Interaction between the Unphosphorylated Receptor with High Affinity for IgE and Lyn Kinase*

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Chinese hamster ovary fibroblasts previously transfected with the high affinity receptor for IgE (FceRI) were further transfected with the α subunit of the receptor for interleukin 2 (Tac) or with chimeric constructs in which the cytoplasmic domain of Tac was replaced with the C-terminal cytoplasmic domain of either the β subunit or the γ subunit of FceRI. Whereas native Tac failed to affect the aggregation-induced phosphorylation of FceRI, both chimeric constructs substantially inhibited this reaction. Alternatively, the FceRI-bearing fibroblasts were transfected with two chimeric constructs in which the cytoplasmic domain of Tac was replaced with a modified short form of Lyn kinase. The Lyn in both of the chimeric constructs had been mutated to remove the sites that are normally myristoylated and palmitoylated, respectively; one of the constructs had in addition been altered to be catalytically inactive. The catalytically active construct enhanced, and the inactive construct inhibited, aggregation-induced phosphorylation of the receptors. All of the chimeric constructs were largely distributed outside the detergent-resistant microdomains, and whereas aggregation caused them to move to the domains in part, their aggregation was neither necessary nor enhanced their effects. These results and others indicate that the receptor and Lyn interact through protein-protein interactions that neither are dependent upon either the post-translational modification of the kinase with lipid moieties nor result exclusively from their co-localization in specialized membrane domains.

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1 The abbreviations used are: DRM, detergent-resistant membrane; DNP, 2,4-dinitrophenyl; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)glycine; Pipes, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; BSA, bovine serum albumin.
Characteristics of Tac and chimeric constructs

| Construct | C terminus of CD25a Base | N-terminus of CD25a, or of FcRI β or γ, or of Lyn Base | Junctional sequence |
|-----------|-------------------------|--------------------------------|---------------------|
| TT-T      | 714 237                 | 715 238                        | LLGLTQWRRL        |
| TT-β      | 714 237                 | 660 202                        | LLGLRIGGEE        |
| TT-γ      | 720 239                 | 150 27                         | SGLTWHLKIQG       |
| TT-Lyn    | 714 237                 | 12 4                           | LLGLIEIKSK        |
| TT-LynKR  | 714 237                 | 12 4                           | LLGLIEIKSK        |

We performed complementary experiments by constructing chimeric constructs consisting of the ecto- and transmembrane domains of Tac to a construct of Lyn that had been mutated to chimeric constructs consisting of the ecto- and membrane spanning domains of Tac fused to the active kinase with the receptor (17, 18).

Preparation of Chimeric Constructs—Table I shows selected sequences of the constructs used for our studies. Dr. J. Oliver (University of New Mexico) generously provided the cDNAs for constructs consisting of the ecto- and membrane spanning domains of Tac fused to the C-terminal 43 residues of the β subunit of FcRI (TT-β or TT-γ) in pcDL-SRα286 (16). They were digested with BamHI and EcoRI and subcloned into pZeo-SV (minimum orientation of the multiple cloning site), which had been digested with the same enzymes. The cDNA for wild-type Tac (TT-T), which contains the Tac cytoplasmic domain of 13 residues (20) in pGM, was obtained from W. Leonard (NHBLI, National Institutes of Health) and similarly subcloned into pZeo-SV. We also prepared a construct in which a stop codon was inserted after that coding for leucine residue 238 so that no cytoplasmic domain would be expressed (TT-0).

For the Lyn-based chimeric constructs, we generated fusion proteins by polymerase chain reaction. cDNA coding for Lyn kinase mutated at its N terminus was prepared as follows: sense and antisense primers for the desired mutations of the intact wild-type short form of Lyn (5'-CTCAGGATTAAAATCAAAAAAAGGAAAAGACAATC-3' and 5'-CTCAGGCCCATTGTCGCTGGATCTAACGCTTCCTGCGGACATG-3') were synthesized and used in polymerase chain reaction with the wild-type Lyn as template. The sense primer encodes a XhoI site at its N terminus that excludes the N-terminal methionine and glycine. The primer also substitutes the codon for cysteine with one for glutamic acid. A construct of this type was digested with XhoI and then ligated to the cytotoxicity inhibitory Lyn generated previously (17). Analysis of each construct was determined using the Big Dye® kit obtained from Applied Biosystems (Foster City, CA) and shown to be as planned.
as for transfectants generated by electroporation. Duplicate wells of each clone to be tested were grown to confluence in 24-well plates (≈ 2.5 × 10^5 cell/well). The cells in one set of wells were incubated with 1 μg/ml of [125I]IgE in 0.1 ml for 1 h at 37 °C, washed five times with phosphate-buffered saline (PBS), and then solubilized with boiling hot 1% SDS, in 62.5 mM Tris, 0.5 mM EDTA, and 0.05% PBS buffer. The lysates were then counted.

