cytochalasin D. Immunoprecipitated IgE-FcεRI were analyzed by nonreducing SDS−PAGE followed by blotting with anti-phosphotyrosine. Immunoblot compares tyrosine phosphorylation of FcεRI stimulated by 1 μg/mL DNP-BSA (Ag; lane 1), 1.2 nM $(DCT)_2$-cys (D2; lanes 2 and 3), and 25 nM $(DCT)_2$-cys (lanes 4 and 5), or unstimulated (lanes 6 and 7). Assignments for the positions of phosphorylated forms of the β and γ_2 subunits of FcεRI are based on reprobing with an anti-β monoclonal antibody and selective loss of the γ_2 bands under reducing conditions. Positions of prestained molecular mass standards for this and subsequent figures are indicated at left in kDa units; F, dye front.

where a second peak in the linear dimers is predicted (Figure 1A; data not shown). The contrasting results with 1.2 and 25 nM $(DCT)_2$-cys are similar in the presence and absence of cytochalasin D during stimulation (Figure 2), indicating that this reagent enhances degranulation (Figure 1B) by means of a more downstream signaling step. These differential tyrosine phosphorylation patterns have been observed consistently in more than 10 independent experiments. In several of these experiments, we reprobed the blots with an anti-FcεRI-β mAb (NB; Rivera et al., 1988) and confirmed equal recoveries of this subunit for both concentrations of $(DCT)_2$-cys used, as well as for Ag-stimulated and unstimulated cells (data not shown). These results lead us to conclude that cyclic dimers of IgE-FcεRI formed with $(DCT)_2$-cys undergo tyrosine phosphorylation of γ without stimulated β tyrosine phosphorylation. This view is further supported by recent results with RBL cells sensitized with a hybrid IgE containing only one anti-DNP combining site (and thus forming only linear dimers with $(DCT)_2$-cys). These cells undergo maximal degranulation at concentrations of $(DCT)_2$-cys that are similar to those predicted for maximal cross-linking (i.e., <10 nM; eq 1). With this hybrid IgE maximal tyrosine phosphorylation of β also occurs at similarly low concentrations of $(DCT)_2$-cys (K. Subramanian, N. T. Harris, D. Holowka, and B. Baird, unpublished experiments).

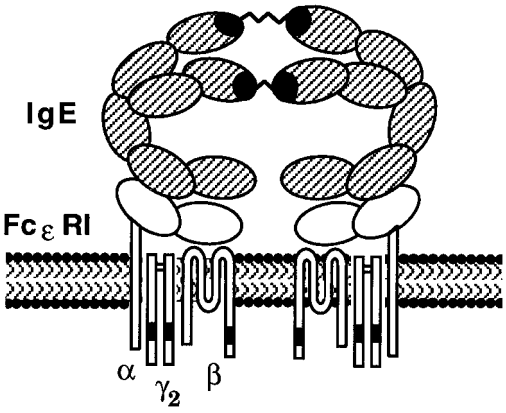

FIGURE 3: Suggested structure for the cyclic dimeric complex of $((DCT)_2$-cys/IgE-FcεRI$)_2$ in the plasma membrane. Wavy lines connecting the IgE combining sites (black circles) represent $(DCT)_2$-cys; hatched ovals represent IgE domains; white structures represent the FcεRI subunits with ITAM motifs indicated by black shading. FcεRI-bound IgE conformation is based on resonance energy distance measurements (Baird et al., 1993). FcεRI subunits α, β, and γ are positioned as described in the text.

