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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 (FcεRI) 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 FcεRI. Whereas native Tac failed to affect the aggregation-induced phosphorylation of FcεRI, both chimeric constructs substantially inhibited this reaction. Alternatively, the FcεRI-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.

Cellular responses mediated by the high affinity receptor for IgE (FcεRI) begin with the phosphorylation of the tyrosines in each of the three immunoreceptor tyrosine activation motifs of the receptor (1). This phosphorylation is stimulated by aggregation of the receptors and in the cells studied in greatest detail, the RBL-2H3 line (2), is effected by the Src family kinase Lyn (3).

Two molecular models have been proposed to explain the role of aggregation in stimulating the phosphorylation of the receptors. The transphosphorylation model we have used to study this system is based on the following experimental findings (4, 5): (*a*) Lyn kinase is constitutively and weakly associated with a small fraction of the receptors; b) *in vivo* and *in vitro*, the constitutively associated kinase is able to phosphorylate the receptors but only when they are aggregated; and (*c*) the initial phosphorylation of the receptors, but not their aggregation *per se*, promotes their association with additional, more firmly bound, Lyn kinase. These findings suggest that aggregation stimulates the phosphorylation of the receptors by stabilizing the juxtaposition of the kinase with its substrate, thereby shifting the balance between phosphorylation and dephosphorylation (6).

A second translocation model is based on the following observations: (*a*) aggregates of receptors, particularly larger aggregates, become localized, at least transiently, in discrete detergent-resistant membranes (DRM)[1] (7–9); (*b*) such microdomains occupy only a small fraction of the total plasma membrane but contain a substantial fraction of the total Lyn kinase (7); (*c*) upon aggregation, those receptors that become phosphorylated are largely found in the DRM; and (*d*) at least in some experiments (see "Discussion"), cholesterol-depleting agents, which interfere with the formation of such microdomains, inhibit the phosphorylation of the receptors (10). It has been proposed that these findings indicate that the primary effect of aggregation is to localize the receptors to regions of the membrane enriched in Lyn kinase (11). Neither of the two models predicates any aggregation-induced increase in the specific activity of the kinase *per se*, consistent with the limited experimental data on this question (5).

We undertook the present studies for two reasons. First, to clarify whether the translocation of the aggregated receptors to DRM was a necessary and sufficient or simply an accompanying aspect of the interaction between Lyn and the receptor. Second, to establish a system with which we could analyze in as physiological a milieu as possible the effect of genetically introduced changes in the structure of the receptor or Lyn on their interaction.

We used genetic constructs of portions of the receptor similar to those that had already been applied to this system by others (12–16). Specifically, we used cDNA constructs in which the ecto- and transmembrane domains of a subunit of interleukin-2 receptors (CD25α, Tac) were fused to the cytoplasmic domains of the β or the γ chains of FcεRI, alternatively. The constructs were stably transfected into Chinese hamster ovary (CHO)

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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.

TABLE I
Characteristics of Tac and chimeric constructs

Columns 2 and 3 show the number for the 3′ base in the 3′ codon of the open reading frame and for the corresponding C-terminal amino acid residue from the transmembrane domain of the Tac protein (CD25α). Columns 4 and 5 show the number for the 5′ base in the 5′ codon of the open reading frame and for the corresponding N-terminal amino acid residue from the cytoplasmic domains of the Tac protein, the β subunit (C-terminal), the γ subunit, and the Lyn protein, respectively. The junctional sequence is given in the last column with the joined residues shown in bold type. In the construct Tac-Lyn^{KR}, arginine substitutes for lysine at residue 279.

Construct	C terminus of CD25α		N-terminus of CD25α, of FcεRI β or γ, or of Lyn		Junctional sequence
	Base	Amino acid	Base	Amino acid	
TT-T	714	237	715	238	..LLSG**LT**WQRR..
TT-β	714	237	660	202	..LLSG**LR**IGQE..
TT-γ	720	239	150	27	..SGLT**WR**LKIQ..
TT-Lyn	714	237	12	4	..LLSG**LE**IKSK....
TT-Lyn^{KR}	714	237	12	4	..LLSG**LE**IKSK....

fibroblasts that we had previously transfected with FcεRI (17). The latter transfectants contain minimal amounts of endogenous Lyn kinase and, therefore, are particularly sensitive to manipulations that either enhance or inhibit the interaction of active kinase with the receptor (17, 18).

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 remove the sites of myristoylation and palmitoylation. We then examined the ability of such constructs to influence the phosphorylation of the aggregated receptors, much as we had previously studied normally anchored Lyn (17, 18).

EXPERIMENTAL PROCEDURES

Materials—Avidin and polyclonal anti-Tac coupled to biotin were purchased from R & D Systems (Minneapolis, MN). Extravidin coupled to horseradish peroxidase was obtained from Sigma. Monoclonal anti-phosphotyrosine antibody (anti-PY) coupled to biotin (4G10), rabbit polyclonal anti-Lyn kinase, and Src family kinase substrate peptide (19) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Dr. T. Waldmann (NCI, National Institutes of Health) provided a hybridoma producing monoclonal anti-Tac (clone HD245). The hybridoma cells were gradually weaned off serum in successive tissue cultures. The antibody was purified from the serum-free supernatants using a kit containing immobilized protein A (Pierce) and labeled with carrier-free [^{125}I]iodine using Iodogen tubes also from Pierce. (Iodination of the anti-Tac with chloramine T resulted in the complete loss of its bindability to Tac-expressing cells.) Monoclonal anti-Tac coupled to biotin (clone B1.49.9) was from Cappel (Durham, NC). Goat anti-mouse IgE, mouse monoclonal anti-DNP IgE (iodinated as appropriate), and covalently cross-linked oligomers of IgE were prepared as described previously (17). Polyclonal anti-caveolin was from Santa Cruz (Santa Cruz, CA). SpinZyme® phosphocellulose filters were from Pierce.

