Evidence Supporting a Role for Microfilaments in Regulating the Coupling between Poorly Dissociable IgE−FcϵRI Aggregates and Downstream Signaling Pathways†

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ABSTRACT: Aggregation of FcϵRI, the high-affinity receptor for IgE, on RBL-2H3 mast cells caused by reversible ligands such as multivalent antigen causes cellular responses that can be halted by subsequent addition of excess monovalent ligand. In contrast, Ca^{2+} and degranulation responses elicited by effectively irreversible streptavidin cross-linking of biotinylated IgE−FcϵRI are not stopped by addition of excess biotin after stimulation is initiated. These results support previous conclusions based on studies with covalent oligomers of IgE that stable cross-links can continue to deliver stimulatory signals for extended periods of time. Dissociation measured in the presence of monovalent hapten reveals two populations of IgE−FcϵRI cross-linked by multivalent antigen that differ in functional effectiveness. Aggregates with readily dissociable cross-links are normally responsible for triggering essentially all of the degranulation response, whereas aggregates with poorly dissociable cross-links apparently do not trigger this response. Treatment of RBL-2H3 cells with cytochalasin D, an inhibitor of actin polymerization, enhances downstream signaling and enables the less readily dissociable aggregates to stimulate Ca^{2+} and degranulation responses. Under these conditions, cytochalasin D does not affect hapten-mediated dissociation of multivalent antigen, nor does it prevent hapten from reversing tyrosine phosphorylation of Syk. Cytochalasin D alone causes tyrosine phosphorylation of a protein at ∼75 kDa, and it reduces hapten-induced reversal of antigen-stimulated tyrosine phosphorylation of several other proteins. Taken together, these results indicate that stimulated actin polymerization normally regulates the coupling of aggregated FcϵRI to downstream signaling pathways, and they provide an explanation for seeming discrepancies between responses to stable and reversible cross-links.

The high-affinity receptor for IgE, FcϵRI,[1] is abundant on the surface of the mast cell line RBL-2H3 (Barsumian *et al.*, 1981). Aggregation of FcϵRI triggers a cascade of signaling events beginning with tyrosine phosphorylation of the β and γ subunits of FcϵRI and activation of several tyrosine kinases, including Lyn and Syk (Scharenberg & Kinet, 1995). The consequent activation of protein kinase C and the rise in the cytoplasmic [Ca^{2+}] result in cellular degranulation and the release of mediators of the allergic response (Beaven & Metzger, 1993). It is well established that aggregation of FcϵRI is required for initiating transmembrane signaling (Metzger, 1992), but the requirements for sustaining the signaling response are incompletely understood. A controversial aspect of this issue has been the relative importance of stable versus dynamic cross-links of FcϵRI, i.e. whether stably aggregated FcϵRI are sufficient for sustained signaling or whether continuous aggregation of additional FcϵRI is necessary to maintain a functional response [reviewed in Holowka and Baird (1996)].

A dynamic cross-linking model was suggested by the observations that cellular degranulation due to multivalent antigen (Ag)-cross-linked IgE−FcϵRI complexes can be rapidly stopped by the addition of a competing monovalent hapten (Sobotka *et al.*, 1979; Fewtrell, 1985; Seagrave *et al.*, 1987). Dissociation measured in the presence of monovalent hapten under these conditions showed that a large amount of Ag can remain bound to the cell surface in a cross-linked state (Seagrave *et al.*, 1987; Erickson, 1988; Holowka & Baird, 1990). One interpretation of these results is that monovalent hapten is not stopping the response by reversing cross-links but, rather, by preventing the formation of new cross-links. In this model, aggregates are transiently active before moving to an inactive or desensitized state [reviewed in Holowka and Baird (1996)]. Consistent with this, evidence for Ag-mediated desensitization in RBL-2H3 cells has been described (Weetall *et al.*, 1993), and McCloskey (1993) has shown that halting lateral diffusion of FcϵRI by addition of wheat germ agglutinin stops the stimulated degranulation response. An alternative interpretation of these results is that addition of monovalent hapten stops signaling by causing dissociation of a subpopulation of bound Ag that is more readily dissociable and also more active in stimulating the early signaling events (Paolini *et al.*, 1991, 1996; Goldstein & Wofsy, 1996).

Evidence against a dynamic cross-linking model comes from studies that utilized covalently cross-linked trimers of IgE to form small, stable aggregates of receptors (Fewtrell & Metzger, 1980; Kent *et al.*, 1994). Because IgE binds to its receptor with high affinity, the addition of monomeric

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[1] Abbreviations: Ag, antigen; BGG, bovine γ globulin; BSA, bovine serum albumin; BSS, buffered saline solution; DCT, [ϵ-[*N*-(2,4-dinitrophenyl)amino]caproyl]-L-tyrosine; DNP, 2,4-dinitrophenyl; FcϵRI, high-affinity receptor for immunoglobulin E; FITC, 5′-fluorescein isothiocyanate; mAb, monoclonal antibody; sAv, streptavidin; TX-100, Triton X-100.

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IgE does not cause dissociation of trimeric IgE, while it does effectively prevent ongoing cross-linking of FcεRI. These studies showed that signaling is not substantially interrupted by the addition of monomeric IgE, indicating that signaling from these small aggregates is sustained without any new receptor cross-links being formed (Kent *et al.*, 1994). These results argue against the generality of a dynamic cross-linking model and suggest that cross-linking-dependent desensitization in these cells is insufficient to efficiently turn off the response under these conditions. However, this result with covalent oligomers is not necessarily general for all stable cross-linking ligands. Covalent IgE oligomers have been shown to bind to Fcγ receptors on mast cells (Takizawa *et al.*, 1992), and this could complicate the interpretation of results with these ligands.

As part of investigating this issue further, we used streptavidin to cross-link monomeric biotinylated IgE bound exclusively to FcεRI, and we found that these stable aggregates of FcεRI remain active without formation of new receptor cross-links. Related experiments showed that, in the presence of the inhibitor of actin polymerization, cytochalasin D, Ca^{2+} and degranulation responses caused by multivalent Ag exhibit markedly decreased sensitivity to reversal by monovalent hapten. Furthermore, in the presence of cytochalasin D, these downstream signaling processes are sustained even though monovalent hapten inhibits some early tyrosine phosphorylation events. These results suggest that microfilaments normally regulate coupling between early and more downstream signaling events activated by FcεRI aggregation.