The tyrosine phosphorylation results suggest that cyclic dimers of $(DCT)_2$-cys and IgE-FcεRI have a sterically restricted conformation that allows access of Lyn but strongly limits its action, preventing it from phosphorylating β. A series of resonance energy transfer measurements revealed a bent conformation for IgE bound to FcεRI (Zheng et al., 1991, 1992) and indicated a conformation for cyclic dimers in which the two Fcε termini are oriented toward each other (Baird et al., 1993; Figure 3). It is possible that, in such a complex, the C-terminal cytoplasmic tails of FcεRI-β are positioned so as to render them inaccessible to Lyn which is anchored to the inner leaflet of the plasma membrane. In Figure 3, the apposed FcεRI-β subunits are depicted as closest in proximity and thereby sequestered in the middle of the cyclic dimeric complex. This postulated orientation of the subunits of FcεRI is based on (1) the much tighter association of β with γ_2 than β or γ_2 with α (Rivera et al., 1988), and (2) the interaction of human FcεRI-α with γ_2 in the absence of β (Miller et al., 1989). The phosphorylation results also suggest that accessibility of Lyn to the ITAM region of β is not required for Lyn phosphorylation of γ. Our results also could be plausibly explained by differential access to the β and γ subunits by phosphotyrosine phosphatases; very few details are currently known about participation of these enzymes in RBL cell signaling. As revealed by phosphorescence anisotropy measurements (Myers et al., 1992) and fluorescence photobleaching recovery measurements (Posner et al., 1995), dimers and higher aggregates of IgE-FcεRI exhibit significantly reduced rotational and lateral mobility, respectively, compared with monomeric IgE-FcεRI. These results indicate that dimeric IgE-FcεRI complexes (cyclic and linear) interact with some larger structure in the plasma membrane that may influence the signaling process (Holowka & Baird, 1996).

We further examined tyrosine phosphorylation patterns in whole cell lysates. Figure 4 shows a representative experiment, in which RBL-2H3 cells sensitized with anti-DNP IgE were stimulated for 5 min with an optimal dose of DNP-BSA or with the selected concentrations of $(DCT)_2$-cys that cause maximal cross-linking and cyclic dimer formation (1.2

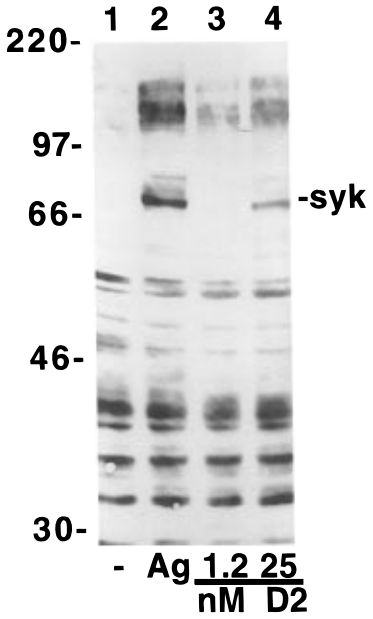

FIGURE 4: Tyrosine phosphorylation in whole cell lysates stimulated by DNP-BSA (Ag; 500 ng/mL) and $(DCT)_2$-cys (D2; 1.2 and 25 nM). RBL-2H3 cells sensitized with anti-DNP IgE were stimulated (lanes 2−4) or not (lane 1) for 5 min at 37 °C, then lysed in 0.2% Triton X-100, and lysates were analyzed by reducing SDS−PAGE and anti-phosphotyrosine blotting. The position of Syk indicated at the right is based on a reprobe of this blot with anti-Syk antibody, which showed similar amounts of Syk in all four lanes (data not shown).

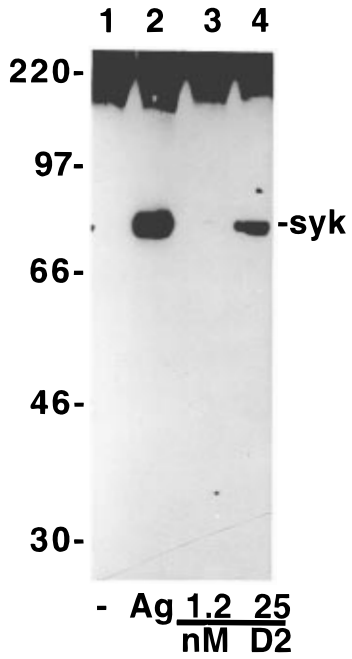

FIGURE 5: Tyrosine phosphorylation of Syk in response to DNP-BSA (Ag; 500 ng/mL) and $(DCT)_2$-cys (D2; 1.2 and 25 nM). RBL-2H3 cells sensitized with anti-DNP IgE were stimulated (lanes 2−4) or not (lane 1) for 5 min at 37 °C with the indicated ligands, and then Syk was immunoprecipitated from cell lysates and analyzed by nonreducing SDS−PAGE and anti-phosphotyrosine blotting. Anti-Syk western blots of parallel samples verified the position of Syk and showed similar amounts of this protein in all four lanes (data not shown). The heavy bands at the 220 kDa marker at the top of each lane correspond to the anti-Syk antibody detected nonspecifically by the anti-phosphotyrosine antibody.

