Chemical Cross-Linking of IgE−Receptor Complexes in RBL-2H3 Cells

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ABSTRACT: Aggregation of the high-affinity receptors for IgE (FcεRI) on mast cells activates nonreceptor kinases and other enzymes, thereby initiating a complex biochemical cascade. We have employed a chemical cross-linker in order to stabilize the association of FcεRI with other cellular proteins that are involved in the early events. We reacted the water-soluble, membrane-impermeant chemical cross-linker 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) with permeabilized rat mucosal mast cells of the RBL line and analyzed immunoprecipitates of the receptor in detergent lysates of cells that had biosynthetically incorporated [^{35}S]cysteine. Gel electrophoresis revealed substantial amounts of nonreceptor components only when the cells had been reacted with DTSSP. Receptors isolated from cells whose receptor-bound IgE had been aggregated with antigen prior to cross-linking yielded a similar pattern of ^{35}S-labeled proteins. However, when the cells had also been exposed to [γ-^{32}P]ATP, the proteins associated with the cross-linked, aggregated receptors revealed enhanced incorporation of ^{32}P compared to those associated with cross-linked, unaggregated receptors. A variety of antibodies were used to try to identify the associated proteins. Of those tested for, two, the src-like kinase p53/56^{lyn} and the δ isoform of protein kinase C, were associated with the cross-linked FcεRI in amounts much greater than could be accounted for by nonspecific cross-linking.

The high-affinity receptor for IgE (FcεRI)[1] stimulates a variety of responses in mast cells and basophils, and plays an essential role in mediating allergic responses (Siraganian, 1988; Dombrowicz et al., 1994; Takai et al., 1994). The interaction of receptor-bound IgE with a multivalent antigen leads to the aggregation of the receptor—the critical step that initiates the subsequent course of events. Much has been learned about the early biochemical pathways that are activated, but many fundamental questions remain. Among these uncertainties is the nature of the entity that is aggregated. Is it only the receptor as defined by its subunits FcεRIα, -β, and the dimer of -γ, or are there other proteins associated with FcεRI before or after it is aggregated?

Evidence for such receptor-associated proteins has recently been obtained from studies in which either the receptor (or receptor analogs) or a candidate protein was immunoprecipitated (Eiseman & Bolen, 1992a,b; Hutchcroft et al., 1992b; Li et al., 1992; Jouvin et al., 1994). However, the findings are markedly influenced by the conditions used for solubilization of the receptors, and the stringency with which the immunoprecipitates are washed (Hutchcroft et al., 1992a; Pribluda et al., 1994). At the most fundamental level, this makes it difficult to distinguish between physiological and artifactual associations. In addition, it is difficult to determine what fraction of the receptors are associated with a particular protein, whether more than one of the candidate proteins is associated with an individual receptor simultaneously, and whether the associations change upon aggregation of the receptors.

We are exploring chemical cross-linking as a potentially useful adjunct to help resolve such ambiguities. In the past, chemical cross-linking assisted us both in discovering the β and γ subunits of the receptor (Holowka et al., 1980; Holowka & Metzger, 1982; Perez-Montfort et al., 1983) and in determining their stoichiometric relationship to the receptor's IgE-binding α subunit. In those earlier studies, we had no persuasive evidence for other components associated with unaggregated FcεRI (Holowka & Metzger, 1982; Perez-Montfort et al., 1983), and we were unable to solubilize sufficient amounts of chemically cross-linked aggregated receptors to analyze (Holowka & Metzger, 1982).

In the current study, we modified our methods in a variety of ways, influenced both by our recent experience on the solubilization of aggregated receptors (Mao et al., 1992), by the report of Sarosi et al. on T lymphocyte receptors (Sarosi et al., 1992), and by the studies already cited, that suggested that FcεRI-associated proteins were there to be found.

MATERIALS AND METHODS

Reagents. The monoclonal anti-DNP murine IgE from the hybridoma Hi-DNP ε 26.82 (Liu et al., 1980) and rat IgE of unknown specificity from the immunocytoma IR162 (Bazin et al., 1974) were purified as described (Holowka & Metzger, 1982; Kulczycki & Metzger, 1974). Amidination of the amino groups on IgE with the methyl acetimidate hydrochloride (Research Organics Inc., Cleveland, OH) was carried out as described (Holowka et al., 1980). [^{125}I]IgE was prepared by the chloramine T method (McConahey & Dixon, 1966). Dinitrophenylated-bovine serum albumin (DNP_{25}-BSA, 25 mol of DNP/mol of protein) was prepared as previously described (Isersky et al., 1974) as was the affinity-purified rabbit IgG specific for the $(Fab')_2$ region of IgE (Taurog et al., 1977). The permeabilizing reagent tetanolysin, a toxin derived from *Clostridium tetani*, was a

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[1] Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenyl; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); EMEM, Eagle's minimal essential medium; FcεRI, high-affinity receptor for IgE; RBL, rat basophilic leukemia cells; SDS−PAGE, electrophoresis on a polyacrylamide gel in sodium dodecyl sulfate.

gift from Dr. W. Habig (CBER, FDA, Bethesda, MD). The chemical cross-linker 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) was from Pierce (Rockford, IL). [γ-^{32}P]-ATP was purchased from Dupont NEN (Wilmington, DE). Prestained molecular weight markers used in SDS−PAGE were purchased from Gibco-BRL (Gaithersburg, MD). ^{14}C-Methylated molecular weight markers were from Amersham (Arlington Heights, IL). Antibodies against ERK1, raf-1, rasGAP, and cdc-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); against lyn, protein tyrosine phosphatase 1C, phospholipase C-γ-1, and phosphoinositide 3-kinase from Upstate Biotechnology, Inc. (Lake Placid, NY); against protein tyrosine phosphatase 1D and ezrin from Transduction Laboratories, Inc. (Lexington, KY); and against protein kinase C δ from Gibco-BRL.

Cells. The 2H3 subline of rat basophilic leukemia (RBL) cells was grown adherent in stationary culture (Barsumian et al., 1981) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were harvested following exposure to 0.05% trypsin/0.02% EDTA in Hanks' buffered salt solution (HBSS; Biofluids), washed, and assayed in suspension.

Biosynthetic Labeling. RBL cells grown in a 150 cm^2 tissue culture flask were grown in 25 mL of cysteine-free EMEM (NIH media laboratory) with 16% dialyzed fetal bovine serum (GIBCO) for 2 h. Two millicuries of L-[^{35}S]-cysteine (Amersham) was added to the culture, and cells were incubated for 2 h at 37 °C. The cells were then grown overnight after the addition of 25 mL of complete growth medium containing 10 μg of amidinated-IgE.

Permeabilization of Cells and Chemical Cross-Linking. RBL cells were sensitized with amidinated IgE, washed, and resuspended at 5×10^7 cells/mL in assay buffer (25 mM HEPES, pH 8.0, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.5 mM $CaCl_2$, and 1 mM $MgCl_2$). For permeabilization, cells were first incubated with tetanolysin at 8 μg/mL for 10 min at 4 °C with agitation, then diluted 4-fold with assay buffer containing 2 mM ATP, and further incubated for 5 min at 37 °C. This protocol resulted in the permeabilization of >90% of cells, as assessed by trypan blue uptake. In experiments where [γ-^{32}P]ATP was used to label phosphoproteins, assay buffer containing 100 μM ATP and 39 nM [γ-^{32}P]ATP (about 70 μCi per 5×10^6 cells) was used during the permeabilization step.