Preparation of Chimeric Constructs—Table I shows selected sequences of the constructs we 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 42 residues of the β or of the γ subunit of FcεRI (TT-β or TT-γ) in pcDL-SRa296 (16). They were digested with *Bam*HI and *Eco*RI and subcloned into pZeo-2SV (minus 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 (NHLBI, National Institutes of Health) and similarly subloned into pZeo-2SV. 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′-**CTCGAG**ATTAAATCAAAAAGGAAAGACAATC-3′ and 5′-**CTCGAG**CCACTATGGCTGCTGCTGATACTGCCCTTCCGTGGCACTG-3′) were synthesized and used in polymerase chain reaction with the wild-type Lyn as template. The sense primer encodes a *Xho*I 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 Tac coding for the ecto- and transmembrane domain was generated by polymerase chain reaction with an *Xho*I site at its C terminus. The two cDNAs were digested with *Xho*I and then ligated to generate the fusion chimera (TT-Lyn) and subsequently subcloned into the expression vector pZeo. TT-Lyn^{KR} was generated similarly using the Lyn cDNA that encodes the catalytically inactive 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.

TABLE II
Expression of Tac and FcεRI on transfected CHO-B12 cells

A CHO cell line previously transfected with rat FcεRI by electroporation and selected with G418 (CHO-B12 (last row)), was further transfected by electroporation or LipofectAMINE either with vector containing the zeocin antibiotic resistance marker only (pZeo) or in addition with Tac chimeras (TT-β, TT-γ, TT-Lyn, or TT-Lyn^{KR}) or the wild-type or truncated Tac (TT-T and TT-0), and selected with zeocin. FcεRI were quantitated using [^{125}I]IgE in duplicate incubations two to seven times for each transfectant except those transfected with TT-0. Tac was quantitated using [^{125}I]anti-Tac from clone HD245 in duplicate incubations two to seven times for each transfectant except those transfected with TT-0. We assumed each molecule of anti-Tac bound two molecules of Tac (22). The values shown are the means ± S.E.

Transfected cDNA	Clone[a]	FcεRI ($\times 10^{-5}$)	Tac ($\times 10^{-5}$)	Tac:FcεRI
TT-β	1A3E	1.2 ± 0.10	2.7 ± 0.50	2.2
	1A4E	1.0 ± 0.01	2.1 ± 0.20	2.1
	2A3E	1.0 ± 0.07	0.9 ± 0.10	0.9
	2C9E	1.3 ± 0.01	0.4 ± 0.08	0.3
TT-γ	A1L	0.8 ± 0.10	2.0 ± 0.40	2.5
	A3E	1.4 ± 0.20	0.3 ± 0.04	0.2
	B1E	1.3 ± 0.09	0.9 ± 0.10	0.7
	C1L	0.9 ± 0.10	2.2 ± 0.10	2.4
	D6E	1.6 ± 0.10	4.0 ± 0.70	2.5
TT-Lyn	D6L	1.5 ± 0.16	0.43 ± 0.04	0.29
	A6L	1.2 ± 0.10	0.5 ± 0.10	0.43
	B1L	0.78 ± 0.01	0.5 ± 0.07	0.64
	A5L	1.5 ± 0.17	0.6 ± 0.06	0.40
	C6L	1.4 ± 0.12	0.64 ± 0.08	0.44
	C4L	0.95 ± 0.12	1.4 ± 0.18	1.53
	42C2E	1.7 ± 0.16	0.02 ± 0.014	0.01
TT-Lyn^{KR}	33A4E	1.2 ± 0.14	0.22 ± 0.03	0.18
	31A4E	1.3 ± 0.15	0.62 ± 0.09	0.48
	34A3E	1.5 ± 0.26	2.18 ± 0.48	1.44
TT-T	21A4E	1.2 ± 0.11	0.68 ± 0.08	0.58
	24B2E	0.96 ± 0.07	0.16 ± 0.05	0.17
TT-0	51B2E	0.80	0.16	0.20
	52C4E	0.93	0.33	0.36
	52C2E	1.05	0.44	0.42
	51C2E	0.93	4.40	4.73
pZeo	4E	1.5 ± 0.08	NA[b]	NA
	5E	1.2 ± 0.04	NA	NA
none	B12	1.7 ± 0.19	NA	NA

[a] Clones with numbers ending in E were transfected by electroporation; those with numbers ending in L were transfected by LipofectAMINE.

[b] NA, Not applicable.