MATERIALS AND METHODS

Reagents. Cytochalasin D and 4-(nitrophenyl)-2-acetamido-2-deoxy-β-D-glucopyranoside (enzyme substrate) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bovine serum albumin (BSA), bovine γ globulin (BGG), *d*-biotin, and streptavidin (sAv) were obtained from Sigma Chemical Co. (St. Louis, MO). Indo-1/AM was from Calbiochem (San Diego, CA). Anti-2,4-dinitrophenyl (DNP) mouse IgE was purified as described previously (Holowka & Metzger, 1982). Biotinylated IgE (biotin-IgE) was prepared as described (Field *et al.*, 1995). Fluorescein isothiocyanate-labeled IgE (FITC-IgE), prepared as described previously (Erickson *et al.*, 1986), contained 5−7 fluoresceins per IgE as determined by UV−vis absorption spectroscopy. The multivalent antigens DNP-BSA and DNP-BGG contained 16−18 DNP groups per BSA and 24−27 DNP groups per BGG, respectively, and were prepared as described previously (Hardy, 1986). The monovalent DNP hapten [[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosine (DCT) was obtained from Biosearch, Inc. (San Rafael, CA). The mouse mAb AA4 recognizes α-galactosyl derivatives of the ganglioside GD_{1b} (Guo *et al.*, 1989) and was from R. Siraganian at the NIH (Bethesda, MD).

Cells. RBL-2H3 cells (Barsumian *et al.*, 1981) were maintained in monolayer culture in Minimum Essential Medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1 mL/L mito+ serum extender, and 10 μg/mL gentamicin. Typically, cells were used 3−5 days after passage. All tissue culture reagents were obtained from Gibco (Grand Island, NY) unless otherwise noted.

Ca^{2+} Experiments. Cells were harvested by treatment with 1.5 mM EDTA, 20 mM HEPES, 135 mM NaCl, and 5 mM KCl at pH 7.4 for 10 min at 37 °C. This treatment was quenched with buffered saline solution (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose, 1 mg/mL BSA, and 20 mM HEPES at pH 7.4), centrifuged at 200*g* for 10 min, and then resuspended at 1×10^7 cells/mL in BSS with 0.25 mM sulfinpyrazone (Sigma Chemical Co.). Cells were usually sensitized with 1 μg/mL IgE for 30 min at 37 °C before loading with indo-1, a fluorescent indicator of intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$), as described (Pierini *et al.*, 1996). Typically, two 2 mL suspensions of indo-1-loaded cells (1.5×10^6 cells/mL) were maintained at 35 °C in acrylic cuvettes (Walter Sarstedt, Inc.) with continual stirring throughout an experiment. One cuvette of cells was the test sample, and the other suspension of cells was the control sample; each sample was treated in parallel as described in the figure legends. $[Ca^{2+}]_i$ was monitored with an SLM spectrofluorimeter operated in ratio mode (excitation, 330 nm; emission, 400 nm). $[Ca^{2+}]_i$ changes are represented as changes in the raw indo-1 fluorescence intensity. For the experiments described here, the fractional indo-1 response as defined previously (Weetall *et al.*, 1993) is ≤0.3. In this range, indo-1 fluorescence intensity is nearly proportional to $[Ca^{2+}]_i$ (Weetall *et al.*, 1993; Grynkiewicz *et al.*, 1985). The peak $[Ca^{2+}]_i$ responses shown in Figures 1, 3, and 4 correspond to a 4−5-fold increase over the basal (unstimulated) $[Ca^{2+}]_i$, and these increases are similar to changes previously observed with these cells and stimuli (Fewtrell *et al.*, 1987). In the absence of stimulus, the indo-1 fluorescence increases at a slow rate due to the leakage of indo-1 from the cells.

β-Hexosaminidase Degranulation Assay. For assays on suspended cells, adherent cells were first sensitized with 1 μg/mL IgE overnight and then harvested on the day of the experiment as described above. Cells were resuspended at 1×10^6 cells/mL in BSS, and 2 mL samples of cells were maintained in suspension by stirring at 37 °C. At various times after the addition of prewarmed solutions of antigen, aliquots of the cell suspension (100 μL) were removed and the reactions quenched in ice cold BSS. The quenched samples were centrifuged at 9000*g* for 2 min to sediment the cells; aliquots of supernatant (50 μL) were then measured for β-hexosaminidase content by a colorimetric enzyme assay as described (Pierini *et al.*, 1996).

For assays on adherent cells, harvested cells were resuspended in their original culture medium, sensitized by addition of IgE at 1 μg/mL, and then plated into a 24-well plate at a density of 2.5×10^5 cells per well. Following overnight incubation at 37 °C in a CO_2 incubator, cells were washed twice with warm BSS and then incubated with or without 2 μM cytochalasin D for 10 min at room temperature. Cell supernatants were replaced with room-temperature solutions of DNP-BSA in BSS with or without cytochalasin D, and then the cells were immediately moved to a 37 °C CO_2 incubator. At 10 min after antigen addition, cells were removed from the incubator, monovalent hapten was added where indicated, and an aliquot (25 μL) of supernatant was removed before returning the cells to the incubator. Over the course of the subsequent 50 min incubation at 37 °C, 25 μL aliquots of cell supernatants were removed to ice at various additional time points. The β-hexosaminidase content of sample aliquots was determined as previously described (Pierini *et al.*, 1996).

Antigen Binding and Dissociation Experiments. The binding of DNP-BSA to cell surface-bound FITC-IgE can be monitored spectroscopically by measuring FITC fluorescence quenching that accompanies DNP occupation of the Ab combining sites (Erickson *et al.*, 1986). Because binding of the monovalent DNP hapten, DCT, quenches FITC fluorescence less than does binding of oligovalent DNP ligands, the dissociation of DNP-BSA from the cell surface can be measured as the fluorescence recovery that occurs when DCT occupies binding sites that were formerly occupied by DNP-BSA (Erickson *et al.*, 1991). Internalization of cross-linked FITC-IgE−FcεRI, which also causes FITC quenching, was prevented by use of hypertonic medium (BSS containing 0.45 M sucrose) which has been shown to inhibit receptor-mediated endocytosis of LDL through the disruption of the clathrin cage of coated pits (Heuser & Anderson, 1989). By comparing the percent of FITC quenching resulting from DNP-BSA binding to FITC-IgE on RBL cells at 35 °C in the presence or absence of sucrose to that at 15 °C where internalization does not occur, we confirmed that internalization of aggregated IgE−FcεRI at 35 °C is completely inhibited in RBL cells in the presence of this hypertonic solution (0.45 M sucrose) (data not shown). Furthermore, fluorescence microscopy also showed no detectable internalized FITC-IgE−FcεRI complexes under these conditions (L. Pierini, unpublished observations).

For these binding experiments, suspended cells were sensitized with 1 μg/mL FITC-IgE for 1−2 h at 37 °C, washed twice, and then resuspended at 2×10^6 cells/mL in BSS. These cells were maintained at 35 °C with continuous stirring in an acrylic cuvette, and 10 min prior to addition of Ag, 0.54 mL of 1.35 M sucrose in BSS was added to 1.06 mL of cells (2.2×10^6/mL). Binding of DNP-BSA to cell surface-bound FITC-IgE was monitored as described previously (Erickson *et al.*, 1986). Briefly, an SLM 8000 spectrofluorimeter, operated in the ratio mode, was used to monitor quenching of FITC fluorescence (excitation, 490 nm; emission, 520 nm) due to DNP occupation of anti-DNP combining sites (Erickson *et al.*, 1986). The Ag dissociated within 7 min after addition of excess DCT was considered to be the rapidly dissociating Ag, and the corresponding percentage was calculated by dividing the fluorescence recovered at this plateau by the maximum possible recovery (see Figure 6). The maximum possible recovery is the absolute difference between the fluorescence quenching caused by DCT binding in the absence of Ag and the total fluorescence quenching immediately following addition of DCT to cells with Ag. Half-times for dissociation of the readily dissociable Ag were determined from the plots as the time required for the fluorescence recovery that was 50% of the plateau occurring by 7 min.