Cells (10^7 cells/mL) were stimulated for 2 min at 37 °C with 1 μg/mL DNP_{25}-BSA, and the reaction was stopped by the addition of ice-cold quench buffer (assay buffer containing 5 mM EDTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, and 2 mM iodoacetate, pH 8.0). The chemical cross-linker DTSSP was then added to the permeabilized cells at the desired concentration, the cells were incubated for 30 min at 4 °C, and the reaction was quenched by further incubation with 40 mM glycine for 20 min at 4 °C.

Solubilization and Immunoprecipitation. Cells were solubilized in lysis buffer containing 0.5% TX-100, 50 mM Tris, pH 7.6, 50 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM iodoacetate, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL each of aprotinin, leupeptin, and pepstatin A, for 30 min at 4 °C. The lysates were clarified by centrifugation (16000g, 5 min) to remove insoluble materials. The supernatants were first "pre-cleared" with 50−100 μL of protein A−Sepharose (Pharmacia, Piscataway, NJ) and then immunoprecipitated with various antibodies for 1 h at 4 °C, followed by incubation with 60 μL of 50% protein A−Sepharose (Pharmacia) overnight at 4 °C. The beads were washed 5 times with 400 μL of lysis buffer, and the proteins were extracted with hot sample buffer (25 mM Tris, pH 6.8, 2% SDS, and 10% glycerol) by boiling for 5 min. For those samples where the chemical cross-links were to be cleaved, the sample buffer contained 50 mM dithiothreitol. The eluted proteins were separated by SDS−PAGE on precast gels (Novex, San Diego, CA).

Immunoblotting. Analysis of protein phosphotyrosine using Western blotting by anti-phosphotyrosine antibody was performed as described previously (Alber et al., 1992). Western blotting by various antibodies was carried out using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence for detection (Amersham). Autophotographs and autoradiographs were quantitatively analyzed with a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Permeabilization. For the studies described here, we planned to react permeabilized cells with a membrane-impermeant chemical cross-linking reagent. Therefore, we first assessed the effect of permeabilization on the cellular responses. RBL-2H3 cells were permeabilized using tetanolysin, a toxin derived from *Clostridium tetani*, whose use for permeabilization of cell membranes has been characterized by Rottem et al. (1990). The RBL cells swelled upon permeabilization, their diameter appearing to increase by about 50%. Under the conditions we used (Materials and Methods), >90% of the cells became permeable as monitored by the uptake of trypan blue (molecular mass = 961 Da), and only a few percent of the cells were lysed.

An early consequence of aggregation of FcϵRI is the enhanced phosphorylation of tyrosines in the β and γ subunits of the receptor and in other cellular proteins (Benhamou & Siraganian, 1992; Paolini et al., 1991). We investigated the effect of permeabilization on this phenomenon in order to test the integrity of the system. Cells were sensitized with anti-DNP-IgE, permeabilized, and reacted with DNP_{25}-BSA, and the total cellular proteins were analyzed for phosphotyrosine by Western blotting of unreduced specimens (Figure 1A). In specimens from intact cells, components at approximately 79, 67, 56/53, and 31 kDa showed substantially increased phosphotyrosine after aggregation of FcϵRI (lanes 1 and 2). The specimens from permeabilized cells showed that the intensity of phosphorylation was sensitive to [Mg^{2+}] in both the unstimulated and stimulated cells (lanes 3−10). The patterns observed with the permeabilized cells were less complex than the patterns seen in the intact cells, but in both preparations, the components at 67 and 53/56 kDa exhibited the principal aggregation-dependent increase in phosphotyrosine.

The pattern of phosphorylation observed in the immunoprecipitated receptors is shown in Figure 1B. In intact cells, aggregation of the receptor stimulates an increase in phosphorylation of the β and γ subunits [Figure 1B, lanes 1 and 2, and Paolini et al. (1991), Pribluda and Metzger (1992), and Li et al (1992)]. Multiple components are seen in the molecular mass range for the γ subunit. After reduction, these bands shifted to lower molecular masses as a group (not shown), so they are likely to reflect variable phosphorylation or other modifications of the γ subunit. In perme-

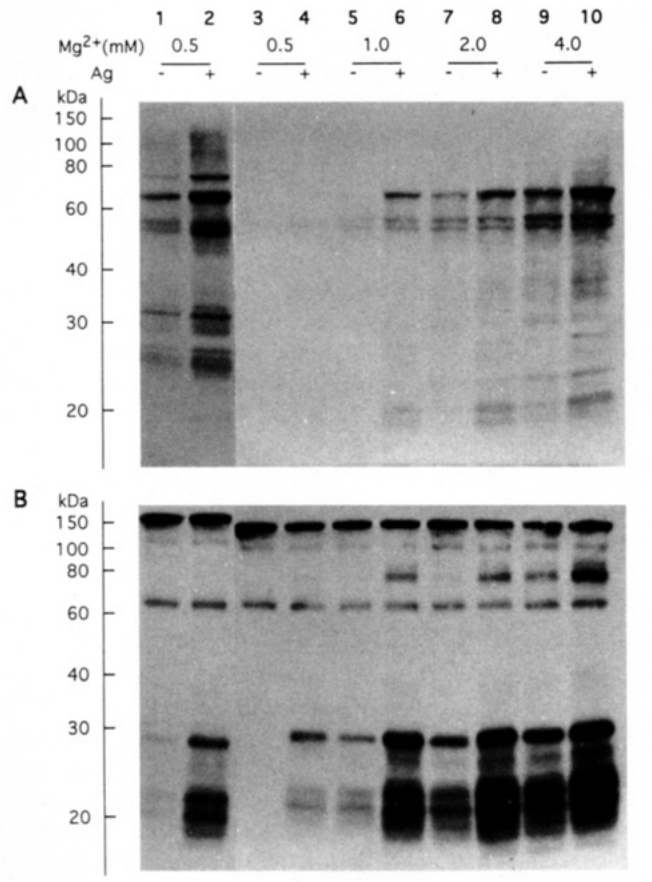

FIGURE 1: Effect of permeabilization on the phosphorylation of protein tyrosines in response to aggregation of FcεRI. The figure shows autophotographs of Western blots of specimens resolved by SDS−PAGE under nonreducing conditions and analyzed for phosphotyrosine. (A) Whole cell extracts (2×10^5 cell equiv/lane). (B) Immunoprecipitated receptors (3×10^6 cell equiv/lane). Lanes 1 and 2, intact cells; lanes 3−10, cells permeabilized by tetanolysin. All the cells had been preincubated with DNP-specific IgE. Those in the odd-numbered lanes were unstimulated (−); those in the even-numbered lanes were reacted with 1 μg/mL DNP_{25}-BSA for 2 min at 37 °C (+). The conditions under which the intact cells were stimulated were the same as those used for the permeabilized cells. The concentrations of $MgCl_2$ in the assay buffer are shown. Immunoprecipitated receptors were isolated and analyzed as described under Materials and Methods. The whole cell extracts were prepared by solubilizing cells directly with hot sample buffer (25 mM Tris, pH 6.8, 2% SDS, and 10% glycerol).

abilized cells, the amount of phosphotyrosine seen in the immune precipitates was also influenced by the [Mg^{2+}] in the incubation medium (Figure 1B, lanes 3−10). The patterns obtained at 1.0 mM [Mg^{2+}] with the immunoprecipitates of the permeabilized cells before and after stimulation (lanes 5 and 6) closely resembled the corresponding samples from intact cells. In these gels, the components at 130 and 65 kDa are artifacts due to the components in the detection system; however, the component at 76 kDa represents the phosphorylation of a cellular protein. It was not detected after reduction (data not shown), but this is difficult to interpret. We have not analyzed it further so far. Together, these results indicate that in the permeabilized cells, the phosphorylation of the receptor is preserved, but that phosphorylation of some of the other cellular proteins is somewhat diminished.