For expressing the Tac constructs, cells were incubated with 0.5 μg/ml of [125I]anti-Tac in 0.5 ml at 4 °C for 30 min, washed four times with ice-cold Iscove’s medium, and then solubilized and counted as for the cells labeled with IgE. To maintain the integrity of cell surface Tac, cells were harvested using 3 mM EDTA in PBS rather than a hypotonic shock treatment. In addition, all CHO transfectants were cryopreserved in growth media supplemented with 5% (CH₃)₂SO, and a new vial of cells was routinely thawed every 2 months to assure a consistent ratio of Tac to FcRI.

**Quantitation of FcRI and Tac Constructs**—CHO transfectants were harvested and incubated at 5 × 10^6 cells/ml with 5 μg/ml [125I]IgE for 1 h at 37 °C. Non-specific binding was assessed by preincubating the cells with 50 μg/ml unlabeled IgE for 30 min. Cells were then centrifuged through a mixture of phthalate oils (21), and the radioactivity in the pellets was counted. Alternatively, CHO transfectants were incubated at 5 × 10^6 cells/ml with 0.5 μg/ml [125I]anti-Tac for 30 min at 4 °C. Non-specific binding was measured in duplicate incubations with 5 μg/ml unlabeled anti-Tac. Cells were then isolated and counted as above, and the pellets were counted. The number of molecules of Tac was calculated from the recovered radioactive counts and the specific activity of the labeled anti-Tac, assuming one molecule of anti-Tac binds two molecules of Tac protein (22).

**Stimulation of Cells**—Stimulation of CHO transfectants with IgE plus antigen or with preformed covalently cross-linked oligomers IgE was conducted as described previously (17). Where aggregation of Tac plus antigen or with preformed covalently cross-linked oligomers IgE was conducted as described previously (17). Where aggregation of Tac plus antigen or with preformed covalently cross-linked oligomers IgE for 30 min at 4 °C. Non-specific binding was measured in duplicate incubations with 5 μg/ml unlabeled anti-Tac. Cells were then isolated and counted as above, and the pellets were counted. The number of molecules of Tac was calculated from the recovered radioactive counts and the specific activity of the labeled anti-Tac, assuming one molecule of anti-Tac binds two molecules of Tac protein (22).

**Isolation of Plasma Membranes**—For monitoring of FcRI, CHO transfectants were sensitized for 1 h at room temperature with 5 μg/ml IgE, 10% of which had been labeled with [125I]. For monitoring of Tac constructs, the CHO cells were labeled with [125I]anti-Tac for 30 min, at 4 °C. After washing the cells three times with buffer A (4), plasma membranes were isolated by Dounce homogenization and sedimented in 30% sucrose (23). Successive 1-ml fractions were removed from the top of the gradient, the location of the visible band was noted, and the radioactive counts in each fraction were determined in a γ-counter.

**Isolation of DRM**—The method we used is similar to that described by Rodgers and Rose (24) and by Field et al. (8). Briefly, cells were detached with 3% EDTA in PBS and resuspended in growth medium at 5 × 10^6/ml. After the desired treatment, 10^7 cells in 0.5 ml were diluted 2-fold with 0.5 ml of 0.1% Triton X-100. The lysate was kept at 4 °C with gentle agitation for 30 min. It was then mixed with an equal volume of 85% sucrose, transferred to a Beckman 344060 clear cefuge tube and overlaid with 6 ml of 30% sucrose prepared in a P7.5 buffer containing 0.05% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO₄, and 2 mM sodium iodoacetate supplemented with protease inhibitors leupeptin, pepstatin, or apronitin, each at 1 μg/ml, and 5 mM 4-(2-aminoethyl)-benzensulfonyl fluoride or phenylmethylsulfonyl fluoride (buffer A). The 30% layer was overlaid with 3.5 ml of 5% sucrose, again prepared with buffer A. The lysate was centrifuged in a swinging bucket rotor at 38,000 rpm for 12–14 h. Successive 1-ml fractions were removed from the top of the gradient. All fractions were counted. Successive 1-ml fractions were removed from the top of the gradient. All fractions were counted. 

**In Vitro Assays for Associated Kinase**—Triplicate immunoprecipitates with anti-Lyn, anti-Tac, or control antibodies were incubated in 25 μl of kinase assay buffer (25 mM Pipes, 150 mM NaCl, 5 mM KCl, pH 7.2, 5 μM MnCl₂, 2 μM CHAPS, 0.5 mM Na₃VO₄ containing 2 mM substrate peptide. Reactions were initiated by addition of ATP (100 μM ATP, 10 μCi of [γ-32P]ATP (PerkinElmer Life Sciences)) at 25 °C and vortexed every 10–15 min. The reactions were quenched by the addition of 10 μl of 50% trichloroacetic acid. The peptide was then isolated using phosphocellulose filters. The filters were washed twice with 0.5 ml of 0.075 M phosphoric acid and then counted in a scintillation counter in Filtron-X mixture.