Immunoblotting. Cells were harvested, sensitized with IgE, and plated into a 6-well plate (2×10^6/well) as described above. Following overnight incubation at 37 °C, cells were washed twice with warm BSS and then incubated at room temperature in BSS with or without 2 μM cytochalasin D for 10 min. Cell supernatants were removed and then replaced with room-temperature solutions of DNP-BSA with or without cytochalasin D, followed by addition of DCT to some of the samples after 8−10 min of incubation at 37 °C. After a further incubation of 2 min at 37 °C, cells were lysed by replacing the cell supernatants with ice cold lysis buffer (Field *et al.*, 1995) containing 0.2% Triton X-100, followed by centrifugation at 4 °C for 5 min at 13000*g* to remove nuclear material. Aliquots of lysates were boiled in reducing SDS sample buffer or immunoprecipitated with rabbit anti-Syk antiserum from J.-P. Kinet (Harvard Medical School), as previously described (Harris *et al.*, 1997). Samples were analyzed by Western blotting as previously described (Harris *et al.*, 1997) using the anti-phosphotyrosine mAb 4G10 (UBI, Lake Placid, NY).

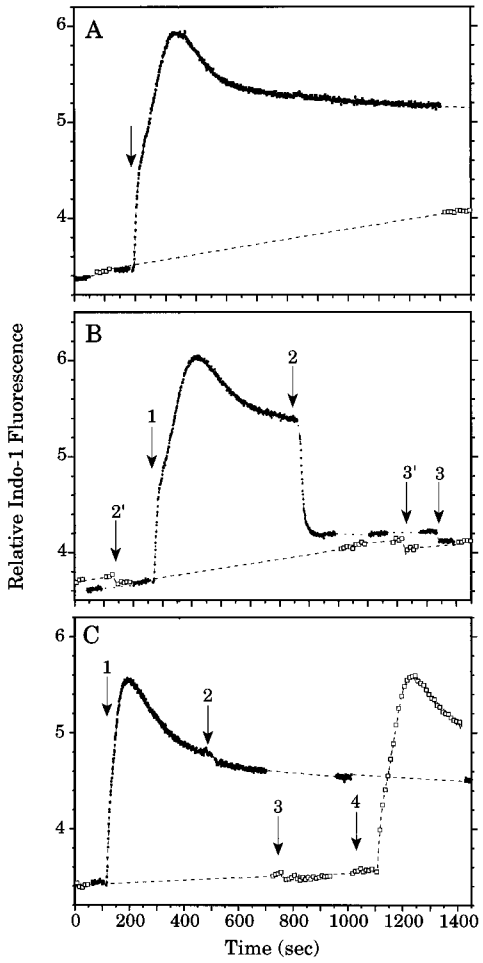

FIGURE 1: Effects of monovalent ligand addition on the Ca^{2+} responses to reversible (A and B) and irreversible (C) cross-linking ligands. (A) Representative experiment showing the typical biphasic Ca^{2+} response elicited by multivalent Ag. Cells, sensitized with IgE and then loaded with indo-1, were triggered by the addition of 100 ng/mL DNP-BGG (filled trace, arrow). The control sample (open squares) was not stimulated. (B) Representative experiment showing the capacity of monovalent hapten to shut off the Ca^{2+} response. DCT (2.0 μM) was added to cells (filled trace, arrow 2) that had been triggered with DNP-BGG (filled trace, arrow 1) as in panel A. The same dose of DCT was added to control cells (open trace, arrow 2′) to account for inner filter effects caused by DCT addition. A second dose of DCT (2.0 μM) was added to both the control sample and the test sample (arrows 3 and 3′). (C) The effect of biotin addition on the Ca^{2+} response induced by sAv-mediated cross-linking. Cells were indo-1 loaded and sensitized with biotinylated IgE. The test sample (filled trace) was first stimulated with 1 nM sAv (arrow 1), followed by the addition of 20 μM biotin (arrow 2). The control sample (open trace) was first challenged with premixed biotin and sAv (20 μM biotin and 1 nM sAv, arrow 3) and then with 100 ng/mL DNP-BGG (arrow 4).

RESULTS

Stable Biotin−sAv Cross-Links Sustain Signaling without Continuous Formation of New Cross-Links. Figure 1A shows the Ca^{2+} response for anti-DNP IgE-sensitized RBL

cells stimulated with an optimal dose of multivalent Ag. Following an initial increase in the intracellular Ca^{2+} concentration that is maximal ~2 min after Ag addition, a plateau phase that depends entirely on the stimulated Ca^{2+} influx (Fewtrell *et al.*, 1989) is sustained for many minutes. As shown in Figure 1B (filled trace), addition of a large excess of the monovalent hapten, DCT, after ~10 min of stimulation with DNP-BGG returns the stimulated Ca^{2+} levels to near baseline with a half-time of less than 1 min. A second addition of DCT to the test sample (Figure 1B, arrow 3) shows that the stimulated Ca^{2+} response can be returned all the way to baseline when a sufficient amount of monovalent hapten is added. These results are similar to those previously described (Weetall *et al.*, 1993; Maeyama *et al.*, 1988), and they indicate that monovalent hapten can shut off the Ag-triggered Ca^{2+} response either because it disaggregates a critical subpopulation of receptors or because it prevents the formation of new receptor cross-links.

To distinguish between these two possibilities, we exploited the high-affinity binding of streptavidin (sAv) and biotin. sAv addition to RBL cells that have been sensitized with biotin-conjugated IgE (biotin-IgE) forms stable, effectively irreversible aggregates of biotin-IgE−FcϵRI (Green, 1975). Figure 1C shows a representative experiment in which the addition of sAv (arrow 1, filled trace) causes a Ca^{2+} response that is qualitatively similar to that elicited by multivalent Ag (compare with Figure 1A). Addition of a large excess of biotin subsequent to sAv (Figure 1C, arrow 2) has no significant effect on the Ca^{2+} response to sAv. Simultaneous addition of sAv and biotin, premixed in the same molar ratio as the used for the sequential additions, elicits no detectable Ca^{2+} response from control cells (Figure 1C, open symbols, arrow 3), indicating that a sufficient amount of biotin has been added to block all biotin binding sites on the sAv. The functional responsiveness of the control cells is verified by their response to multivalent antigen (Figure 1C, arrow 4). These results show that these stable, irreversible cross-links of IgE−FcϵRI, unlike reversible, Ag-mediated cross-links of IgE−FcϵRI, can sustain signaling without ongoing formation of new cross-links.