Stable CHO Transfectants—Plasmid expression constructs (10 μg) were electroporated (0.4 cm gap, 200 volts, 500 millifarad) into 1×10^7 CHO-B12 cells, a cell line stably expressing 170,000 FcεRI under G418 selection (Ref. 17 and Table II). After 72 h, the transfected cells were placed under selection with 250 μg/ml zeocin. Clones were picked after 5–7 days, expanded, and screened for expression of FcεRI and the Tac construct. A second set of transfectants for TT-γ and all of the TT-Lyn transfectants were prepared using LipofectAMINE (Life Technologies, Inc.). In a 6-well plate, 3×10^5 CHO-B12 cells were plated and grown overnight to 60–70% confluence. 1 μg of plasmid DNA was diluted into 100 μl of Opti-MEM serum-free medium (Life Technologies, Inc.) and mixed with a second solution containing 6 μl of LipofectAMINE in 100 μl of Opti-MEM serum-free medium. The mixture was incubated at room temperature for 30 min., diluted with 0.8 ml of Opti-MEM, and overlaid onto the cells. After 5 h at 37 °C, 1 ml of CHO-B12 growth medium containing 20% (2×) fetal bovine serum was added to the cells. The transfectants were then selected with zeocin, cloned and expanded

as for transfectants generated by electroporation. Duplicate wells of each clone to be tested were grown to confluence in 24-well plates ($\approx 2.5 \times 10^5$ cell/well). The cells in one set of wells were incubated with 1 μg/ml of [^{125}I]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 a 62.5 mM Tris, 0.5× PBS buffer. The lysates were then γ-counted. For assessing expression of the Tac constructs, cells were incubated with 0.5 μg/ml of [^{125}I]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 trypsin for passaging and stimulation. In addition, all CHO transfectants were cryopreserved in growth media supplemented with 5% $(CH_3)_2SO$, and a new vial of cells was routinely thawed every 2 months to assure a consistent ratio of Tac to FcεRI.

Quantitation of FcεRI and Tac Constructs—CHO transfectants were harvested and incubated at 5×10^6 cells/ml with 5 μg/ml [^{125}I]IgE for 1 h at 37 °C. Nonspecific 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 [^{125}I]anti-Tac for 30 min at 4 °C. Nonspecific binding was measured in duplicate incubations with 5 μg/ml unlabeled anti-Tac. Cells were then isolated 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 was employed, the cells were incubated first with 2 μg/ml monoclonal anti-Tac-biotin (clone B1.49.9) at room temperature and then with 25 μg/ml avidin, for 10 min at 37 °C. FcεRI were aggregated with oligomers of IgE for 30 min at 37 °C when appropriate. Alternatively, Tac constructs were aggregated using a monoclonal anti-Tac followed by a polyclonal anti-mouse IgG.

Immunoprecipitation and Western Blotting—FcεRI were solubilized and immunoprecipitated with anti-IgE, and phosphorylation of tyrosines on their β, and the γ subunits was determined as described previously (18). Tac constructs were extracted with 1% Nonidet P-40 and immunoprecipitated with monoclonal anti-Tac. Immunoprecipitates were analyzed on 10% Tricine gels followed by immunoblotting first with biotinylated polyclonal anti-Tac or 4G10 and then with horseradish peroxidase-coupled avidin.

Isolation of Plasma Membranes—For monitoring of FcεRI, CHO transfectants were sensitized for 1 h at room temperature with 5 μg/ml IgE, 10% of which had been labeled with ^{125}I. For monitoring of Tac constructs, the CHO cells were labeled with [^{125}I]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% isotonic Percoll (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 mM 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 centrifuge tube and overlaid with 6 ml of 30% sucrose prepared in a pH 7.5 buffer containing 0.05% Triton X-100, 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, and 2 mM sodium iodoacetate supplemented with protease inhibitors leupeptin, pepstatin, aprotinin, 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. Tubes were centrifuged in a swinging bucket rotor at 38,000 rpm for12–14 h. Successive 1-ml fractions were removed from the top of the gradient. All fractions were counted and then diluted with Triton X-100 to a final concentration of 0.5% prior to immunoprecipitations of FcεRI or 1% Triton X-100 for immunoprecipitation with anti-Tac.

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 mM $MnCl_2$, 2 mM CHAPS, 0.5 mM Na_3VO_4) containing 2 mM substrate peptide. Reactions were initiated by addition of ATP (100 μM ATP, 10 μCi of [γ-^{32}P]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% (v/v) acetic 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.

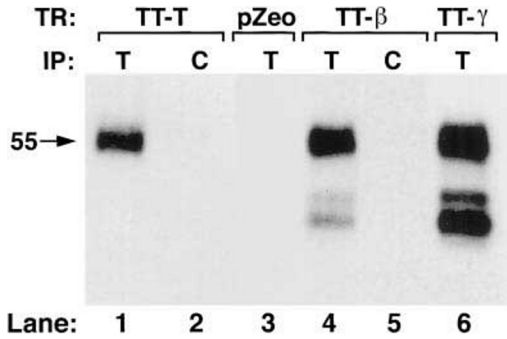

FIG. 1. **Expression of Tac and Tac-FcεRI subunit constructs in CHO-B12 cells.** CHO cells (4×10^6 cell equivalents) previously transfected with FcεRI (CHO-B12) were further transfected either with Tac (clone 21A4E), pZeo vector (4E), TT-β (2A3E), or TT-γ (A3E). Tac constructs were solubilized in 1% Triton X-100, immunoprecipitated (*IP*) with monoclonal anti-Tac (*T*) or an irrelevant control, anti-hemagglutinin (*C*) antibody and Western blotted with polyclonal anti-Tac. The apparent molecular mass (in kDa) of the upper band is shown on the *left*.