**RESULTS**

**Transfection with Tac-FcRI Subunit Constructs**—CHO cells previously transfected with FcRI (17) were further transfected by electroporation with Tac (TT-T) or a chimeric construct containing the extracellular and transmembrane domains of Tac and the C-terminal cytoplasmic domain of either the β chain (TT-β) or the γ chain of FcRI (TT-γ). Stable clones that had been selected with zeocin and characterized for their expression of FcRI and the Tac constructs are listed in Table II. The ratio of TT-β to FcRI ranged from 0.3 to 2.2, and the that of TT-γ to FcRI ranged from 0.2 to 2.5. When analyzed by Western blotting, anti-Tac immunoprecipitates of the detergent extracts of such transfectants revealed a diffuse band of 55 kDa (Fig. 1, lanes 1, 4, and 6).

Although the peptide molecular mass of the constructs is only ~30 kDa, glycosylation of the ectodomain of Tac is known to retard its mobility. The bands with apparently lower molecular mass (38, 40 kDa) likely represent non- or hypo-glycosylated constructs not expressed on the cell surface. Such species were previously described for chimeric constructs of Tac fused to the cytoplasmic portion of the T cell receptor ε chain or the γ chain of FcRI (13).

**TT-β Inhibits the Phosphorylation of FcRI**—Fig. 2 shows the results of an experiment in which cells from clone 2A3E expressing on their surface approximately 0.9 TT-β per FcRI (Table II) were first reacted with anti-Tac, or not, to aggregate the Tac constructs. They were then reacted with 500 ng/ml of either monomeric IgE or a mixture of trimeric and tetrameric IgE for 30 min. Cells transfected with receptors only (CHO-B12) were treated similarly. The receptors were immunoprecipitated and Western blotted with anti-phosphotyrosine. The odd numbered lanes show that there is only minimal phosphorylation of the receptor on cells reacted with the monomeric IgE, whereas substantial phosphorylation of the β and γ subunits is apparent in cells reacted with the oligomers. Similar results were obtained in the cells transfected with TT-β, but in this and repeated similar experiments analyzed quantitatively, the phosphorylation was substantially less (~70% in the experiment illustrated). Prior aggregation of the Tac-β constructs.
neither enhanced nor diminished the inhibition (lane 6 versus lane 8).

Additional studies examined the relationship between the ratio of TT-β to FcεRI and the inhibition of phosphorylation. The IgE receptors on cells with TT-β to FcεRI ratios of either 0.3 or 0.9 were aggregated with increasing concentrations of antigen, and the phosphorysorine on the receptor was compared with identically stimulated control cells (cells transfected solely with the zeocin marker-containing plasmid). A clone expressing a TT-β to FcεRI ratio of 0.3 (clone 2C9E) and stimulated with 80, 150, or 300 ng antigen/ml showed 40, 60, and 60% less phosphorysorine in their β chains and 60, 40, and 30% less phosphorysorine in their dimer of γ chains compared with the control cells (▲ versus ●, Fig. 3). Somewhat greater expression of the construct (clone 2A3E, TT-β to FcεRI ratio of 0.9) showed further inhibition (Fig. 3, ■), the corresponding reductions being 50, 70, and 70% for β and 60, 70, and 50% for the dimer of γ chains. However, the clones expressing a TT-β to FcεRI ratio of −2.0 showed no substantially greater inhibition (results not shown).

Clones containing the TT-β construct were also compared with clones transfected with unmodified Tac (TT-T). Compared with clone 21A4E, clone 2A3E, which showed comparable expression of the Tac epitope (Table II), showed only ½ to ½ as much phosphorysoration in response to a paucivalent antigen (Fig. 4). Cells transfected with the construct coding for a truncated Tac (TT-0) did show repeated although quite variable decreases in phosphorysoration of FcεRI, but notably the cells grew quite poorly compared with all the other transfectants.