nM) or near-maximal degranulation where linear dimers are significant (25 nM). No stimulated tyrosine phosphorylation of proteins in the Syk region is detectable with 1.2 nM $(DCT)_2$-cys, whereas one major and several minor bands in this region are tyrosine phosphorylated with 25 nM $(DCT)_2$-cys and with multivalent antigen. The position of the major band is coincident with Syk as indicated by reprobing this blot with anti-Syk antibody (data not shown); other tyrosine phosphorylated proteins are also present in this band (Minoguchi et al., 1994). In contrast, stimulated tyrosine phosphorylation of several higher molecular mass bands in the region of ∼100−150 kDa is detected with both 25 and 1.2 nM $(DCT)_2$-cys, as well as with DNP-BSA. Stimulated phosphorylation of several minor bands is also detectable in the region of 30−46 kDa, and at least one of these (at 33 kDa) is seen in all three stimulated lanes but not the control lane. Thus, a subset of tyrosine phosphorylation substrates, including Syk, is not detectably phosphorylated in response to the formation of cyclic dimers of IgE-FcεRI, whereas other proteins are tyrosine phosphorylated with both concentrations of $(DCT)_2$-cys in parallel with phosphorylation of FcεRI-γ (compare Figures 4 and 2).

In order to determine more directly whether Syk is activated by the cyclic dimers, anti-Syk immunoprecipitates were analyzed by anti-phosphotyrosine immunoblotting after SDS−PAGE under nonreducing conditions. As shown in Figure 5, tyrosine phosphorylation of Syk is detected after stimulation with antigen or with 25 nM $(DCT)_2$-cys, but not with 1.2 nM $(DCT)_2$-cys. Similar results were obtained in 2 additional experiments of this design. Tyrosine phosphorylation of Syk has been previously shown to correlate with activation of this tyrosine kinase (Shiue et al., 1995b; Rowley et al., 1995), suggesting that linear chains of IgE-FcεRI formed with $(DCT)_2$-cys can activate Syk, whereas cyclic dimers formed with this ligand cannot. These results are consistent with those from analysis of tyrosine phosphorylation in whole cell lysates (Figure 4), and they indicate that the tyrosine phosphorylation of FcεRI-γ stimulated by cyclic dimers does not lead to Syk activation.

It is not yet clear whether the phosphorylated ITAMs of FcεRI-γ in these cyclic complexes cannot bind Syk, or whether Syk binds but is not activated. In initial experiments we have not detected $(DCT)_2$-cys-stimulated association of Syk with immunoprecipited FcεRI at any concentration of this ligand. Studies on FcεRI- and B cell receptor-mediated activation of Syk have shown that most activated Syk is cytosolic (Shiue et al., 1995a; Peters et al., 1996), suggesting that association with receptor ITAMs may be transient. The manner in which Syk is activated after binding to phosphorylated ITAM sequences is not yet understood, and it may involve a conformational change in the kinase that leads to autophosphorylation (Shiue et al., 1995; Rowley et al., 1995), transphosphorylation of adjacent Syk kinases (Kolanus et al., 1993), or phosphorylation of Syk by proximal Lyn (Scharenberg et al., 1995). Previous studies showed that aggregation of chimeric transmembrane proteins containing the cytoplasmic portions of FcεRI-γ can stimulate degranulation (Letourneur & Klausner, 1991) and activate Syk in the absence of associated β (Jouvin et al., 1994; Wilson et al., 1995). Subsequent evaluation of FcεRI variants reconstituted with Lyn and Syk in NIH-3T3 cells indicated that β tyrosine phosphorylation serves to amplify the tyrosine phosphorylation of γ_2 in response to multivalent antigen (Lin et al., 1996). Considering these other results, our observation that cyclic dimers of IgE-FcεRI are deficient in activating Syk,

even though γ is tyrosine phosphorylated, seems more likely due to steric limitations within these complexes rather than an absolute requirement for proximal, tyrosine phosphorylated FcϵRI-β. A better understanding of the structural basis for this limitation should provide important clues to the mechanism of Syk activation by FcϵRI and other members of the Fc receptor family. In this regard, the present observations are reminiscent of the altered tyrosine phosphorylation of TCR-ζ that is stimulated by antagonist peptide−MHC complexes (Madrenas et al., 1995; Sloan-Lancaster et al., 1994). In both situations, subtle structural alterations in the receptor−ligand complex lead to dramatically different signaling outcomes.

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