RESULTS

Transfection with Tac- FcεRI Subunit Constructs—CHO cells previously transfected with FcεRI (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 FcεRI (TT-γ). Stable clones that had been selected with zeocin and characterized for their expression of FcεRI and the Tac constructs are listed in Table II. The ratio of TT-β to FcεRI ranged from 0.3 to 2.2, and the that of TT-γ to FcεRI 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 ζ chain or the γ chain of FcεRI (13).

TT-β Inhibits the Phosphorylation of FcεRI—Fig. 2 shows the results of an experiment in which cells from clone 2A3E expressing on their surface approximately 0.9 TT-β per FcεRI (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 receptors 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

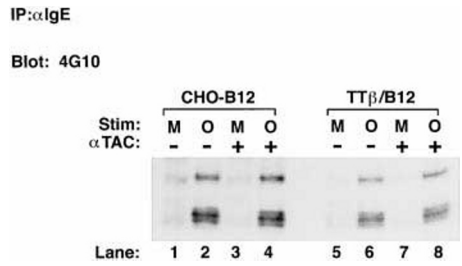

FIG. 2. **Effect of aggregation on the inhibitory action of TT-β.** CHO cells transfected with FcεRI alone (CHO-B12) or co-transfected with TT-β (clone 2A3E) were treated with 2 μg/ml anti-Tac-biotin in growth media (+) or media alone (−) for 20 min at 25 °C. Cells were washed and stimulated with 25 μg/ml avidin or buffer for 10 min at 37 °C and then stimulated with 0.5 μg/ml monomeric (*M*) or oligomeric (*O*) IgE for 30 min at 37 °C. FcεRI were solubilized, immunoprecipitated (*IP*) with anti-IgE, resolved by PAGE, and Western blotted with anti-PY. The results are representative of three experiments conducted, one with TT-β clone 2A3E and two with clone 2C9E.

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 phosphotyrosine 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 phosphotyrosine in their β chains and 60, 40, and 30% less phosphotyrosine 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 ¼ as much phosphorylation 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 phosphorylation 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 (Fig. 5, ▲ *versus* ●). 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. Duplicate samples were either solubilized with 1% Triton X-100 and immunoprecipitated with anti-TAC or solubilized with 0.5% Triton X-100 and immunoprecipitated with anti-IgE. Both sets of immunoprecipitates were then analyzed by Western blotting with anti-phosphotyrosine. In the cells stimulated with antigen, tyrosines on the subunits of the receptor became phosphorylated, but no phosphorylation of the chimeric construct was observed (data not shown). Stripping the blots and reprobing them with anti-FcεRI β or anti-TAC antibodies confirmed the equivalent immunoprecipitation in the samples. Western blots of immunoprecipitated TT-γ from either stimulated or unstimulated cells likewise failed to show any phosphotyrosine on the chimeric constructs. The experiments also showed that there was no co-precipitation of the Tac constructs with the transfected FcεRI.

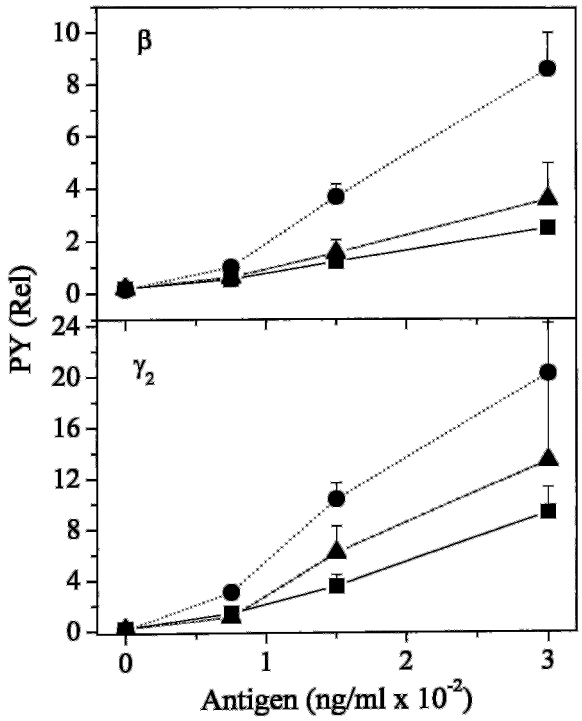

FIG. 3. **Effect of expression of TT-β on phosphorylation of FcεRI.** CHO-B12 cells transfected with vector containing only the pZeo antibiotic resistance marker (clones 4E and 5E (●)) or with TT-β (clones 2A3E (■) and 2C9E (▲)) were sensitized with anti-DNP-specific IgE (trace-labeled with [^{125}I]IgE), washed, and stimulated with DNP_6-BSA for 2 min. FcεRI were solubilized, immunoprecipitated, resolved by PAGE, and Western blotted with anti-PY. The blots were stripped, reprobed with antibody to the FcεRI β chain, and scanned to quantitate the PY per receptor β (*upper panel*) and γ chain dimer (*lower panel*). A fixed amount of phosphorylated human Lyn was loaded onto each gel and the densitometric values for PY normalized to each other. 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 [^{125}I]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.