Cross-Linking of Receptors. Figure 2 reproduces autoradiographs of polyacrylamide gels used to analyze immunoprecipitated receptors from biosynthetically labeled, permeabilized cells, reacted with increasing concentrations of cross-linking reagent. The unreduced immunoprecipitates of un-cross-linked receptors revealed only the three bands characteristic of the receptor's α, β, and γ_2 subunits at 60, 30, and 22 kDa, respectively (Figure 2A, lane 1). Virtually no associated proteins were observed. Upon reduction, the bands at 60 and 30 kDa were unchanged (as expected for the α and β subunits), and the 22 kDa component shifted to a molecular mass consistent with disulfide cleavage of the cystine-linked dimer of γ chains (Figure 2B, lane 1). The [^{35}S]cysteine incorporated into these bands was in the ratio 1 (α):1.1 (β):0.6 (γ) (average from three experiments). If one assumes complete equilibration of the [^{35}S]cysteine, the ratio expected from the amino compositions of the subunits is 1:1.2:0.81 (Blank et al., 1989). The results suggest that about 20% of the β and γ chains had dissociated from the α subunit under the conditions we used. [An alternative solubilization protocol using 10 mM CHAPS and 2 mM lipids, that was found to be optimal for stabilizing FcεRI (Rivnay et al., 1982; Kinet et al., 1985), led to very low recovery of receptors from cells reacted with the cross-linking reagent, and so was not used further.]

With increasing doses of DTSSP, the receptor's subunits became cross-linked. This led to the disappearance of the bands representing the subunits of the receptor from their expected position in the gels when the samples had not been reduced (Figure 2A); after reductive cleavage of the cross-linking reagent, the bands representing the subunits reappeared in more or less the expected position although the more heavily modified β subunits show a somewhat higher molecular mass than the wild-type subunit [Figures 2B, 3B, and 4 (left panel), lanes 3 and 4; and Figure 5 (left panel), lane 4]. The substantial increase in total radioactivity seen on both gels indicates that other cellular proteins had become cross-linked to the receptor (below). The amounts of the β and γ subunits recovered after reduction of samples prepared from cells reacted with varying amounts or for varying times with the chemical cross-linker were compared. It is apparent that higher concentrations of cross-linking reagent and longer incubation times resulted in lower recoveries. In these experiments, 11 mM DTSSP for 30 min appeared to be optimal, with more than 90% of the β chains becoming cross-linked as judged from the gels (compare lanes 2 in Figure 2A and Figure 2B). In three such experiments, recovery of the receptor averaged 55 ± 5% (ave ± SEM) relative to the un-cross-linked sample (Figure 2B, lane 1 vs lane 2). Doses of DTSSP lower than 11 mM were also tested, and the results are reported below.

Recovery of Cross-Linked Aggregated Receptors. In prior studies with a membrane-permeant reagent and unpermeabilized cells, recovery of aggregated receptors was a particular problem (Holowka & Metzger, 1982). In the present study, we used a membrane-impermeant reagent (DTSSP) which had been previously demonstrated not to cross-link the subunits of *unaggregated* FcεRI when applied to unpermeabilized cells (Lee & Conrad, 1985). (The absence of a lysine in the ectodomain of the γ chains would in any case preclude the cross-linking of these subunits.) We performed a limited number of experiments on unpermeabilized cells in this study also. Surprisingly, when cross-linking was performed on unpermeabilized cells, the recovery of the *aggregated* receptors, monitored by the [^{125}I]IgE bound to the α subunit, was very poor: With 0.5% TX-100, only

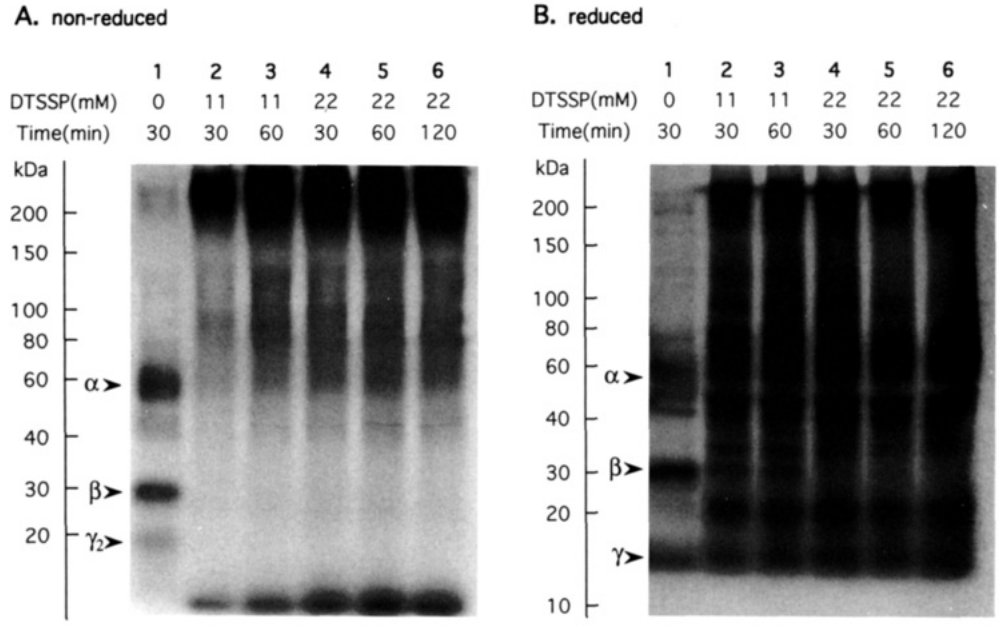

FIGURE 2: Association of FcεRI with cellular proteins stabilized by chemical cross-linking. RBL cells grown in L-[^{35}S]cysteine were reacted with IgE, permeabilized, and reacted with varying concentrations of DTSSP for different lengths of time as indicated. The specimen in lane 1 was from cells that had been permeabilized but that were not exposed to DTSSP. After solubilization of the cells with TX-100, the receptors were isolated by immunoprecipitation with the anti-IgE antibody and analyzed by SDS−PAGE (A) before and (B) after reduction. The figure shows an autoradiograph of the gels. The bands representing the subunits of FcεRI are indicated. The lanes were loaded with all of the receptors recovered from 5×10^6 cells. On the basis of densitometry of the gel at the position of the β component, the relative yields were 1, 0.57, 0.58, 0.33, 0.25, and 0.23 for lanes 1−6, respectively. On the basis of five experiments where [^{125}I]IgE was used, the yield of the material in lane 1 would be expected to be 63 ± 4%; therefore "1" equaled approximately 3.2×10^6 cell equiv in this experiment.

about 35% of the receptor-bound [^{125}I]IgE was extractable, whereas with tetanolysin-permeabilized cells, the recovery was greater than 90%. We have no experimental data that directly explain the difference in detergent solubility for the DTSSP-treated intact and permeabilized cells. We have already noted that after permeabilization the cells swell so that their diameter increased by about 50%. Possibly the reduced local protein concentration and other changes in the plasma membrane of the permeabilized cells reduce the cross-linking of the aggregated receptors with detergent-insoluble cellular components. Almost no phosphorylated β or γ could be recovered from the unpermeabilized cells (data not shown) whereas the recovery was appreciable from the permeabilized cells.