**TT-γ Inhibits the Phosphorylation of FcεRI—** Analogous studies were performed on cells transfected with the corresponding chimeric constructs containing the cytoplasmic extension of the FcεRI γ subunit (Table II). When stimulated with 75, 150, 300, or 600 ng/ml antigen, clones expressing a TT-γ to FcεRI ratio of 0.2–0.7 (A3E and B1E) revealed 3, −2, 25, and 22% inhibition of antigen-induced phosphorylation on the β chain, and 8, 5, −14, and 51% inhibition on the dimer of γ chains relative to clones transfected with the plasmid containing solely the zeocin marker (▲ versus ●, Fig. 5). The corresponding inhibitions observed on clones expressing equal numbers of TT-γ and FcεRI (clones A1L, C1L, and D6E; Table II) were 30, 70, 60, and 80% on the β subunit and 30, 70, 40, and 70% on the dimer of γ subunits (Fig. 5, ■ versus ●). The time dependence of inhibition was unremarkable. Compared with cells transfected with TT-T (clone 21A4E), the TT-γ transfectant C1L showed 40–50% less phosphorylation over the 4-min time period studied (data not shown).

**Inhibitory Constructs Are Not Phosphorylated—** In experiments similar to those reported previously (17), we tested whether the inhibitory constructs themselves became phosphorylated. Cells transfected with TT-β were sensitized with IgE, and aliquots were stimulated with 0, 50, or 150 ng/ml DNP-BSA for 2 min. FcεRI were solubilized, immunoprecipitated, resolved by PAGE, and Western blotted with anti-PY. The results shown are the means ± S.E. from five experiments conducted with clones 2A3E and 2C9E.

**Mechanism of Inhibition—** Because a fundamental aim of this study was to investigate further the role of membrane microdomains in promoting the interaction of Lyn kinase with FcεRI, we analyzed the distribution of the inhibitory chimeric constructs. TT-β transfectants were sensitized with [125I]IgE and then stimulated or not with antigen.

Wilson et al. (16) had previously shown that TT-γ and TT-β chimeric constructs are expressed independently on the surface of RBL transfectants by failing to observe co-localization when one or the other fluorescently labeled species was aggregated.
We observed a similar independence on the CHO cell transfectants. In one type of experiment we tested whether the constructs co-immunoprecipitated with the FcRI and failed to observe any co-immunoprecipitation. We also looked for codistribution on sucrose gradients in which the detergent-insoluble plasma membrane domains were separated from the remainder of the cellular components after treating the cells with 0.05% Triton X-100 (8). The gradient fractions were γ-counted to quantitate the IgE receptors and then adjusted to 1% Triton X-100 and divided equally. One aliquot was precipitated with anti-Tac and Western blotted with anti-TAC to localize the TT-β subunit. The results shown are the averages of three determinations from two separate experiments, i.e. single samples in one experiment and duplicate samples in the second experiment. All the data have been normalized relative to the PY on the β subunit of the FcRI in the TT-T transfectants stimulated with 75 ng/ml antigen.

The simplest explanation for the results described so far is that the chimeric constructs competed with the FcRI for the limited amount of endogenous Lyn kinase in the CHO cells. To investigate further the ability of Lyn kinase to interact with TT-β, we modified a protocol we previously used for assaying co-immunoprecipitated Lyn and FcRI (4). To test several variables we first assayed the kinase immunoprecipitated from CHO cells expressing transfected rat Lyn kinase (clone A11 (17)) using rabbit polyclonal anti-Lyn serum or serum from unimmunized rabbits as a control. The immunoprecipitates were washed as described under “Experimental Procedures” and then subjected to an in vitro kinase assay in the presence of [γ-32P]ATP and a peptide that is a relatively specific substrate for Src family kinases (19). The incubation mixtures were applied to phosphocellulose spin filters, which were washed and counted in a γ counter. The activity associated with the anti-Lyn immunoprecipitates led to a linear increase in phosphorylation of the substrate peptide between 0.5 and 2 h, whereas control immunoprecipitates induced only negligible modification (data not shown). More importantly, the amount of phosphorylated peptide recovered was linearly proportional to the amount of cell extract utilized, whereas there was no significant increase in the amount of activity detected in the control preparations.

![Figure 4](http://www.jbc.org/)

**Fig. 4. Specificity of inhibitory action of TT-β.** Clone 2A3E containing the TT-β construct (■) was compared with clone 21A4E expressing a comparable amount of transfected unmodified CD25α (TT-T) (□). The open symbols represent the data for the dimer of γ subunits; the filled symbols represent the data for the β subunit. The results shown are the averages of three determinations from two separate experiments, i.e. single samples in one experiment and duplicate samples in the second experiment. All the data have been normalized relative to the PY on the β subunit of the FcRI in the TT-T transfectants stimulated with 75 ng/ml antigen.

![Figure 5](http://www.jbc.org/)

**Fig. 5. Effect of expression of TT-γ on phosphorylation of FcRI.** FcRI from pZeo transfectants (clones 4E and 5E (○)) and TT-γ FcRI transfectants (clones A1L, C1L, D6E, B1E and A3E (■)) were aggregated with DNP6-BSA for 2 min. The relative amounts of photophosphorylation on the β (top panel) and γ chains (bottom panel) of FcRI are depicted (means ± S.E.) for the five experiments conducted. The data for clones A1L, C1L, and D6E were averaged (■), as were the data for clones A3E and B1E (●).
Structs and Fc.