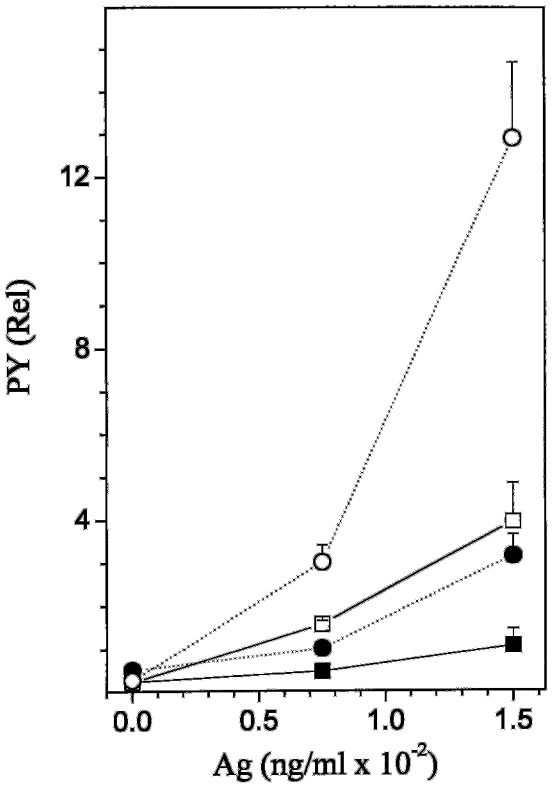

FIG. 4. **Specificity of inhibitory action of TT-β.** Clone 2A3E containing the TT-β construct (□, ■) was compared with clone 21A4E expressing a comparable amount of transfected unmodified CD25α (TT-T) (○, ●). The *open symbols* represent the data for the dimer of γ subunits; the *filled symbols* represent 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 FcϵRI in the TT-T transfectants stimulated with 75 ng/ml antigen.

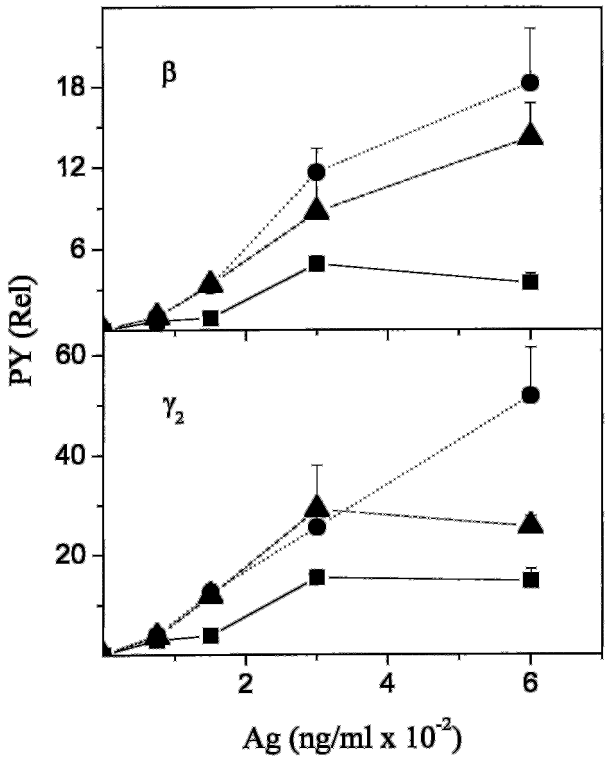

FIG. 5. **Effect of expression of TT-γ on phosphorylation of FcϵRI.** FcϵRI from pZeo transfectants (clones 4E and 5E (●)) and TT-γ FcϵRI transfectants (clones A1L, C1L, D6E, B1E and A3E (■, ▲)) were aggregated with DNP_6-BSA for 2 min. The relative amounts of phosphotyrosine on the β (*top panel*) and γ chains (*bottom panel*) of FcϵRI 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 (▲).

We observed a similar independence on the CHO cell transfectants. In one type of experiment we tested whether the constructs co-immunoprecipitated with the FcϵRI and failed to observe any co-immunoprecipitation. We also looked for co-distribution 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. Samples from the second set were directly Western blotted for both Lyn and caveolin.

The results are described in Fig. 6 (*top panel*). In the unstimulated cells (*open symbols*) both 100% of the recovered TT-β and 98% of the recovered FcϵRI were found in the detergent-sensitive plasma membrane fractions (9–12). Similarly, 100% of the endogenous Lyn kinase was recovered in these fractions (not shown). After stimulation with antigen (*filled symbols*), the percentage of FcϵRI in fractions 3–5 containing the DRM increased from 2 to 17% without a corresponding movement of TT-β or Lyn kinase.

Two similar analyses of a TT-γ transfectant (clone C1L) gave similar results (Fig. 6, *lower panel*). In the unstimulated cells, 100% of TT-γ and 96% FcϵRI were confined to fractions 9–12; the corresponding values after stimulation were unchanged for both TT-γ and FcϵRI. For purposes of clarity the distribution of the caveolin is not shown in the figure, but approximately 60% of the caveolin was localized in fractions 3–5 and 30% in fractions 9–12 in each of the gradients analyzed.