The capacity of irreversibly cross-linked IgE−FcϵRI to sustain downstream signaling events was further investigated by monitoring stimulated exocytosis of β-hexosaminidase. In a set of experiments analogous to the Ca^{2+} experiments described above, the effectiveness of monovalent ligand in halting degranulation was compared for reversible cross-linkers and irreversible cross-linkers. Figure 2A shows the time-dependent exocytosis of β-hexosaminidase resulting from stimulation with DNP-BSA (arrow 1, ○). As shown previously (Fewtrell, 1985; Seagrave *et al.*, 1987; Erickson, 1988) for RBL cells and consistent with the above Ca^{2+} experiments, subsequent addition of a monovalent hapten, DCT (arrow 2), to DNP-BSA-stimulated cells rapidly halts degranulation (Figure 2A, ●). In contrast, for cells that have been sensitized with biotin-IgE and stimulated with sAv (Figure 2B), addition of a large excess of biotin (arrow 2, ●) has no effect on the degranulation response. Consistent with results from Ca^{2+} experiments, addition of premixed biotin and sAv does not cause β-hexosaminidase release (□) that is significantly different from the spontaneous level (△). These results corroborate those from studies with oligomeric IgE (Kent *et al.*, 1994) which conclude that stable, irreversible cross-links of FcϵRI sustain signaling in the absence of the ongoing formation of new cross-links.

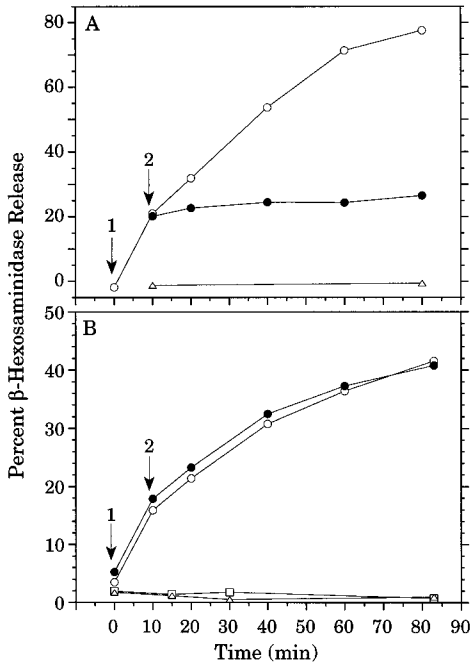

FIGURE 2: Effects of monovalent ligand addition on the degranulation response to reversible (A) and irreversible (B) cross-linking ligands. (A) DNP-BSA (100 ng/mL) was added (arrow 1) to IgE-sensitized cells in suspension at $t = 0$ min, and the stimulated (○ and ●) and spontaneous (△) release of β-hexosaminidase was monitored as a function of time as described at 37 °C. The results are plotted as the percent of total cellular β-hexosaminidase. At $t = 10$ min, DCT (2.0 μM) was added to one of the samples (●), and aliquots of cells were removed at the indicated time points. (B) Cells were sensitized with biotin-IgE and then not stimulated (△) or stimulated with either 1 nM sAv (○ and ●) or 1 nM sAv premixed with 20 μM biotin (□) at $t = 0$ min (arrow 1). After 10 min, excess biotin (20 μM) was added to one of the sAv-stimulated samples (arrow 2, ●). Aliquots were removed, and β-hexosaminidase release was determined.

Cytochalasin D Decreases the Sensitivity of Ag-Stimulated Downstream Signaling to Reversal by Monovalent Hapten. Previous studies have shown that inhibitors of actin polymerization, including cytochalasins B, D, and E (Urata & Siraganian, 1985), as well as dihydrocytochalasin B (Pfeiffer *et al.*, 1985), enhance Ag-dependent degranulation in RBL-2H3 cells under conditions in which they inhibit Ag-stimulated actin polymerization (Oliver *et al.*, 1988; Apgar, 1990). At least part of this effect on degranulation is due to cytochalasin-dependent enhancement of the terminal steps in exocytosis (Narasimhan *et al.*, 1990). Urata and Siraganian (1985) established that the enhancement of stimulated degranulation and $^{45}Ca^{2+}$ influx by cytochalasin B, D, and E exhibits a similar dose dependence that is maximal at concentrations of these inhibitors greater than or equal to 10^{-6} M. As represented in Figure 3, 2 μM cytochalasin D enhances the cytoplasmic Ca^{2+} response to an optimal dose of Ag, both at the peak and in the plateau phase. Consistent with the dose dependence for cytochalasin D determined by Urata and Siraganian (1985), 1 μM cytochalasin D causes a similar amount of enhancement and 0.25 μM causes a partial effect (data not shown). In six independent experiments with 2 μM cytochalasin D, the average enhancement of the peak Ca^{2+} response was 40 ± 8%. This is consistent with an enhancing effect of cytochalasin D on Ca^{2+} influx, a

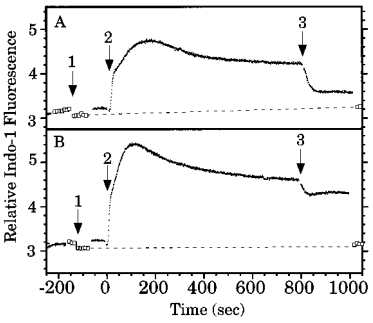

FIGURE 3: Effects of cytochalasin D on the Ag-stimulated Ca^{2+} response of RBL-2H3 cells. For both experiments, either in the absence (A) or in the presence (B) of 2 μM cytochalasin D, cells in the test sample were stimulated with 200 ng/mL DNP-BSA (filled traces, arrows 2), while the cells in the control sample (open squares) were not stimulated. DCT (0.5 μM) was added to both the test sample (filled traces, arrows 3) and the control sample (open traces, arrows 1).

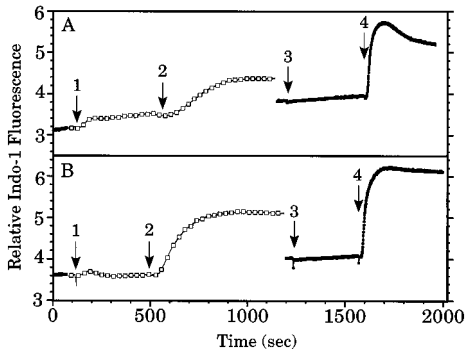

FIGURE 4: Ca^{2+} response elicited by aggregation of derivatives of the GD_{1b} ganglioside in the absence (A) or presence (B) of 2 μM cytochalasin D. In both experiments, RBL cells were sensitized overnight with excess IgE and then harvested and loaded with indo-1. For the test sample (open symbols), AA4 was added to a final concentration of 0.5 μg/mL (arrow 1), followed by the addition of rabbit anti-mouse γ (2.5 μg/mL, arrow 2). The control sample (filled trace) was stimulated with 5 ng/mL DNP-BSA (arrow 4) after the addition of rabbit anti-mouse γ (2.5 μg/mL, arrow 3).

relatively early step in the sequence of downstream signaling events that follow Ag-stimulated tyrosine phosphorylation.