Therefore, we explored how to maximize the yield of aggregated receptors using permeabilized cells. We first tested the effect of varying the order of addition of the cross-linking reagent, DTSSP, and the aggregating agent, DNP_{25}-BSA. RBL cells were sensitized with anti-DNP-IgE. In some samples, DTSSP was added to the cells simultaneously with DNP_{25}-BSA. After 2 min at 37 °C, the cells were diluted with ice-cold "quench" buffer and incubated for a further 30 min before addition of glycine to stop the cross-linking. In other samples, the cells were first activated by antigen and 2 min later the cold quenching buffer was added and only after that the DTSSP. In these samples, the cross-linking reaction was carried out entirely at 4 °C for 30 min. The cells in all of the samples were solubilized with 0.5% TX-100, and the immunoprecipitated receptors were analyzed by Western blotting for phosphotyrosine.

Almost no receptors were recovered from the samples to which DNP_{25}-BSA and DTSSP were added simultaneously (data not shown), but when the antigen and cross-linker were added sequentially, recovery was considerably improved. A representative experiment is shown in Figure 3. The cross-linking efficiency can be calculated from the disappearance of the band representing the β subunit (at ≈30 kDa) in Figure 3A, relative to the corresponding band in Figure 3B. The recovery of the phosphorylated β chain can be assessed by comparing lanes 3−6 with lane 2 in Figure 3B. The quantitative analyses are plotted in Figure 3C. In these experiments, 11 mM DTSSP appeared to be optimal, with about 90% of the β chains becoming cross-linked and 90% of the β chains recovered relative to the un-cross-linked samples. In addition to the bands at the positions of the β and γ subunits, several other bands with which anti-phosphotyrosine reacted more weakly were seen in the cross-linked but not in the un-cross-linked samples (Figure 3B, lane 7). (Lane 7 is simply a 5-fold longer exposure of lane 6 to demonstrate more clearly for a reduced sample the components at the higher molecular masses that had become cross-linked to the receptor.)

In the course of the present study, we have observed a considerable lot-to-lot variation of DTSSP as measured by the efficiency of cross-linking and the recovery of the receptor subunits, although qualitatively it did not alter the pattern of proteins cross-linked to the receptors. We have also noted that the reactivity of anti-phosphotyrosine antibody toward the γ subunit and receptor-associated proteins was sharply diminished after reduction of the cross-linked samples (compare the total radioactivity in lanes 3−6 for Figure 3A,B). We think it likely that this reflects the higher affinity with which the anti-phosphotyrosine antibody reacts with multivalent epitopes (Pribluda & Metzger, 1992). Therefore, the amount of tyrosine-phosphorylated, receptor-associated proteins may be underestimated compared with that detected by ^{32}P labeling particularly for the reduced samples (below).

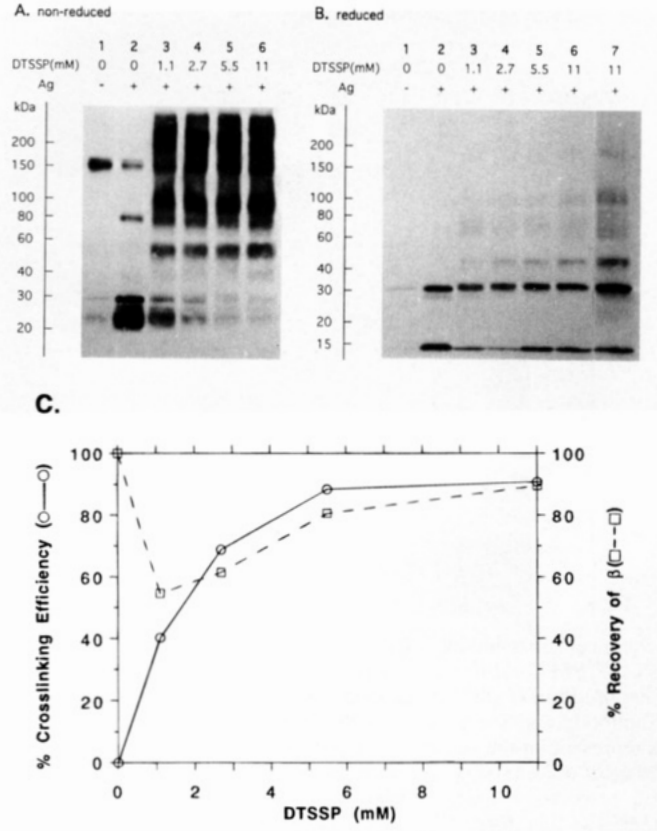

FIGURE 3: Phosphorylation of protein tyrosine residues in receptor-associated proteins. RBL cells were permeabilized, stimulated (+) or not (−) with 1 μg/mL DNP_{25}-BSA for 2 min at 37 °C, prior to the addition of DTSSP at varying concentrations for 30 min at 4 °C (lanes 3−6). The cells were then solubilized in TX-100, and the receptor was immunoprecipitated with anti-IgE antibody. The phosphotyrosine on proteins resolved by SDS−PAGE before (A) and after reduction (B) was detected with anti-phosphotyrosine antibody. (C) Efficiency of cross-linking (○) and relative recovery (□) were calculated (see text) on the basis of densitometric measurements of the band on the gel representing the β subunit. In (B), lane 7 is the same as lane 6 but exposed 5 times longer.

Specificity of Chemical Cross-Linking. It was important to assess whether the multiple components that had become chemically cross-linked to FcεRI (Figure 2) were normally associated with the receptor or had become artifactually cross-linked. Although the latter possibility must be specifically assessed for any particular protein, we explored whether this might be a general problem. The FcεRI itself can serve as an appropriate test for artifactual cross-linking. These receptors are abundant (≈300 000/cell), are known to diffuse in the plasma membrane independently of each other (Schlessinger et al., 1976), and are obviously reactive with the cross-linking reagent. We took advantage of the fact that the rodent receptor binds rat and mouse IgE equivalently (Mendoza & Metzger, 1976) and that these IgEs can be distinguished with specific antibodies. Because the rate constant for dissociation of the IgE from the receptor is $\leq 10^{-5}$ s^{-1} (Kulczycki & Metzger, 1974), exchange of the species-specific IgE during the course of the experiment was not a concern. One aliquot of RBL cells was incubated with approximately equimolar amounts of ^{125}I-labeled nonspecific rat IgE and unlabeled DNP-specific mouse IgE; a second aliquot was reacted with the same IgEs but with the radiolabel on the alternative species. Each sample of cells was handled identically but separately. After the cells were washed, they were subjected to chemical cross-linking, and the solubilized IgE−receptor complexes were immunoprecipitated with either anti-rat or anti-mouse IgE antibody. The results are shown in Table 1.

Table 1: Specificity of Cross-Linking

[^{125}I]IgE[a]	anti-IgE	antigen DTSSP	recovery (%)[b] −−	+−	−+	++
mouse	anti-mouse		55.5	54.3	44.1	37.4
	anti-rat		1.5	1.4	2.4	1.9
rat	anti-mouse		0.2	0.2	0.9	0.6
	anti-rat		58.4	62.0	37.1	39.8

[a] RBL cells were reacted with approximately equimolar amounts of nonspecific rat IgE and DNP-specific mouse IgE in which either the mouse or the rat IgE had been labeled with ^{125}I. [b] The radioactivity recovered in the immunoprecipitates as percentage of the total counts.