Lower panel

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tatively. Substantially more kinase activity co-precipitated with the fraction was measured in a top of the gradient, the location of the visible band was noted, and each on sucrose gradients. Successive 1-ml fractions were removed from the supernatant from cells lysed with 0.05% Triton X-100 was fractionated to phosphorylation of the kinase. Notably also, in the absence of peptide, the recovery of counts was only equal to that found in the assay blanks containing no lysate. This shows that the radioactivity we monitored was due to phosphorylation of the peptide and not due to autophosphorylation of the kinase.

We applied the same procedure to assay the endogenous Lyn in immunoprecipitates from the TT-β or TT-T transfectants solubilized under conditions that gave a ratio of micellar detergent to lipid (p) (25) of 3. These conditions were previously shown to stabilize the association of the kinase with FcεRI in RBL cells (4). To minimize nonspecific precipitation of kinase with the protein A-Sepharose beads used for the isolation, the immunoprecipitating or control antibody was prebound to the beads, and the lysates were extensively precleared prior to the specific immunoprecipitation. Fig. 7 (upper panel) shows the averaged results of two in vitro kinase assays of anti-Tac immunoprecipitates from TT-β and TT-T transfectants, respectively. Substantially more kinase activity co-precipitated with the TT-β than with the TT-T. Control immunoprecipitates using an irrelevant antibody showed only a small difference between the two lysates (Fig. 7, lower panel).

Transfection with Tac-Lyn Constructs—Wild-type rat Lyn has the N-terminal sequence MGCIKSK... (17, 26). During its biosynthesis the methionine is removed, the glycine is myristoylated, and the cysteine is palmitoylated (27). The Tac-Lyn chimeric construct we prepared (TT-Lyn) (Table I) truncates the Tac protein after its SGL sequence, substitutes a glutamic acid residue for the N-terminal glycine-cysteine sequence of the mature Lyn, and then continues with the remaining 489 residues of the short form of the rat enzyme (Lyn B (17, 28)). The fourth construct (TT-LynKR), is exactly like Tac-Lyn except that the lysine in the catalytic site (residue 279 in wild-type Lyn) was replaced by arginine, rendering the kinase inactive.

Table II lists the clones examined most intensively. As can be seen, the apparent expression of either FcεRI, Tac, or the chimeric Tac was variable even within a clone (relatively high standard errors), but there was no marked tendency for the expression of FcεRI to be affected differentially by any of the constructs. As noted in Table II, we assumed that one molecule of anti-Tac bound two molecules of the surface expressed Tac construct (22).

Enhanced Response in Transfectant with Chimeric Construct of Lyn (TT-Lyn)—We previously reported that FcεRI on CHO cells transfected with receptor alone (CHO-B12) show virtually no phosphorylation of tyrosines when exposed to dimerized IgE and only a modest response to small doses of a paucivalent dinitrophenylated antigen (DNPε-BSA) after sensitization with anti-DNP IgE. Both the responses to the dimers of IgE and the responses to antigen were progressively enhanced in the cells express increasing amounts of transfected wild-type Lyn kinase (17, 18). The top panels in Fig. 8 shows a similar comparison between CHO-B12 cells and the two clones of cells expressing the highest amount of the transfected catalytically active or inactive Tac-Lyn constructs (clones C4L, 34A3E; Table II). In these panels, the extent of phosphorylation of the β and γ subunits of the receptors 3 min after the addition of a variable amount of DNPε-BSA are depicted separately. All the data were normalized to the amount of anti-phosphotyrosine antibody bound to the β subunit on receptors from the cells reacted with 200 ng/ml of antigen. It is clear that at this dose the extent
of phosphorylation of the β to γ ratio is 1:2, as expected from prior studies (29). Within experimental error, this was equally true at other doses and with the alternative clones depicted in the figure. It can be seen that at all doses of antigen, there is a roughly 2-fold enhancement in the amount of phosphorylation of both the β and γ subunits of the receptors in clone C4L compared with the cells not transfected with the Tac-Lyn construct.