Figure 3 also compares the effect of a limited excess of monovalent hapten, DCT, on the Ca^{2+} response in the presence (Figure 3B) and absence (Figure 3A) of cytochalasin D. This hapten binds to the anti-DNP IgE employed with an affinity that is 10-fold greater than that of ϵ-DNP-L-lysine (Erickson *et al.*, 1986). Figure 3A shows that 0.5 μM DCT decreases the plateau phase of the Ca^{2+} response caused by 200 ng/mL DNP-BSA (with 40 nM DNP groups) by 65%, whereas in the presence of cytochalasin D, this response is only decreased by 15% (Figure 3B). In six experiments in which this ratio of monovalent hapten to multivalent Ag was maintained, the average reduction due to DCT addition in the absence of cytochalasin D was 60 $\pm$ 7%, whereas in the presence of cytochalasin D, the average reduction was 20 $\pm$ 9%. This difference is statistically significant ($p < 0.001$) on the basis of a Student's *t* test. At larger ratios of DCT to DNP-BSA, cytochalasin D was usually less effective at preventing the reversal of the response by monovalent hapten, and at a sufficiently large excess of DCT (15 μM), cytochalasin D was ineffective, probably because dissociation of Ag was complete (data not shown). These results suggest that cytochalasin D preferentially enhances the capacity of poorly dissociable aggregates to stimulate Ca^{2+} responses.

Our laboratory recently presented evidence for the involvement of detergent-resistant plasma membrane domains in FcεRI-mediated activation of RBL cells (Field *et al.*, 1995, 1997; Pierini *et al.*, 1996). We postulate that coalescence of these membrane domains with aggregated FcεRI initiates the signaling process. Because both a cell surface IP_3 receptor (Fujimoto *et al.*, 1992) and a calcium ATPase (Fujimoto, 1993) have been localized to caveolae, a subset of detergent-resistant membrane domains in other cell types (Parton & Simons, 1995), we investigated whether direct aggregation of membrane domain components could cause Ca^{2+} mobilization by a similar, but FcεRI-independent, process. The α-galactosyl derivatives of the ganglioside GD_{1b} that are recognized by the mAb AA4 (Basciano *et al.*, 1986) are co-isolated almost entirely with these membrane domains (Field *et al.*, 1995) and are co-redistributed with aggregated FcεRI on the cell surface in parallel with another domain marker, the fluorescent lipid probe $DiIC_{16}$ (Thomas *et al.*, 1994; Pierini *et al.*, 1996). Binding of AA4 alone does not cause a significant Ca^{2+} response from RBL-2H3 cells (Figure 4A, arrow 1), but subsequent addition of a secondary Ab (arrow 2) does elicit a Ca^{2+} response that is somewhat smaller in magnitude than the response to cross-linking of IgE−FcεRI by a near-optimal dose of DNP-BSA (arrow 4). To eliminate the possibility that AA4 could activate tyrosine phosphorylation by simultaneous binding to the ganglioside derivatives via AA4 combining sites and FcεRI via the AA4 Fc region, we added excess monomeric IgE prior to AA4 addition (Swaim *et al.*, 1994). Also, the secondary Ab causes no detectable response from RBL cells in the absence of AA4 (Figure 4A, arrow 3), indicating that it is not stimulating via cross-reactivity with IgE.

Similar to the response to FcεRI aggregation, the Ca^{2+} response to aggregation of bound AA4 is enhanced by treatment with cytochalasin D. As shown in Figure 4B (arrow 2, open symbols), the Ca^{2+} response caused by cross-linking of AA4 by secondary Ab is increased by ~100% over the response of cells in the absence of cytochalasin D (Figure 4A). The response of control cells (Figure 4B, filled trace) to DNP-BSA (arrow 4) is also increased after treatment with cytochalasin D as shown above (Figure 3). The secondary Ab (Figure 4B, arrow 3) does not elicit a response in the absence of AA4. These results are representative of three separate experiments and demonstrate that direct aggregation of a component of detergent-resistant membrane domains can cause Ca^{2+} mobilization in RBL-2H3 cells in a microfilament-regulated process. The implications of these results for the mechanism of FcεRI-mediated Ca^{2+} responses are discussed below.

Because cytochalasin D treatment affects Ca^{2+} mobilization in Ag-stimulated RBL cells in the presence of excess monovalent hapten, we investigated possible effects on degranulation. Similar to the results in Figure 2A, addition of 2.5 μM DCT 10 min after 100 ng/mL DNP-BSA substantially reduces the degranulation response of RBL-2H3 cells in the absence of cytochalasin D (Figure 5, ○ versus ●). In contrast, the addition of the same dose of DCT to Ag-stimulated cells that have been treated with cytochalasin D has little effect on the degranulation response (Figure 5, □ versus ■). The kinetics of degranulation in this experiment were purposely slowed down by initiating IgE−FcεRI aggregation when the cells were at room temperature,

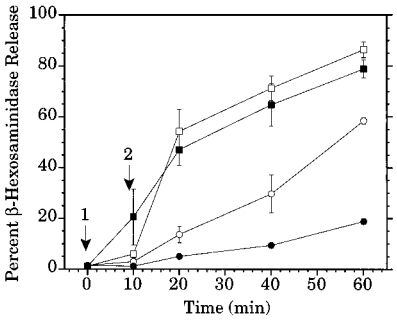

FIGURE 5: Cytochalasin D prevents monovalent hapten from stopping the degranulation response. Adherent cells were either treated with 2 μM cytochalasin D (□ and ■) or not (○ and ●) for 10 min at room temperature. DNP-BSA (arrow 1, 100 ng/mL) was added at $t = 0$ min, and then the cells were warmed to 37 °C for 10 min prior to the addition of DCT (2.5 μM, arrow 2, ■ and ●) and maintained at this temperature. Error bars represent the range of duplicate samples when that range is larger than the width of the data points.