In the samples reacted with neither cross-linking reagent nor antigen, 1.5% of the receptors that had bound the labeled mouse IgE were coprecipitated with those that had bound rat IgE; correspondingly, 0.2% of the receptors that had bound the labeled rat IgE were coprecipitated with those that had bound mouse IgE. The coprecipitations could have resulted either from residual physical trapping or from incomplete specificity of the anti-IgE antibodies. In the samples reacted with DTSSP only, the corresponding values had increased 1.6-fold and 4.5-fold, respectively. In the samples reacted only with antigen, no increase in coprecipitation over that in the untreated samples was seen. In the samples reacted with cross-linking reagent and the antigen (in which the antigen will aggregate the receptors bound to mouse IgE but not those bound to rat IgE), there was no further increase in the amount of heterologous coprecipitation above that seen with cross-linking reagent alone. These results indicate that although the cross-linking reagent increased the background, at least for the component we monitored, i.e., FcεRI, the increase was modest compared to the increases seen with other cellular components whose association with FcεRI we are attempting to probe (below). Notably, aggregation of the receptors by antigen did not lead to significant entrapment of unassociated proteins, because such samples showed no increase in the cross-contamination.

Unaggregated vs Aggregated Receptors. We next investigated whether aggregation of the receptors would lead to a change in the spectrum of proteins that became chemically cross-linked to the receptor. Biosynthetically labeled cells were reacted or not reacted with antigen, and thereafter with or without DTSSP; their solubilized FcεRIs were immunoprecipitated. The left side of Figure 4 shows the autoradiograph of a gel on which the reduced immunoprecipitates were resolved, and the right side of Figure 4 shows densitometric traces of the individual lanes. Panel I shows the pattern obtained with the unaggregated, un-cross-linked receptors. The receptor's α, β, and monomeric γ are readily identified, but little radioactivity is seen elsewhere on the gel. Below it, in panel III, is the densitometric trace of the corresponding sample that had been chemically cross-linked (Figure 4, left side, lane 3). Significant amounts of associated proteins are apparent; when normalized as best as possible for equivalent amounts of β, the ^{35}S in the nonreceptor components in the lower trace was almost 40-fold higher than in the upper trace. The average ratio in three experiments was 31.2 ± 8.4% (mean ± SEM). The tracing from the sample of cells stimulated with multivalent antigen but no cross-linking reagent is shown in panel II of

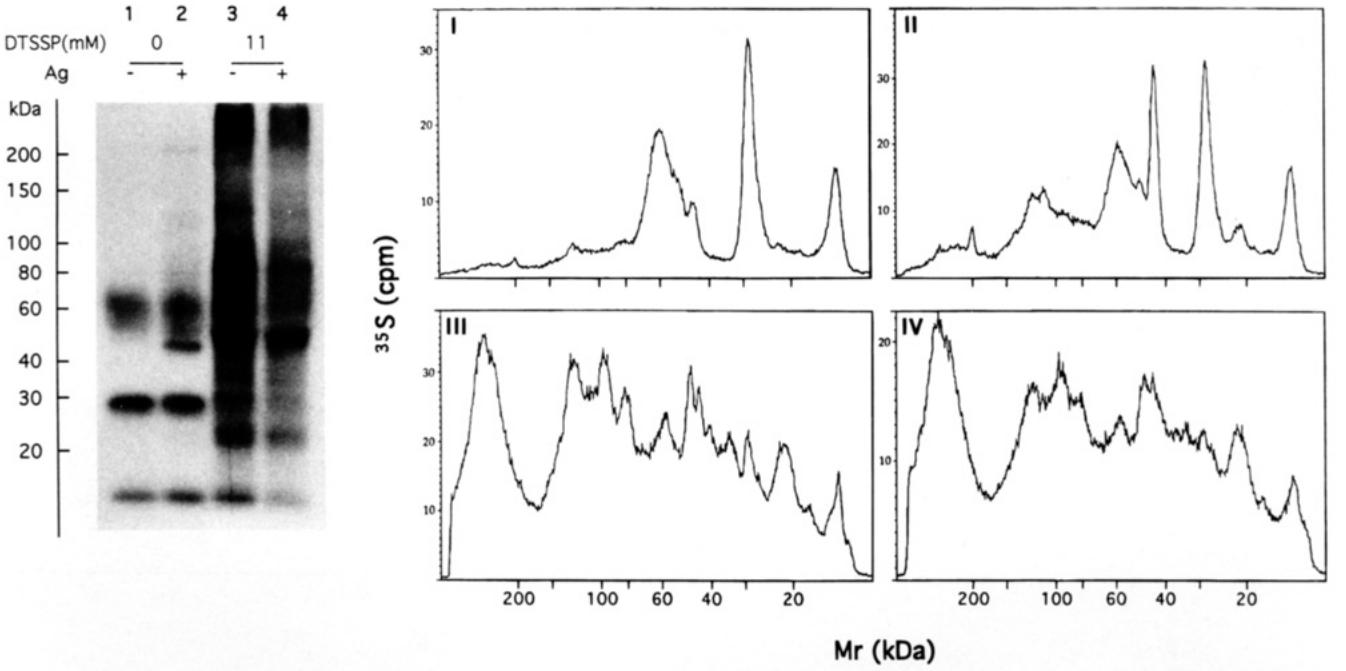

FIGURE 4: Effect of receptor aggregation on receptor-associated proteins. Cells grown in L-[^{35}S]cysteine were permeabilized, stimulated (+) or not (−) with 1 μg/mL DNP_{25}-BSA for 2 min at 37 °C, and then reacted with DTSSP at 11 mM for 30 min at 4 °C (lanes 3 and 4). The cells were then solubilized with TX-100, and the FcϵRI was precipitated with anti-IgE antibody. On the basis of densitometry of the gel at the position of the β component, the relative yields were 1, 0.99, 0.62, and 0.26 for lanes 1−4, respectively. On the basis of five experiments where [^{125}I]IgE was used, the yield of the material in lane 1 would be expected to be 63 ± 4%; therefore, "1" equaled approximately 7.9×10^6 cell equiv in this experiment. The precipitated proteins were resolved by SDS−PAGE under reducing conditions. An autoradiograph (left panel) and densitometric analysis of the gel (right panel) are shown. Panels I−IV correspond to lanes 1−4.

the right side of the figure. Modest amounts of associated proteins were detected with the aggregated non-cross-linked receptors, particularly at higher molecular masses. Similar patterns were seen in repeated experiments except that the sharp spike between the broad peak of the α chain and the β subunit at 30 kDa was not regularly observed. The lower trace (panel IV) is from the corresponding sample that had been chemically cross-linked. (Note that the scale has been expanded to facilitate a comparison with panel III.) The pattern of associated proteins appeared identical whether the receptors were aggregated or not (left side of Figure 4, lanes 3 and 4; right side of Figure 4, panels III and IV). When normalized for recovery of the β chain, the ratio of ^{35}S in the nonreceptor components recovered from the aggregated, cross-linked receptors to the amount recovered from the unaggregated cross-linked receptors was 1.09 for the experiment shown, and 0.82 in a second experiment. These data suggest that, overall, aggregation of the receptors led neither to substantial recruitment of other cellular proteins nor to release of preassociated ones.

The recovery of β (as well as γ) was relatively lower than that of the α subunit-bound [^{125}I]IgE in the chemically cross-linked samples: Setting the yield of β relative to [^{125}I]IgE in the samples from the unstimulated, un-cross-linked cells as 1.0, the corresponding ratios were 1.1 for the stimulated, un-cross-linked cells, 0.8 for the unstimulated, cross-linked cells, and 0.6 for the stimulated, cross-linked specimens. Under the conditions we used, the efficiency of intersubunit cross-linking is less than 100% even for the unaggregated receptors, and the efficiency could be further reduced by steric interference in the antigen-aggregated samples. Thus, the difference between the aggregated and unaggregated receptors from the cross-linked cells may have resulted from cross-linking of β and γ, but not the α subunit, with cytoskeletal elements which preferentially interacted with the aggregated receptors.