Clone C4L was assessed multiple times (Fig. 8, *left middle panel*). To compare the experiments, the amount of anti-phosphotyrosine bound to the β and γ subunits combined from the cells untransfected with the chimeric construct and stimulated with 200 ng/ml antigen was set at 1, and the amount of phosphorylation determined on all the other samples was normalized to it. Although there was a moderate variability, it is apparent that clone C4L reproducibly shows an enhanced phosphorylation of the receptors compared with the clone untransfected with the Lyn construct. Five additional clones of cells transfected with the same construct (Table II) were examined. The two clones expressing the next highest amounts of the construct (A6L and C6L) showed enhancements closely similar to those observed with clone C4L (Fig. 8, *right middle panel*). However, three clones expressing still lesser amounts (clones B1L, A6L, and D6L) showed only irregular increases in phosphorylation of their receptors at any dose (Fig. 8) or time (not shown). The enhancement was not limited to a particular point in time. As seen in the *bottom panel* of Fig. 8, stimulation with 200 ng/ml of the antigen led to a progressively enhanced phosphorylation of the receptor over the time period examined time.

**Inhibitory Activity of Inactive Construct of Lyn (TT-LynKR)—** In our previous studies, we observed an inhibition of aggregation-induced phosphorylation of FcεRI in cells that had been transfected with a construct of Lyn kinase in which the lysine in the catalytic site had been mutated to arginine. Analogous transfections using Tac-LynKR were assessed in the present study. The data shown by the filled circles and dashed lines in the *top panels* of Fig. 8 are for clone 34A3E, which expressed the highest amount of the construct and somewhat more than the clone expressing the catalytically competent TT-Lyn. It is apparent that in clone 34A3E the receptors were only one-third or less phosphorylated than in the cells transfected with the catalytically active TT-Lyn construct. In six further experiments this clone gave similar results (Fig. 8, *left middle panel*). The time course of phosphorylation of the receptors in this clone is shown in the *bottom panel* of Fig. 8. Finally, the right *middle panel* of Fig. 8 shows our findings with the three clones transfected with Tac-LynKR studied in greatest detail as a function of dose of antigen.

**Topological Distribution of Tac-Lyn Constructs and Effect of Aggregation—** Several of these transfectants were also examined with respect to the distribution of both the Tac constructs and the FcεRI, by density gradient centrifugation in sucrose of Triton X-100 extracts of the cells. To some cell samples 0.2 μg/ml of 125I-labeled murine anti-Tac was added, and the cells were incubated for 20 min on ice and then washed in buffer A. They were resuspended at 1 × 10^7 cells/ml and incubated further with or without 25 μg/ml of anti-mouse IgG Fab fragment for 10 min at 37 °C. Similarly, cells were examined with or without having their receptor-bound IgG aggregated by addition of 200 ng/ml DNP-antigen for 2 min at 37 °C. In the absence of anti-Tac, the TT-Lyn and TT-LynKR constructs, like those containing cytoplasmic domains of the receptor, were distributed virtually exclusively in the fractions containing both soluble and membrane proteins outside the DRM (Fig. 9, ■). For those four clones, ~83% of the Tac was within fractions 9–12 and <5% in fractions 3–5. The distribution was very similar in the cells stimulated with antigen (96 and 1% in the same fractions). As expected the Tac constructs partially translocated to the DRM when they themselves were aggregated either with only the monoclonal anti-Tac (Fig. 9, ○) and even more so when reacted in addition with a second anti-antibody (Fig. 9, △).

Aggregation of either the enhancing TT-Lyn or inhibitory TT-LynKR constructs failed to affect phosphorylation of FcεRI.
Table III shows a detailed analysis of one such experiment in which two clones transfected with LynKR constructs were compared with clone 42C2E. Although transfected with Lyn, the latter clone expressed little of it (Table II) and showed no enhanced phosphorylation of FceRI compared with those transfected with TT-T or vector alone (data not shown). If, in the penultimate column of Table III, one first compares the relative amounts of phosphorylation of the receptors in the cells in which only the FceRI were aggregated, it is apparent that the inhibition observed in clones 31AE (sample 2B) and 34AE (sample 2C) was about 35 and 55%, respectively. The ratio of the relative phosphorylation of the receptors in the respective samples 3 versus 2 (column 9) is in each case not appreciably different than 1, indicating that aggregation of the chimeric constructs did not appreciably enhance their effect.

**DISCUSSION**

**Previous Studies**—Several studies have utilized chimeric constructs in which the cytoplasmic domain of either the β or γ subunits of FceRI were fused to the ecto- and transmembrane domain of an irrelevant protein, such as CD4 (12) or CD25a (13–16). The chimeric constructs generally have been transfected into the rat mucosal type mast cell line RBL-2H3 and challenged with antigens. Yeast two-hybrid studies also showed a weak interaction between Lyn kinase and the C terminus of the β chain; contrariwise, reaction of the γ cytoplasmic domain with Lyn, if any, was too feeble to be observed by this method (17).