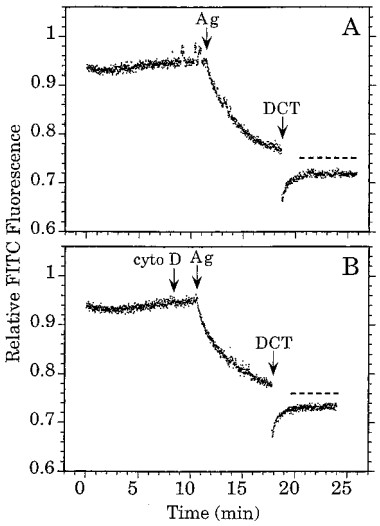

FIGURE 6: Effect of cytochalasin D on DCT-mediated dissociation of DNP-BSA from the cell surface. Suspended cells, sensitized with FITC-IgE and maintained in BSS with 0.45 M sucrose, were either treated with 2 μM cytochalasin D for 3 min at 37 °C (B) or not (A) prior to stimulation with 500 ng/mL DNP-BSA (Ag) and subsequent addition of 10 μM DCT. The binding of DNP-BSA to FITC-IgE on RBL cells is shown by the quenching of the fluorescence caused by the filling of the IgE binding sites; DNP-BSA dissociation after DCT addition (DCT arrow) is shown by fluorescence recovery. Quenching of FITC-IgE fluorescence by the addition of DCT in the absence of Ag was determined in a parallel experiment (dotted line). The readily dissociable Ag is considered to be that corresponding to the fluorescence recovery plateau occurring within 7 min after DCT addition. The difference between this plateau and the maximal possible recovery (dotted line) corresponds to the poorly dissociable Ag.

then allowing the cells to warm to stimulatory conditions at 37 °C for 10 min prior to the addition of monovalent hapten. Under these conditions, we have observed results qualitatively similar to those in Figure 5 for four separate experiments. However, we found that cytochalasin D treatment does not overcome the effect of DCT addition under all conditions. When Ag is allowed to bind to the cells at 15−22 °C for 10 min prior to addition of DCT and then the cells are warmed to 37 °C, the degranulation response of both cytochalasin D-treated and untreated cells is halted by the presence of DCT (data not shown). Together, these results suggest that it is necessary to initiate a temperature-dependent signaling pathway, possibly Ca^{2+} influx, prior to addition of monovalent hapten in order for cytochalasin D to overcome the reversing effect of DCT on the subsequent degranulation response.

Cytochalasin D Does Not Affect DCT-Mediated Dissociation of Ag from the Cell Surface. We used the FITC-IgE fluorescence quenching method to determine whether cytochalasin D has any effect on the binding and dissociation properties of DNP-BSA. At temperatures above 15 °C, cross-linking of FITC-IgE−FcϵRI with multivalent Ag generally leads to internalization and consequent reduction in FITC fluorescence in the low-pH environment of acidic endosomes (Menon *et al.*, 1986). However, in the presence of 0.45 M sucrose, this internalization is prevented, and Ag dissociation at 35 °C from cytochalasin D-treated versus untreated RBL cells can be compared directly.

Figure 6A shows the time-dependent binding of DNP-BSA to FITC-IgE on the surface of RBL cells at 35 °C as monitored by FITC fluorescence quenching. Addition of DCT ~8 min after Ag, in a molar ratio similar to that of DNP-BSA as used for the degranulation experiments described above, causes some immediate further fluorescence quenching due to filling of unoccupied sites by DCT, followed by fluorescence recovery as DCT displaces DNP-BSA. These data and those from two other independent experiments under the same conditions indicate that, following DCT addition, 65 ± 13% of the bound DNP-BSA dissociates from cell-bound IgE−FcϵRI with a half-time of 73 ± 10 s, and the remainder dissociate at a much slower rate. As shown in Figure 6B, treatment of the cells with cytochalasin D prior to the addition of DNP-BSA has no effect on either the binding kinetics or the extent of binding of DNP-BSA. Likewise, cytochalasin D does not affect the dissociation of DNP-BSA caused by DCT addition; in this case, 65 ± 8% of the DNP-BSA dissociates with a half-time of 78 ± 10 s.

Effects of Cytochalasin D on Tyrosine Phosphorylation of Syk and Other Cellular Proteins. One of the earliest known signaling steps to follow FcϵRI aggregation is tyrosine phosphorylation of multiple cellular proteins (Benhamou & Siraganian, 1992; Beaven & Metzger, 1993), including the protein tyrosine kinase Syk (Hutchcroft *et al.*, 1992; Benhamou *et al.*, 1993). Thus, we investigated whether cytochalasin D affects DCT-mediated reversal of the tyrosine phosphorylation events. For these experiments, sensitized, adherent RBL cells were stimulated with DNP-BSA in the presence or absence of cytochalasin D in a protocol similar to that for the degranulation experiment shown in Figure 5. After 8−10 min of stimulation at 37 °C, DCT was added to some samples and cells were lysed 2 min later. As shown in Figure 7A, immunoblotting of whole cell lysates reveals that cytochalasin D causes tyrosine phosphorylation of a protein at ~75 kDa in the absence of antigen stimulation (compare lanes 1 and 2). In the absence of cytochalasin D, antigen stimulates tyrosine phosphorylation of a number of different bands (compare lanes 1 and 3), and tyrosine phosphorylation in the presence of cytochalasin D and antigen (lane 4) is approximately additive. Monovalent hapten (DCT) added after antigen substantially reduces the stimulated tyrosine phosphorylation of several bands in the range of 66 to ~150 kDa, but other stimulated bands are less affected (compare lanes 3 and 5). Apparently, the remaining tyrosine phosphorylation is not adequate to maintain a full degranulation response (Figure 5). In the