If the receptor and any proteins associated with it are more or less in a 1:1 ratio to each other and have roughly equivalent numbers of cysteines per unit molecular mass, one would expect to see increased amounts of radioactivity at the higher molecular masses. Just such a trend is in fact seen.

Several experiments in which the associated proteins were detected on the basis of incorporated [^{35}S]cysteine yielded patterns that were similar to those shown in Figure 4 (right). Although the autoradiographs suggested that discrete components were being cross-linked, the patterns were sufficiently complex that the apparent peaks in the pattern of the nonreceptor components shown in Figure 4 (right side) may not accurately represent the number of discrete species of receptor-associated proteins. More refined analyses will be required to analyze these components.

Studies on Phosphorylated Proteins. As already noted, aggregation of FcϵRI stimulates the phosphorylation of the receptor itself and of other cellular components. It was of interest to see whether some of the latter might be included among those proteins that can be chemically cross-linked to the receptor. Phosphorylation was detected either on the basis of incorporated ^{32}P in those instances where the cells had been exposed to [γ-^{32}P]ATP or by their phosphotyrosine content using anti-phosphotyrosine and Western blotting (Figure 3).

Figure 5 illustrates the results from an experiment in which the cells were incubated with [γ-^{32}P]ATP. In resting cells, the β and γ subunits of FcϵRI were minimally phosphorylated (Figure 5, left, lane 1, and Figure 5, right, panel I). Upon aggregation with antigen, the phosphorylation of the receptor increased substantially, but no major additional

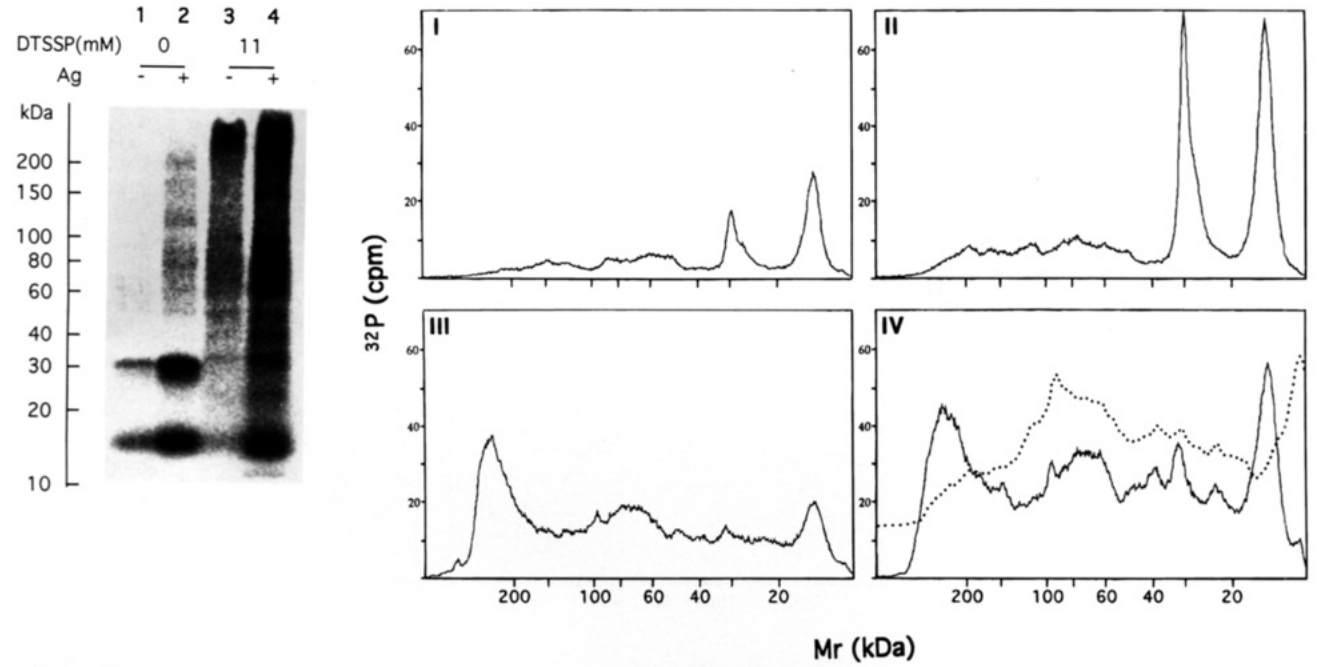

FIGURE 5: Phosphorylation of receptor-associated proteins. RBL cells were permeabilized in the presence of [γ-^{32}P]ATP, stimulated (+) or not (−) with 1 μg/mL DNP_{25}-BSA for 2 min at 37 °C, and the cross-linker DTSSP was added at 11 mM for 30 min at 4 °C (lanes 3 and 4). The cells were then solubilized with TX-100 and the receptors immunoprecipitated with the anti-IgE antibody. On the basis of three to five similar experiments in which [^{125}I]IgE was used, the relative yields would be expected to be 1, 0.94 ± 0.05, 0.69 ± 0.03, and 0.53 ± 0.04 for lanes 1−4, respectively, where "1" equals 3.2×10^6 cell equiv. The precipitated proteins were resolved by SDS−PAGE after reduction, and an autoradiograph of the gel was prepared (left panel). The latter was analyzed by laser densitometry (right panel). Panels I−IV correspond to lanes 1−4. A densitometric trace of the whole cell extract prepared from 1.5×10^5 stimulated cells was rescaled for comparison (dotted line, panel IV).

phosphorylated components were associated with the receptor (Figure 5, left, lane 2, and Figure 5, right, panel II). The chemically cross-linked receptors isolated from unstimulated cells were associated with multiple phosphorylated components (Figure 5, left, lane 3, and Figure 5, right, panel III). Receptors from antigen-stimulated cells reacted with cross-linking reagents showed an overall ≈3-fold increase in associated phosphorylated components after correction for differences in recovery (Figure 5, left, lane 4, and Figure 5, right, panel IV). Figure 5, right, panel IV, also shows the densitometric trace of [γ-^{32}P]ATP-labeled total cellular proteins. Their pattern differs from that of the receptor-associated proteins. Thus, the proteins that became cross-linked to the receptor do not simply represent a nonselective sampling of the more abundant or more extensively labeled proteins in the cellular pool.

As shown above, with the protocol we used, neither the monomeric nor the aggregated receptors become cross-linked to each other artifactually. This allowed us to investigate whether proteins associated with unaggregated FcϵRI would become phosphorylated when only a fraction of the total FcϵRI on the same cell were aggregated. Cells were loaded with approximately equimolar amounts of nonspecific rat IgE and DNP-specific mouse IgE. After stimulation with DNP_{25}-BSA and chemical cross-linking, the solubilized IgE−receptor complexes were immunoprecipitated by either anti-rat or anti-mouse IgE antibody. The proteins associated with each fraction of receptor were analyzed for incorporated [γ-^{32}P]ATP. Whereas substantially enhanced phosphorylation was observed for the proteins associated with the aggregated receptors, no such enhanced phosphorylation was detected on the proteins associated with the monomeric receptor (data not shown).

These data indicate that the nonreceptor proteins that become chemically cross-linked to the receptor become phosphorylated only if the particular receptor with which they are associated is aggregated; i.e., there is no bystander effect for these proteins just like there is none for the receptor itself (Paolini et al., 1991; Pribluda & Metzger, 1992). It provides additional evidence that the proteins found associated with the cross-linked receptors were not just random membrane proteins entrapped during the cross-linking.