We were particularly interested in the observations of Wilson et al. (16), which suggested an experimental way of distinguishing between the two models referred to in the Introduction. Wilson et al. noted that in transfected RBL-2H3 cells, the TT-β construct could inhibit responses stimulated by aggregation of FceRI. They hypothesized that although it could not independently activate downstream events, the β-construct sequestered a critical component required by FceRI, likely Lyn. Their suggestion anticipated our subsequent findings that the Lyn available to the FceRI in RBL cells is in short supply and shuttles between different FceRI (32). We were particularly intrigued that Wilson et al. (16) only observed inhibition when the TT-β construct was aggregated. We hypothesized that this might be explained using the model proposed by Sheets and co-workers (11) as follows: The monomeric TT-β construct would be distributed outside the specialized microdomains but like wild-type CD25a would translocate to the microdomains upon aggregation (33). Then if the interaction between aggregated FceRI and Lyn preferentially occurred in these domains, that could explain why the aggregated but not the monomeric TT-β inhibited the responses triggered by FceRI. The experiments described in this paper were designed to test this explanation.

The system we used differs only somewhat, but we think significantly from that used by Wilson et al. (16). First, rather than RBL cells, we used CHO cells transfected with FceRI. The only response to aggregation of the receptors exhibited by these transfectants is phosphorylation of the receptors themselves. Likely this is because the cells contain no Syk kinase, an essential component for all downstream responses (34). The CHO cells contain only a small amount of Lyn kinase, and therefore their response to aggregation of FceRI is particularly sensitive to manipulations that affect the availability of active Lyn to the receptors (17, 18). Furthermore, by using either small oligomers or relatively low doses of antigens modified only lightly with haptenic groups, we stimulated the cells less vigorously than is generally done. Thus, the aggregates of
TT-β and TT-γ Inhibit the Phosphorylation of FcεRI——Like Wilson et al. (16), we observed inhibition of the FceRI-induced response in those cells transfected with TT-β. The inhibition was specific for the β portion of the construct because the responses of cells transfected with plasmids containing only the antigenic marker were indistinguishable from those transfected with CD 25α, i.e., TT-T.

TT-γ also inhibited the phosphorylation of FcεRI, but three times more TT-γ than TT-β was required to see more or less equivalent inhibition. As already noted, prior studies appeared to yield contradictory results with respect to the capacity of the γ chains to interact with Lyn. That Fc receptors lacking β as well as chimeric constructs of γ can stimulate responses mediated by Lyn strongly favors such coupling, but evidence for a direct interaction could not be obtained from either in vitro kinase assays (15) or the yeast two-hybrid complementation studies (17). Likewise, with TT-γ, Wilson et al. (16) failed to see the same inhibitory effect they observed with TT-β. We believe that all of the results are consistent with this interaction being relatively weak. One likely reason we were able to observe such an interaction in our current studies is that our in vitro assay preserves the normal high mutual concentration and co-localization of Lyn and the cytoplasmic domain of γ at the cytoplasmic face of the plasma membrane. A likely second reason is that as already noted, various features of our protocol make the system particularly sensitive to perturbations of the available Lyn kinase.

Mechanism of the Inhibition——We found direct support for the hypothesis that TT-β inhibits the action of FceRI because it associates with Lyn by comparing the kinase activity in immunoprecipitates of chimeric and control constructs. Utilizing a Src family kinase-specific substrate, the results strongly support such a specific interaction and, being consistent with related studies by others (15, 16), were not surprising.

What was unexpected was that the ability of the constructs to inhibit the response did not require and was not enhanced by the secondary aggregation of the construct with biotinylated anti-Tac and avidin as had been found by Wilson et al. (16). Again, we believe this difference results from the sensitivity of the system we employed and the more limited aggregation of FcεRI we used to stimulate the cells.

Our studies with the chimeric constructs of Lyn yielded complementary results. In previous studies on CHO-B12 cells, we observed enhanced aggregation-induced phosphorylation of tyrosines on the β and γ subunits of the receptors in cells that had been transfected with catalytically active Lyn kinase (17, 18). On the other hand, cells transfected with the catalytically inactive constructs of the kinase showed diminished aggregation-induced phosphorylation of FcεRI. We interpreted those results in the context of a model in which the initial aggregation-dependent phosphorylation of FcεRI results from a transphosphorylation by Lyn kinase constitutively associated with a small percentage of receptors. The transfection with wild-type Lyn was thought to have enhanced the probability that any given aggregate included at least one molecule of active kinase, whereas transfection with incompetent Lyn would decrease that probability.

The experiments with the Lyn constructs were in part aimed to overcome some of the difficulties in interpreting prior experiments in which the interaction of a Src family kinase with a multi-subunit immune response receptor was probed. In a prior study in which we utilized both the yeast two-hybrid methodology and CHO cell transfectants, we obtained evidence that the constitutive interaction occurs between the membrane proximal, unique domain of Lyn and principally the C-terminal cytoplasmic domain of the receptor β chain (17). Those results were consistent with analogous observations by others (16, 31).