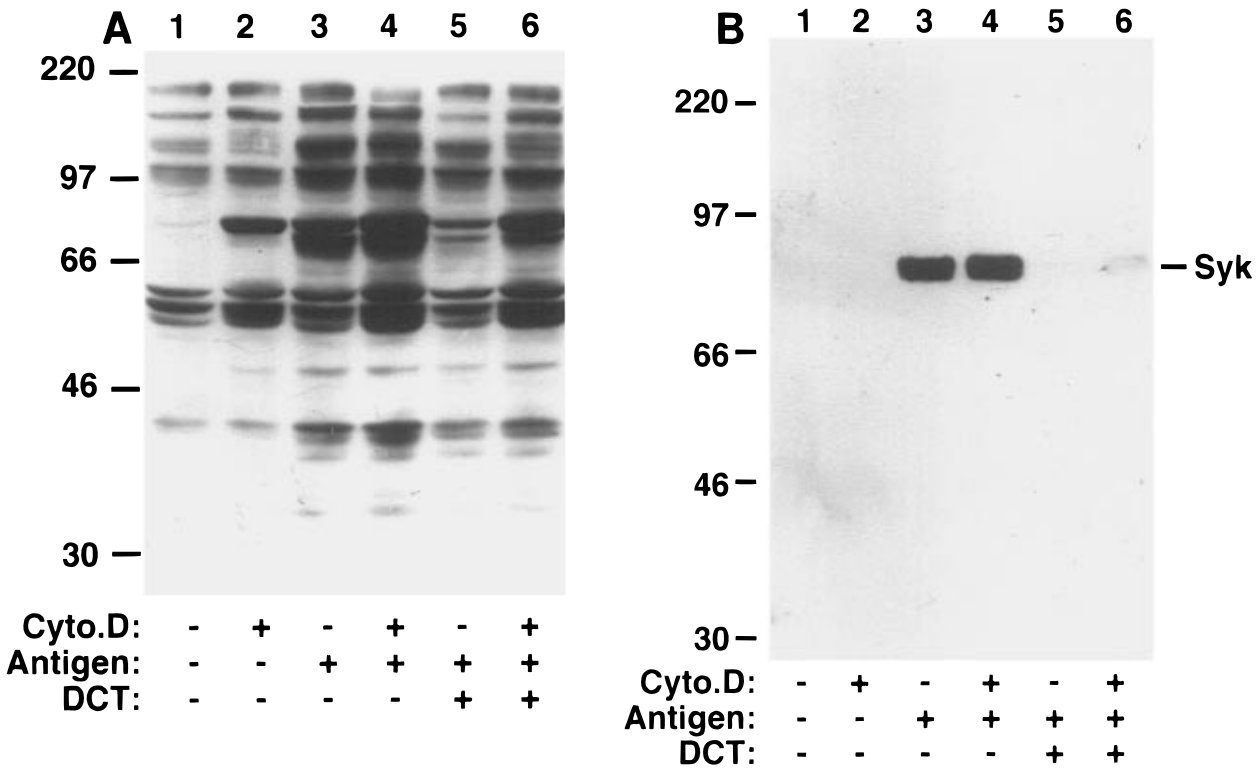

FIGURE 7: Effect of cytochalasin D on the capacity of monovalent hapten to reverse Ag-stimulated tyrosine phosphorylation in whole cell lysates (A) and of Syk (B). Cells were incubated for 8 min at 37 °C with 200 ng/mL DNP-BSA (lanes 3−6) or no stimulus (lanes 1 and 2) in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 2 μM cytochalasin D prior to the addition of 4.0 μM DCT (lanes 5 and 6) for 2 min. (A) Whole cell lysates were analyzed by reduced SDS−PAGE and immunoblotted with anti-phosphotyrosine mAb. (B) Anti-Syk immunoprecipitates were analyzed by SDS−PAGE in the absence of reducing agents and immunoblotted as in panel A. Numbers (kilodaltons) on the left-hand side indicate the positions of the molecular mass standards.

presence of cytochalasin D, monovalent hapten has only a small inhibitory effect on some antigen-stimulated tyrosine phosphorylation bands (compare lanes 4 and 6), consistent with its minimal effect on antigen-stimulated degranulation under these conditions (Figure 5).

To determine whether the antigen-stimulated tyrosine phosphorylation and degranulation that is maintained in the presence of cytochalasin D and monovalent hapten is dependent on the Syk tyrosine kinase, we immunoprecipitated this protein and determined its state of tyrosine phosphorylation as a measure of its kinase activity (Rowley *et al.*, 1995; Shiue *et al.*, 1995). As shown in Figure 7B, antigen-stimulated tyrosine phosphorylation of Syk is not significantly affected by cytochalasin D (compare lanes 3 and 4), and addition of monovalent hapten subsequent to antigen causes nearly complete loss of Syk tyrosine phosphorylation both in the presence and in the absence of cytochalasin D (lanes 5 and 6). As discussed below, this result is surprising in view of the strong dependence of stimulated whole cell tyrosine phosphorylation on Syk in the absence of cytochalasin D (Zhang *et al.*, 1996). Apparently, cytochalasin D reduces the dependence of tyrosine phosphorylation, Ca^{2+} responses, and degranulation on sustained Syk activation. Cytochalasin D-mediated tyrosine phosphorylation of the ~75 kDa protein(s) (Figure 7A, lane 2) may be important for this effect, possibly by reducing the dependence of a more downstream tyrosine kinase on sustained Syk activation.

DISCUSSION

Evidence for Functional Heterogeneity among Aggregated IgE−FcεRI. The present study showed that stable FcεRI cross-links formed by sAv-mediated cross-linking of biotin-IgE−FcεRI elicit strong Ca^{2+} and degranulation responses that are not halted by the addition of excess biotin (Figures 1C and 2B). These results are consistent with those obtained previously with covalent oligomers of IgE (Kent *et al.*, 1994), as well as with recent studies employing surface-attached photoaffinity ligands that covalently couple IgE and immobilize FcεRI (Tamir *et al.*, 1996). Together, they demonstrate that ongoing formation of new FcεRI cross-links is not an essential requirement for sustained signaling via FcεRI. Thus, it appears that stably aggregated FcεRI can be active for extended periods of time, in contrast to some G-protein-coupled receptors (Benovic *et al.*, 1988) and ligand-gated ion channels (Hess *et al.*, 1987) that desensitize following transient activation in response to ligand binding.

On the other hand, several studies indicate that desensitization of FcεRI *can* limit the response of these receptors under some conditions. Our laboratory previously described heterologous desensitization of FcεRI in RBL cells in which aggregation with oligovalent DNP-containing Ag of a subpopulation of FcεRI sensitized with anti-DNP IgE causes unresponsiveness to dansyl-BSA by anti-dansyl IgE−FcεRI on the same cells (Weetall *et al.*, 1993). Partial desensitization of FcεRI responses in RBL cells due to excess cross-linking has also been described (Fewtrell, 1985; Seagrave & Oliver, 1990). In the present experiments, addition of monovalent DNP hapten rapidly halts multivalent DNP-Ag-mediated Ca^{2+} and degranulation responses of RBL cells (Figures 1B and 2A), whereas dissociation experiments indicate that significant amounts of multivalent Ag remain bound to cell surface IgE−FcεRI long after the functional

response is halted (Figure 6). Thus, these and similar results described previously (Seagrave *et al.*, 1987; Erickson, 1988; Holowka & Baird, 1990) demonstrate that some of the aggregated FcεRI are desensitized under conditions where full cellular responses are normally observed.

How can these seemingly different results with different types of cross-linkers be reconciled? Part of the answer to this question comes from the fact that only a small fraction of FcεRI in active aggregates is required to sustain a signaling response (Fewtrell, 1985; Maeyama *et al.*, 1988). Thus, both for stable cross-links and for reversible cross-links in the absence of binding competitors, a full signaling response can be sustained even if a substantial fraction of receptors has been desensitized. However, for a reversible cross-linker, such as DNP-BSA, addition of a monovalent competitor can stop the functional response. Two possible explanations for this are the following. (1) A subset of aggregated FcεRI is responsible for all of the signaling, and this subset is preferentially disaggregated by addition of monovalent hapten. (2) All FcεRI are only transiently active before moving to a desensitized state, so monovalent hapten addition stops signaling by preventing the continuous recruitment of new FcεRI into the existing aggregates. The results obtained with irreversible FcεRI cross-linkers, oligomeric IgE (Kent *et al.*, 1994), covalently attached antigen (Tamir *et al.*, , 1996), and sAv, described above, are most consistent with the first explanation, and thus indicate functional heterogeneity among aggregated IgE−FcεRI. We recently described a similar situation for T cells conjugated to cell-sized beads that were coated with anti-TCR antibodies; addition of excess soluble anti-TCR Fab halts the Ca^{2+} response to the beads without disrupting the conjugates (Hashemi *et al.*, 1996). This suggests that functional heterogeneity among cross-links may be a general property of multichain immune response receptors.