Association of FcϵRI with Specific Proteins. Antibodies to candidate proteins were used to probe the cross-linked receptors. The cross-linked FcϵRIs were immunoprecipitated with anti-IgE, the precipitates reacted with $NaDodSO_4$, and the extracts electrophoresed on gels after reductive cleavage of the cross-links. Blots of the gels were probed by Western blotting with the specific antibody. In separate tests, $NaDodSO_4$ extracts of the chemically cross-linked and un-cross-linked cells were similarly probed both before and after reduction. These assays permitted us to compare the total cellular content of a particular protein relative to the fraction that had become cross-linked to some other protein, as well as the integrity of the epitope(s) after exposure to the cross-linking reagent.

Because its association with FcϵRI has been implicated from other studies (Eiseman & Bolen, 1992a,b), our initial experiments utilized antibodies to the src-related p53/56^{lyn} kinase. Under the conditions we used for solubilization and washing, virtually no lyn kinase was observed in the immunoprecipitates of FcϵRI isolated from un-cross-linked cells (Figure 6, lane 1). However, when the cells were reacted with increasing amounts of cross-linking reagent, increased amounts of the 53−56 kDa doublets that correspond to the two isoforms of lyn kinase (Yi et al., 1991)

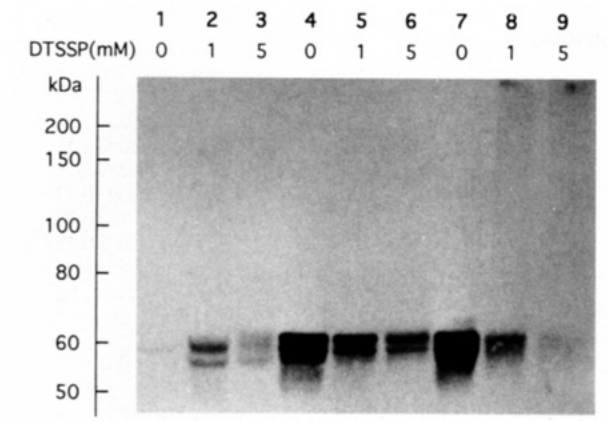

FIGURE 6: Association of lyn kinase with FcεRI. The figure shows a blot of electrophoresed immunoprecipitates of FcεRI (lanes 1−3) or whole cell extracts (lanes 4−9) probed with anti-lyn kinase. On the basis of the precipitated [^{125}I]IgE, the relative numbers of receptors in the first three lanes are in the ratio of 1, 0.87, and 0.77 where "1" equals 3.1×10^6 cell equiv. Lanes 4−9 each contained 1.5×10^5 cell equiv of whole cell extract. The samples were analyzed by SDS−PAGE either with (lanes 1−6) or without reduction (lanes 7−9). None of the cells had been stimulated with antigen. The concentration of DTSSP with which the cells were reacted is indicated.

were detected (Figure 6, lanes 2, 3, and Table 2). The maximum intensity of the bands was obtained when 2 mM DTSSP was used to cross-link 6.7×10^6 cells/mL. Densitometric quantitation of the film reproduced in Figure 6 showed that the cross-linked receptors were associated with over 30-fold more lyn kinase than the un-cross-linked receptors. From the Western blots of the immune precipitates, we estimated that over 3.5% of the total cellular lyn was associated with the cross-linked FcεRI, under conditions where most of the lyn had reacted with the cross-linking reagent (Figure 6, lanes 8, 9).

We performed similar studies with antibodies directed against other proteins present in RBL cells. The proteins were selected either because they participate in early pathways stimulated by FcεRI or because their molecular masses approximate those of proteins that appeared to become cross-linked to the receptor. Table 2 summarizes the results of a detailed analysis of several proteins in addition to lyn. For each protein, we have indicated the reactivity of the antibody used in Western blotting with the whole cell extract of cells cross-linked with 1 or 5 mM DTSSP relative to the un-cross-linked cells (columns 3 and 4 vs 2). The second set of data in columns 5−7 shows the percent of the particular component associated with the immunoprecipitates of the FcεRI from cross-linked samples relative to the amount detected in the FcεRI from un-cross-linked cells. The numbers in parentheses show the absolute percent of the component found in the immune precipitates of the most heavily cross-linked cells relative to the amount estimated for the whole cell extract from the same cells.

As shown by the data in columns 3 and 4, the immunoreactivity of several proteins in the cross-linked samples was moderately impaired. For one in particular, ezrin, the exposure to chemical cross-linker appeared to enhance the immunoreactivity on Western blots, possibly by protecting the protein against the denaturing effects of $NaDodSO_4$.

Of all the proteins tested, only protein kinase C δ showed an enhanced association with FcεRI after cross-linking similar to that observed for p53/56^{lyn} kinase. A blot of one of the three experiments in which the association of protein kinase C δ was probed is shown in Figure 7. Lanes 1−6 were loaded with approximately 4×10^6 cell equiv of immunoprecipitated FcεRI. It is evident that only those specimens from cells treated with 1 or 5 mM DTSSP reacted with the antibody to the kinase and that the results for the samples from cells that had also been reacted with antigen (lanes 4−6) were equivalent to those from unreacted cells. Lanes 7−9 were loaded with 7.5×10^4 cell equiv of the whole cell extracts from the unreacted cells. Densitometric analysis of the Western blot when corrected for the receptor equivalents loaded per lane (based on the counts of receptor-bound [^{125}I]IgE) showed that FcεRI had become cross-linked with up to about 5% of the cell's protein kinase C δ. The average for the three experiments was just under 3% (Table 2). It is interesting that Germano et al. have recently found that protein kinase C δ is primarily associated with the β subunit of FcεRI and is the PKC isozyme that is uniquely able to phosphorylate threonine on the γ subunit of the receptor (Germano et al., 1994).

For each of the proteins shown in Table 2 other than p53/56^{lyn} kinase, similar results were obtained for the immunoprecipitates from cells either stimulated or not stimulated with antigen. Only for the p53/56^{lyn} kinase was an increase regularly observed, and this, as well as a more complete analysis of the interaction between the p53/56^{lyn} kinase and FcεRI, is presented elsewhere (Yamashita et al., 1994).

DISCUSSION

In evaluating the present results, it is useful to recall our earlier experience with the characterization of FcεRI. With the purification procedures we and others employed initially, only the IgE binding α subunit of the receptor was uncovered (Kulczycki et al., 1976; Rossi et al., 1977; Conrad et al., 1976). The associated β and γ polypeptides were only detected when either the conditions for solubilization and subsequent washing were carefully controlled (Rivnay et al., 1982; Kinet et al., 1985), distinctive labeling reagents were employed (Holowka et al., 1981; Perez-Montfort et al., 1983), or chemical cross-linking was used (Holowka et al., 1980; Holowka & Metzger, 1982; Perez-Montfort et al., 1983). With biosynthetic labeling, it became increasingly apparent that the stoichiometry of the new components to the α chain was 1:1:2 (α:β:γ) (Alcaraz et al., 1987). In addition, coordinate synthesis and degradation of α, β, and γ were observed (Quarto et al., 1985), and still later we discovered that in order to get efficient surface expression of the rodent α chain, it had to be cotransfected with the cDNA for β and γ (Blank et al., 1989). Together, these findings form the basis for considering the β and γ chains as authentic subunits of a tetrameric receptor rather than as "receptor-associated proteins" or simply copurifying contaminants.

The problem arises when one begins to detect oth components for which such convincing quantitative data a lacking. In part, this is a conceptual problem: it is as if one is at a stage of ignorance where one is still uncertain whether one is dealing with a subunit of an enzyme, its macromolecular substrate, or even an impurity. Obviously, the quantitative expectations are very different for such alternatives.