In separate experiments we exposed cells to either ^{125}I-labeled IgE or anti-Tac and then fractionated Dounce homogenates of the TT-β transfectants or CHO-B12 cells on Percoll gradients. The visible band observed after centrifugation of the Percoll gradients contained >90% of TT-β and FcϵRI, verifying that the corresponding proteins were largely in the plasma membrane.

Interaction of Kinase with Constructs—The simplest explanation for the results described so far is that the chimeric constructs competed with the FcϵRI 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 FcϵRI (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-^{32}P]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.

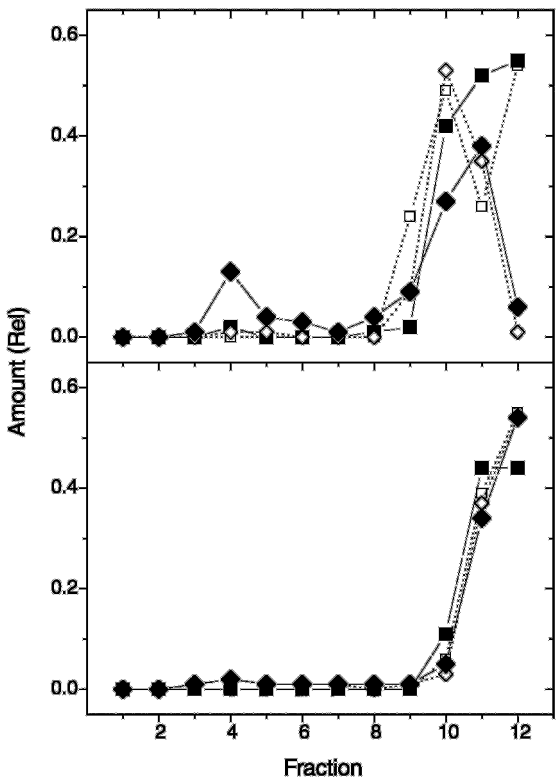

FIG. 6. **Distribution in membranes of Tac-β and and TT-γ constructs and FcεRI.** CHO-B12 cells transfected with TT-β (clone 2A3E) and TT-γ (clone C1L) were sensitized with [^{125}I]IgE (◇, ◆, and stimulated or not with 100 ng/ml DNP_6-BSA for 2 min. The low speed supernatant from cells lysed with 0.05% Triton X-100 was fractionated on sucrose gradients. Successive 1-ml fractions were removed from the top of the gradient, the location of the visible band was noted, and each fraction was measured in a γ-counter. Aliquots of each fraction were then further solubilized in 1% Triton X-100, immunoprecipitated with anti-Tac, and analyzed by Western blotting with either anti-Tac (□, ■), anti-Lyn (not shown), or anti-caveolin (not shown). *Top panel*, TT-β unstimulated with antigen (*open symbols*) or stimulated (*filled symbols*). *Lower panel*, duplicate experiments with TT-γ.

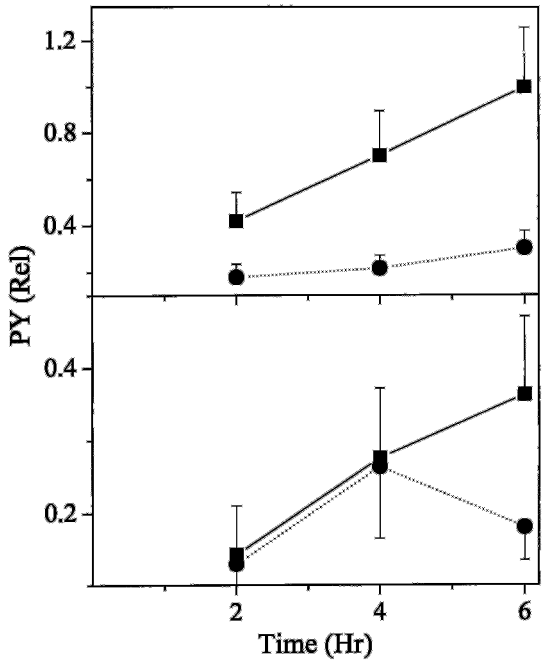

FIG. 7. **Kinase activity associated with TT-β.** Lysates from 3 × 106 CHO-B12 cells transfected with Tac (TT-T, clone 21A4E (●), or TT-β, clone 2A3E (■)) were reacted with anti-Tac or an isotype-matched control antibody, and the immunoprecipitates were assayed for co-precipitating endogenous kinase using a Src family kinase substrate. The average of two such experiments with each time point of the assay measured in triplicate is shown. *Top panel*, anti-Tac immunoprecipitates. *Lower panel*, control (anti-hemagglutinin) immunoprecipitates. Two of the six samples assayed after 4 h gave aberrantly high values, and these data were not included in the averages. *Error bars* show ranges of values.