Actin Polymerization Affects the Capacity of Poorly Dissociable Ag Cross-Links to Stimulate Ca^{2+} and Degranulation Responses. Insight into the molecular basis for sensitivity to monovalent hapten-mediated reduction of functional responses has come from the use of cytochalasin D in inhibiting Ag-stimulated actin polymerization. In these experiments, the reversing effects of monovalent hapten addition on DNP-BSA-stimulated Ca^{2+} and degranulation responses are substantially diminished in the presence of cytochalasin D (Figures 3 and 5). Cytochalasin D does not affect the capacity of monovalent hapten to dissociate DNP-BSA from the cell surface under these conditions (Figure 6), consistent with the previous study of Seagrave *et al.* (1987), in which dihydrocytochalasin D only modestly accelerated dissociation of multivalent DNP-phycoerythrin in the presence of excess monovalent hapten. The most critical parameter in the capacity of cytochalasin to overcome the inhibitory effects of monovalent hapten is the ratio of monovalent hapten to multivalent antigen; insufficient monovalent hapten causes only small inhibitory effects with or without cytochalasin D, whereas very large amounts of monovalent hapten can cause dissociation of all bound antigen (K. Xu, D. Holowka, and B. Baird, unpublished observations) and results in complete reversal of Ca^{2+} and degranulation responses (L. Pierini, D. Holowka, and B. Baird, unpublished observations). These observations indicate that the responses maintained in the presence of monovalent hapten and cytochalasin D (Figures 3 and 5) must be due to the poorly dissociable aggregates which contribute very little to the functional response in the absence of cytochalasin D. Similar trends observed for both suspension cells (Figure 3) and adherent cells (Figure 5) indicate that the effects of cytochalasin D characterized here are not critically dependent on the state of attachment of the cells.

Surprisingly, cytochalasin D does not prevent monovalent hapten from dramatically reducing the stimulated tyrosine phosphorylation of Syk (Figure 7B) under the same conditions where there is relatively little reversal in tyrosine phosphorylation of whole cell substrates (Figure 7A, lane 6). Because tyrosine phosphorylation of Syk contributes only a small amount to the stimulated response in the 72 kDa region of the whole cell blots (Benhamou *et al.*, 1993), this nearly complete inhibition of Syk tyrosine phosphorylation (Figure 7B, lane 6) causes only a modest reduction in p72 tyrosine phosphorylation in lane 6 of Figure 7A. Variability among experiments cannot account for these different trends, as panels A and B of Figure 7 are derived from the same lysate samples. The results indicate that treatment with cytochalasin D can substantially compensate for inhibition of Syk activation as measured by Syk tyrosine phosphorylation. Stimulation of ~p75 tyrosine phosphorylation by cytochalasin D in the absence of antigen has been consistently observed in more than six separate experiments, and this may be relevant to the compensatory effect. Our results do not imply that treatment with cytochalasin D can substitute for Syk activation in FcεRI-mediated responses, as this treatment alone does not stimulate Ca^{2+} or degranulation responses. Rather, it is likely that tyrosine phosphorylation (and activation) of Syk is necessary for the *initiation* of antigen-stimulated responses, but not for the maintenance of the acitvation state under these conditions.

In a recent study, Paolini *et al.* (1996) showed that FcεRI in both readily dissociable aggregates and poorly dissociable aggregates exhibit tyrosine phosphorylation, but only the former complexes activate Syk efficiently. They proposed that larger aggregates formed later in the response to multivalent antigen are less readily dissociable and do not activate Syk, possibly because of interactions between these complexes and the cytoskeleton. Their results and ours are consistent with a model in which cross-linking by multivalent Ag normally leads to active aggregates that reversibly associate with relevant signaling molecules, and also to poorly dissociable aggregates that do not make or maintain these associations and therefore do not signal. Ag-mediated cross-links break and reform continuously, but in the presence of monovalent hapten, reformation of active cross-links is prevented and signaling ceases because poorly dissociable aggregates are normally desensitized as discussed above. A likely explanation for our present findings is that inhibition of stimulated actin polymerization by treatment with cytochalasin D permits the poorly dissociable aggregates to maintain productive associations with signaling molecules that are sufficient to sustain Ca^{2+} and degranulation responses. In this model, treatment with cytochalasin D reduces the interaction of these aggregates with polymerized actin and thereby facilitates their interaction with signaling molecules. Consistent with this, Seagrave *et al.* (1991) showed by backscattered electron imaging that dihydrocytochalasin B prevents the formation of large clusters containing tightly packed IgE−FcεRI aggregates that may not efficiently couple to certain signaling molecules.

Plasma membrane domains may be involved in microfilament-mediated regulation of FcεRI signaling. We find that cytochalasin D enhances the Ca^{2+} responses caused by both FcεRI and the GD_{1b} gangliosides recognized by mAb AA4, indicating a microfilament-regulated process (Figures 3 and 4). The association of Lyn with these GD_{1b} gangliosides in solubilized cell extracts (Minoguchi *et al.*, 1994b) and in isolated detergent-resistant membrane domains (Field *et al.*, 1995) suggests a mechanism by which aggregation of the ganglioside via AA4 mAb causes Lyn to aggregate and thereby facilitate its activation by transautophosphorylation (Schuh & Lublin, 1995). Aggregation of FcεRI may activate Lyn by a similar process, as aggregated FcεRI associate with membrane domains containing both the ganglioside and Lyn (Field *et al.*, 1997). Poorly dissociable aggregates of IgE−FcεRI may be those surrounded by gel-like (Thomas *et al.*, 1994) membrane domains and therefore slow to diffuse away from each other upon breakage of a cross-link, whereas more readily dissociable aggregates may be those on the periphery of these membrane domains.

In summary, it is now clear from the results of this study and others that stable aggregates of FcεRI can continue to deliver stimulatory signals for extended periods of time. Nevertheless, ample evidence also exists that individual IgE−FcεRI can become desensitized. It appears that there is heterogeneity with regard to the transition from an active to a desensitized FcεRI aggregate. Some aggregates formed by multivalent Ag are desensitized relatively early in the degranulation response, whereas others remain active for extended periods of time unless they are disrupted by monovalent hapten competition. The present results also indicate that not all signaling pathways stimulated by FcεRI aggregation are desensitized in parallel. In the presence of cytochalasin D, tyrosine phosphorylation of Syk is halted by the addition of monovalent hapten which leaves only the poorly dissociable aggregates. However, Ag-stimulated phosphorylation of many other proteins is maintained, as are Ca^{2+} and degranulation responses. The most likely interpretation of these results is that hapten-sensitive aggregates of FcεRI are responsible for Syk tyrosine phosphorylation but, in the presence of cytochalasin D, poorly dissociable aggregates can maintain more downstream signaling once cellular activation has been initiated. Apparently, the microfilament cytoskeleton normally regulates cellular responses by decreasing the effectiveness of poorly dissociable complexes in downstream signaling. Other mechanisms of desensitization of FcεRI-mediated responses are also likely to contribute to the overall regulation of this response. Further experiments will be necessary to understand more fully the structural and mechanistic bases for these complex regulatory processes.

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