In part, it is a methodological problem. That is, the reagents and methods necessary to isolate and purify the

Table 2: Reactivity and Extent of Association with FcεRI of Candidate Proteins before and after Chemical Cross-Linking[a]

protein	relative reactivity[b] at [DTSSP] (mM)			relative receptor associated (% associated)[c] at [DTSSP] (mM)		
	0	1	5	0	1	5
p53/56^{lyn} kinase	1.00	0.55	0.34	1.00	25.8	33.0 (3.63)
protein kinase C δ	1.00	0.76	0.36	1.00	7.35	12.2 (2.81)
phospholipase C-γ	1.00	1.04	0.69	1.00	1.00	1.69 (0.27)
phosphoinositol 3-kinase	1.00	1.03	0.37	1.00	1.33	2.17 (0.26)
raf-1	1.00	1.40	1.10	1.00	0.94	1.00 (0.16)
rasGAP	1.00	1.00	0.84	1.00	1.43	2.43 (0.17)
MAP kinase	1.00	1.05	0.89	1.00	1.00	1.50 (0.06)
phosphotyrosine phosphatase 1C	1.00	0.49	0.32	1.00	1.70	2.26 (0.61)
phosphotyrosine phosphatase 1D	1.00	0.78	0.49	1.00	1.00	1.25 (0.05)
cdc-2 kinase	1.00	0.94	0.49	1.00	0.57	1.00 (0.07)
ezrin	1.00	5.16	6.69	1.00	0.18	0.12 (0.04)

[a] The results shown are from a minimum of two completely separate analyses. [b] The reduction in reactivity toward each antibody as a consequence of the chemical cross-linking was estimated by densitometric analysis of autophotographs of Western blots. In these analyses, whole cell lysates of cells treated with 0, 1, or 5 mM DTSSP for 30 min at 4 °C were compared. An example is shown in Figure 6, lanes 4−6, for reactivity toward antibody to lyn kinase. [c] The relative amount of a candidate protein that was associated the FcεRI, with or without prior reaction of the cell with DTSSP, was assessed by comparing the amounts in the whole cell extract and in the immunoprecipitated receptors by Western blotting. The data are presented as relative values for the untreated and cross-linked samples. The numbers in parentheses are the absolute percents obtained at the highest dose of DTSSP. The cell equivalents loaded per lane were determined on the basis of the receptor-bound [^{125}I]IgE, and the densitometric readings were normalized appropriately.

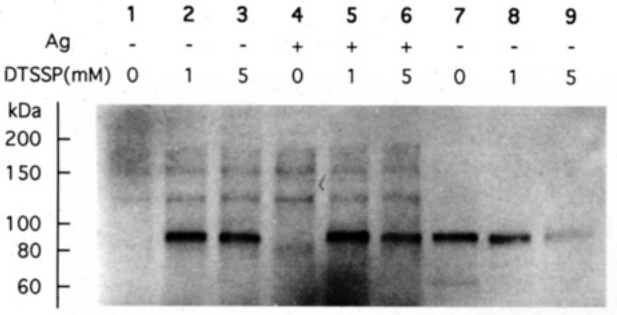

FIGURE 7: Association of protein kinase C δ with FcεRI. The figure shows a blot of electrophoresed immunoprecipitates of FcεRI (lanes 1−6) or whole cell extracts (lanes 7−9) probed with anti-protein kinase C δ. Each of the first six lanes was loaded with an amount of receptors equivalent to about 4 × 10^6 cells. Where indicated, the cells were stimulated with antigen for 2 min. The last three lanes contained 7.5 × 10^4 cell equiv of whole cell extract from unstimulated cells. The concentration of DTSSP to which the cells had been exposed is shown. Densitometric analyses of the amount of the kinase in the sample were corrected for the receptors loaded per lane based on the receptor-bound [^{125}I]IgE in the sample. All the specimens showed ≥90% solubilization and ≥80% immunoprecipitation of the FcεRI.

receptor may themselves disrupt the associations one is attempting to define. Additionally, the extraordinary sensitivity of contemporary methods that make it possible to detect vanishingly small amounts of material also make it increasingly difficult to distinguish between gold and garbage. Thus, in our experiments we could readily detect 1 molecule of lyn kinase per 1000 molecules of receptor; similar values have been estimated in un-cross-linked samples for fyn kinase relative to the T-cell antigen receptor by Samelson et al. (1990) and by Benhamou et al. (1993) for syk kinase associated with FcεRI.

There is no simple way to resolve these dilemmas. Rather, one must apply a variety of structural and functional approaches in order to ascertain the physiological state. The findings reported here lead us to conclude the following:

(1) Whatever components may be associated with FcεRI physiologically, the components interact much less firmly with the receptor than the α, β, and γ subunits of the receptor do with each other.

(2) Following chemical cross-linking, multiple components are found to be covalently attached to FcεRI in significantly greater amounts than are likely to have resulted from artifactual cross-linking. Thus, after reaction with cross-linking reagent, the increase in receptor-associated [^{35}S]-cysteine-labeled proteins, the p53/56^{lyn} kinase, and the δ isoform of protein kinase C exceeded almost by an order of magnitude the increase observed with a control protein (other FcεRI) known to be independent of the receptor (Table 1) or with other cellular proteins we examined (Table 2). Interestingly, the increase in the association of lyn kinase with FcεRI after chemical cross-linking was quantitatively very similar to the increase in total protein as assessed by biosynthetic labeling. The most obvious explanation is that the chemical cross-linker stabilized the association of the receptor with a discrete, multicomponent "signaling particle" (Ullrich & Schlessinger, 1990; Paolini et al., 1992), a possibility we are currently exploring. The rotational mobility of FcεRI is substantially less than is predicted from theory and would be consistent with receptor interacting with other cellular components even prior to aggregation (E. Y. Chang, S.-Y. Mao, H. Metzger, D. Holowka, and B. Baird, unpublished results).

(3) If a "signaling particle" exists, our data suggest its association with FcεRI is not substantially promoted by aggregation of the receptor. However, we cannot exclude the possibility that aggregation promotes the association or disassociation of particular nonreceptor components. Indeed, we have evidence for an increased association for one component, p53/56^{lyn} kinase [see Results and Yamashita et al. (1994)]. Eisman and Bolen (1992b) provided evidence that this kinase is preassociated with FcεRI but could not determine whether the enhanced lyn kinase activity they observed following aggregation represented activation of the kinase or recruitment of additional kinase by the aggregated receptor. Benhamou et al. (1993) concluded p72^{syk} becomes associated only after the receptors are aggregated and phosphorylated.

(4) The phosphorylation of the receptor-associated components is confined to those associated with the receptor being aggregated. As with the phosphorylation of the

receptors themselves (Paolini et al., 1991; Pribluda & Metzger, 1992), there appears to be no "bystander" effect.

(5) The protocol we used for chemical cross-linking, which closely resembles that described by Sarosi et al. (1992), provides a useful adjunct for studying the complex macromolecular machinery utilized by the class of membrane receptors we are investigating. In favorable circumstances, it can detect proteins that are associated in vivo but whose interaction is difficult to sustain during isolation. A variety of methodological problems need to be overcome, however. Immunological assays for a candidate protein may be foiled if the chemical cross-linking reduces too drastically the immunoreactivity of the candidate protein. The use of polyclonal antibodies directed toward a variety of regions on the protein should minimize this problem. It is helpful if one has independent evidence that the candidate protein becomes cross-linked to other cellular proteins (and not just internally) in order to be sure that, in principle, an association of interest could have been detected. If these minimal criteria can be demonstrated, they help to make a "negative" result more meaningful.

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