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 (ρ) (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-Lyn^{KR}), 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 (DNP_6-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 DNP_6-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

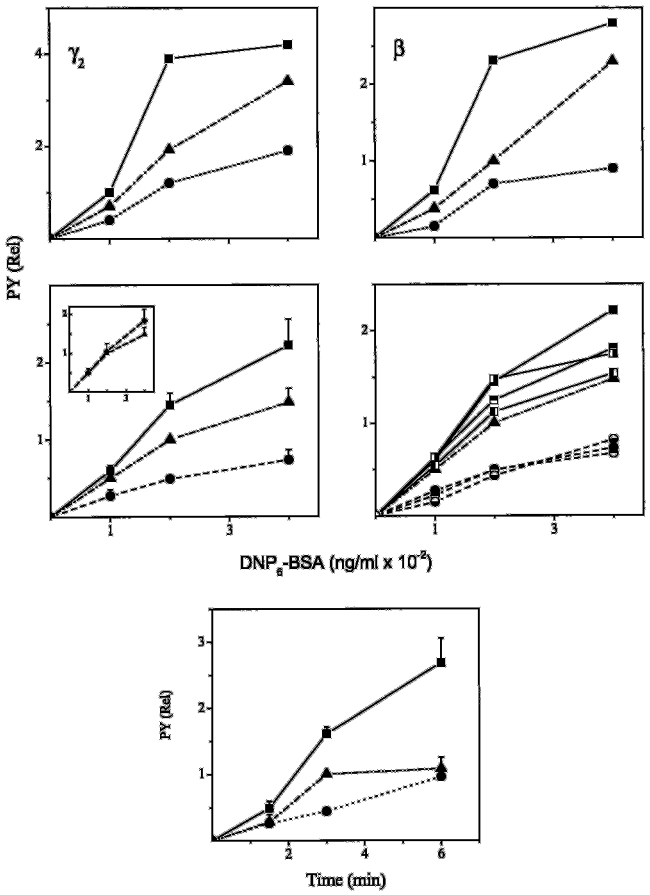

FIG. 8. **Aggregation-induced phosphorylation of tyrosines on FcεRI in CHO-B12 cells and transfectants.** *Top panels*, phosphorylation of the dimer of γ subunits and of the β subunit of FcεRI, 3 min after addition of several different doses of DNP-BSA to clone C4L (■), 34A3E (●), and CHO-B12 (▲) cells. *All* of the data have been normalized to the amount of phosphotyrosine determined for the β subunit on receptors of the CHO-B12 cells exposed to 200 ng/ml antigen. Each data point shows the mean of duplicate assays. *Left middle panel*, phosphorylation of the combined β and γ subunits of FcεRI, 3 min after addition of several different doses of DNP-BSA in five separate experiments on clones C4L(■), 34A3E (●), and CHO-B12 (▲) cells. The data have been normalized to the amount of phosphotyrosine determined for the receptors on the CHO-B12 cells exposed to 200 ng/ml antigen. The *error bars* show the positive standard error of the mean; where *error bars* are not shown they were smaller than the symbol. *Inset*, summary of five experiments in which clone 21A4 (TT-T) (♦) and CHO-B12 (▲) were compared. *Right middle panel*, comparison of multiple cloned transfectants. Phosphorylation of the combined β and γ subunits of FcεRI, 3 min after addition of several different doses of DNP-BSA to 10 different clones (Table II) transfected either with TT-Lyn (C4L, ■; A5L, □; C6L, ♦; A6L, B1L, and D6L, ▼) or with TT-Lyn^{KR} (34A3E, ●; 31A4E, ○; 33A4E, △); CHO-B12, ▲) cells. The data have been normalized to the amount phosphotyrosine on the FcεRI of CHO-B12 cells exposed to 200 ng/ml antigen. The *error bars* show the positive standard error of the mean; where *error bars* are not shown they were smaller than the symbol. *Bottom panel*, time course of phosphorylation of the combined β and γ subunits of FcεRI. Results are from 5–12 separate experiments on clones C4L (■), 34A3E (●), and CHO-B12 (▲) cells 1.5, 3, and 6 min after addition of 200 ng/ml of DNP-BSA. The data have been normalized to the amount phosphotyrosine on the FcεRI of CHO-B12 cells exposed to antigen for 3 min.

of phosphorylation of the β to γ_2 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 (A5L 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-Lyn^{KR})—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-Lyn^{KR} 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-Lyn^{KR} 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 ^{125}I-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 IgE 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-Lyn^{KR} 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-Lyn^{KR} constructs failed to affect phosphorylation of FcεRI.