A related study in which the association of p59Fyn with T-cell receptors was explored, it was concluded that the N-terminal 10 amino acid residues in the unique domain of the kinase were critical for the coupling. However, the role of certain of these residues in the protein-protein interaction could not be distinguished from those residues involved in localizing the enzyme correctly (35). Thus, the myristoylated glycope polymer anchors the enzyme to the inner leaflet of the plasma membrane, and the palmitoylated cysteine appears to localize it preferentially within microdomains (7).

A related study employed a chimeric construct of CD8 fused to the cytoplasmic domain of the T cell receptor ζ chain and examined its interaction with p59Fyn (36). However, that work examined the interaction between the kinase and phosphorylated ζ immunoreceptor tyrosine activation motifs, whereas what we wish to define is an earlier interaction involving the constitutive association between a Src family kinase and the unphosphorylated receptor. Varying the sequence of the Tac-Lyn chimera used here should now allow us to examine the role of the sequence of the unique domain of the kinase without concern about the extent of localization on the plasma membrane, which can confound the interpretation of the results.

We also wanted to test whether the interaction of the kinase with the receptor could be examined independently of their co-localization in specialized microdomains or “rafts.” All our results suggest that they can be. By employing biochemical fractionation we clearly demonstrated that under the conditions in which TT-Lyn or TT-LynKR amplify or inhibit the phosphorylation of FcεRI, the critical components are largely localized outside the DRM (Fig. 9 and Table III). Wilson et al. (16) did not examine the phosphorylation of FcεRI per se, and

**TABLE III**

**Effect of aggregation on the inhibitory action of construct TT-LynKR**

| Sample | Clone | Antigen | Avidin | PY | JRK | Gross | Net | Ratio samples (3:2) |
|--------|-------|---------|--------|----|-----|-------|-----|-------------------|
| 1A     | 42C2E(TT-Lyn)  | -      | +      | 53 | 1400| 0.038 | 0   |                   |
| 2A     | 16A2E(TT-Lyn)  | +      | -      | 1640| 1385| 1.334 | 1.296 |                   |
| 3A     | 1740 | +      | +      | 1740| 1244| 1.399 | 1.361 | 1.05             |
| 1B     | 31A4E(TT-LynKR) | -      | +      | 70 | 1690| 0.039 | 0   |                   |
| 2B     | +      | -      | 1500 | 1707| 0.879| 0.840 |     |                   |
| 3B     | +      | +      | 1420 | 1778| 0.799| 0.760 | 1.11 |                   |
| 1C     | 34A3E(TT-LynKR) | -      | +      | 30 | 1800| 0.018 | 0   |                   |
| 2C     | +      | -      | 600  | 990 | 0.606| 0.588 |     |                   |
| 3C     | +      | +      | 905  | 1420| 0.637| 0.620 | 1.0  |                   |
we cannot rule out that the later events that they monitored involved receptors localized to the DRM. Possibly, they were able to inhibit these distal events only by driving the chimeric constructs into the DRM through vigorous aggregation. Alternatively, we think it more likely that the more extensive aggregation of FcRII they employed, coupled with the greater supply of Lyn available to the receptors in the RBL cells they transfected, made it more difficult to observe the inhibition by the unaggregated constructs.

It could be argued that in view of the small amount of endogenous Lyn in the CHO cells, the relevant fraction responsible for the phosphorylation of FcRII we monitored was in the DRM but went undetected. Likewise, it was the tiny amounts of the transfected constructs in the DRM that were solely responsible for the effects we observed. In principle, such a thesis is virtually impossible to reject. Nevertheless, our experimental findings cannot be explained simply by the co-localization per se and are only explicable on the basis of protein-protein interactions.

In the experiments of Sheets et al. (10) in which they reported a sharp decline in the initial phosphorylation of FcRII following disruption of the microdomains, large correction factors were required to compensate for major depletions of the receptors that accompanied the experimental procedures. Our observations are more consistent with the recent observations by Yamashita et al. They found that later events, but not the initial phosphorylation of the receptor tyrosines, were inhibited when DRM were disrupted by depleting the cells of cholesterol. Likewise, using different approaches to prevent localization of Lyn kinase to DRM, Kovarova et al. observed no reduction in the initial phosphorylation of the receptor in Syk kinase. Taken together, the data suggest that the constitutive association between Lyn and FcRII can occur outside discrete membrane microdomains and that it is mediated principally by the protein-protein interactions rather than principally by induced co-localization based on surrounding or covalently attached lipids. On the other hand, it seems likely that under normal conditions these critical interactions can, and perhaps more often do, occur in the specialized membrane domains.

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Interaction between the Unphosphorylated Receptor with High Affinity for IgE and Lyn Kinase

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