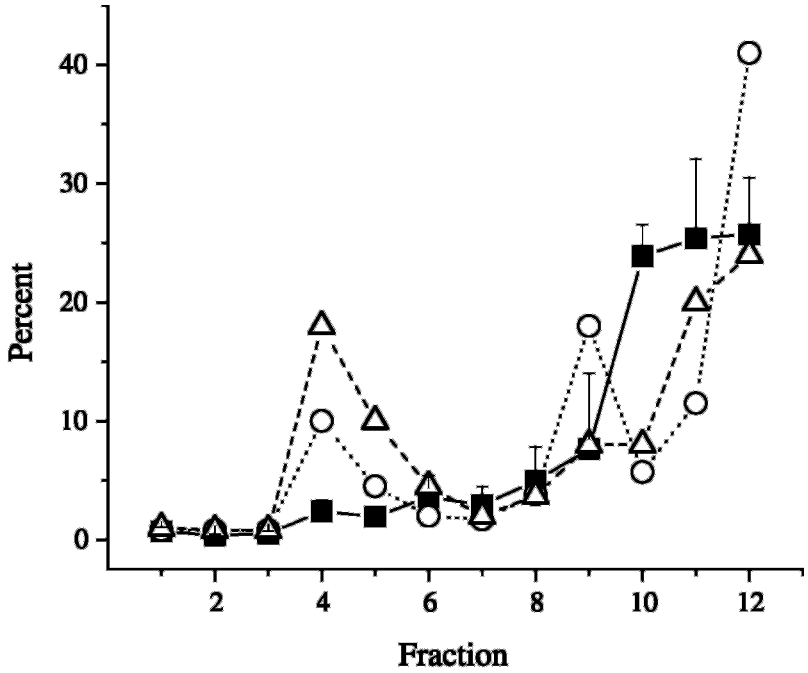

FIG. 9. **Distribution on membranes of Tac-Lyn constructs.** Transfected cells were lysed in 0.05% Triton X-100, and the extracts were separated on sucrose gradients. Individual fractions were either counted for radioactivity or analyzed by Western blotting. ■, composite results from four separate experiments on cells transfected either with TT-Lyn or with TT-Lyn^{KR}. The cells were otherwise not treated. The points show the average of the densitometric readings of Western blots, and the *error bars* show the standard deviations. ○, distribution of counts from extract of TT-T transfectant reacted with radioiodinated monoclonal anti-Tac. △, distribution of counts from extract of TT-T transfectant reacted with radioiodinated anti-Tac and then a polyclonal anti-mouse Fab.

Table III shows a detailed analysis of one such experiment in which two clones transfected with Lyn^{KR} 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 FcεRI 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 FcεRI 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 FcεRI were fused to the ecto- and transmembrane domain of an irrelevant protein, such as CD4 (12) or CD25α (13–16). The chimeric constructs generally have been transfected into the rat mucosal type mast cell line RBL-2H3 and yielded the following observations. Aggregation of the γ-containing construct stimulates a variety of cellular responses that resemble an anemic version of the responses induced by aggregation of FcεRI. Tyrosines on downstream proteins, but neither the construct nor the unaggregated FcεRI are phosphorylated following aggregation of the construct (15, 16). Although there is reason to think that the action of the γ constructs is mediated via Lyn, under the conditions used, Lyn is not detectable in immunoprecipitates of that construct (15). Experiments with the construct containing the C-terminal cytoplasmic domain of the β subunit gave virtually the opposite results; that is, although aggregation of the β construct failed to trigger phosphorylation of it or of other cellular components, immunoprecipitates of it revealed associated Lyn. The amount of Lyn recovered appeared to be independent of whether the β-construct had been aggregated or not. These results were consistent with earlier observations that the C-terminal domain of both the β and γ subunits are required for full activation but that β-less receptors can nevertheless stimulate responses (30, 31). 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 FcεRI. They hypothesized that although it could not independently activate downstream events, the β-construct sequestered a critical component required by FcεRI, likely Lyn. Their suggestion anticipated our subsequent findings that the Lyn available to the FcεRI in RBL cells is in short supply and shuttles between different FcεRI (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 CD25α would translocate to the microdomains upon aggregation (33). Then if the interaction between aggregated FcεRI and Lyn preferentially occurred in these domains, that could explain why the aggregated but not the monomeric TT-β inhibited the responses triggered by FcεRI. 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 FcεRI. 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 FcεRI 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

TABLE III
Effect of aggregation on the inhibitory action of construct TT-Lyn^{KR}

Cells were sensitized with [^{125}I]IgE and biotinylated monoclonal anti-Tac. Some aliquots were reacted with antigen or with avidin or with both. Detergent extracts were then immunoprecipitated with anti-IgE and analyzed by computerized densitometry of Western blots with an antibody directed to phosphotyrosine (PY) and with JRK an anti-β antibody.

Sample	Clone	Antigen	Avidin	PY	JRK	Gross	Net	Ratio samples (3:2)
1A	42C2E(TT-Lyn)	−	+	53	1400	0.038	0	
2A		+	−	1847	1385	1.334	1.296	
3A		+	+	1740	1244	1.399	1.361	1.05
1B	31A4E(TT-Lyn^{KR})	−	+	70	1690	0.039	0	
2B		+	−	1500	1707	0.879	0.840	
3B		+	+	1420	1778	0.799	0.760	1.11
1C	34A3E(TT-Lyn^{KR})	−	+	30	1800	0.018	0	
2C		+	−	600	990	0.606	0.588	
3C		+	+	905	1420	0.637	0.620	1.0

receptor that form are likely to be smaller than with more vigorous stimuli.

TT-β and TT-γ Inhibit the Phosphorylation of FcϵRI —Like Wilson *et al.* (16), we observed inhibition of the FcϵRI-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 antibiotic 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 vivo* 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 FcϵRI 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). In 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 glycine 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 chimeras 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-Lyn^{KR} 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

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 FcεRI 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 FcεRI we monitored was in the DRM but went undetected. Likewise, that 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 FcεRI 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.*[2] 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.*[3] observed no reduction in the initial phosphorylation of the receptor or in Syk kinase. Taken together, the data suggest that the constitutive association between Lyn and FcεRI 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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