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The Unique Domain as the Site on Lyn Kinase for Its Constitutive Association with the High Affinity Receptor for IgE*

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Aggregation of the high affinity receptor for IgE (FcεRI) leads to the phosphorylation of tyrosines on the β and γ chains of the receptor by the Src family kinase Lyn. We have studied the interaction between Lyn and the FcεRI *in vivo* using a transfection-based approach. FcεRI were stably transfected into Chinese hamster ovary cells. The small amount of endogenous Src family kinase was sufficient to phosphorylate receptor tyrosines upon extensive aggregation of FcεRI but not after addition of dimers of IgE. Upon stable co-transfection of Lyn kinase into the cells, dimers were now able to stimulate receptor phosphorylation and the response to more extensive aggregation was enhanced. In contrast, co-transfection with catalytically inactive Lyn inhibited the aggregation-induced phosphorylation by the endogenous kinase, and a quantitatively similar inhibition was observed in cells transfected with the SH4-containing unique domain of Lyn. Consistent with the results of others using alternative approaches, our additional studies using a yeast two-hybrid system detected a direct interaction between intact Lyn or its unique domain and the C-terminal cytoplasmic domain of the β chain but not with the receptor's other cytoplasmic domains.

The family of proteins known as the "multichain immune recognition receptors" includes the antigen receptors on B and T-lymphocytes and Fc receptors including the receptor with high affinity for IgE (FcεRI)[1] (1). Highly homologous in structure, all these receptors utilize, at least in part, a common mechanism to initiate cellular responses; multivalent interactions with antigen lead to aggregation of the receptors and is followed by enhanced phosphorylation of tyrosines (in the "ITAM" motifs within the cytoplasmic domains) of the receptor itself by a receptor-associated Src family kinase (2). For FcεRI, we recently presented direct evidence for a "transphosphorylation" mechanism that accounts for the earliest events (3, 4). The data showed that a small fraction of receptors are constitutively associated with the Src family kinase Lyn (4, 5) and that the enhanced phosphorylation that follows aggregation of the receptors is likely to result simply from the apposition of the kinase with its substrate. We have also shown that when the kinase available to the receptor is limited, shuttling of the enzyme between individual aggregates can regulate the intensity of the signal (6).[2]

The experiments described in this paper mainly explored the sites of interaction between Lyn kinase and FcεRI. For the most part, the prior studies of others explored the interaction between Lyn and isolated portions of the receptor (7–10). The yeast two-hybrid system (11) used in some of our studies is an analogous approach. We also employed transfection techniques, which allowed us to examine the kinase-receptor interactions in a more physiological setting. The latter experiments also allowed us to test the effect of varying the level of Lyn on the responsiveness of the receptors to discrete stimuli, and thereby to test certain quantitative predictions made by the current model.

EXPERIMENTAL PROCEDURES

Materials—The yeast strains (CG1945 and Y187) and cloning vectors (pAS2-1 and pACT) were obtained from CLONTECH (Palo Alto, CA); the expression vectors pBlueBac, pCDM8, and pZeo, as well as a baculovirus MAXBAC expression kit from Invitrogen (Carlsbad, CA); polyacrylamide gels used for electrophoresis (PAGE) from NOVEX (San Diego, CA); the antibiotics (G418, zeocin) from Life Technologies, Inc. and Invitrogen, respectively; and plasmid DNA purification kits from Qiagen (Santa Clarita, CA).

Antibodies—Monoclonal anti-phosphotyrosine (anti-Tyr(P)) antibodies were obtained conjugated to horseradish peroxidase from Transduction Laboratories (PY-20) or Upstate Biotechnology, Inc. (4G10). Polyclonal antibodies to human Src family kinases Lyn and Fyn were purchased from Upstate Biotechnology, Inc.; antibodies to c-Src and c-Yes were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-DNP IgE (12) and rat IgE (of unknown specificity) (13) were purified as described previously (14, 15) and labeled with carrier-free ^{125}I using chloramine T (16). Goat anti-mouse IgE was purchased from ICN (Costa Mesa, CA); rabbit anti-rat IgE was purified as described (17). Covalently cross-linked IgE oligomers were prepared and analyzed as described (6).

Cell Lines—Rat basophilic leukemia (RBL-2H3) cells were maintained as described previously (18). Chinese hamster ovary cells (CHO) were grown in stationary flasks at 37 °C in a humidified atmosphere containing 5% CO_2 in Iscove's modified Eagle's minimum essential medium, 10% fetal calf serum, 25 mM HEPES, and the appropriate antibiotics to maintain expression of the transfected genes. *Spodoptera frugiperda* (Sf9) insect cells were maintained in spinner culture at 27 °C as described previously (19).

DNA Sequencing—The nucleotide sequence of each expression construct was confirmed by automated DNA sequencing using a dye terminator kit obtained from Applied Biosystems (Foster City, CA).

Isolation of Rat Lyn Kinase cDNAs—A 5′-stretch cDNA library was prepared from mRNA isolated from RBL cells. Two separate priming reactions with either oligo(dT) or random primers were performed to generate the first strand. The reactions were pooled prior to second

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF000300 and AF000301.

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[1] The abbreviations used are: FcεRI, high affinity receptor for IgE; bp, base pair(s); BSA, bovine serum albumin; CHO, Chinese hamster ovary; DNP, dinitrophenyl; ITAM, immuno-recognition tyrosine-based activation motif; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Pipes, 1,4-piperazinediethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

[2] C. Wofsy, C. Torigoe, U. M. Kent, H. Metzger, and B. Goldstein, submitted for publication.

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strand synthesis. The cDNA library was then prepared in the expression vector pCDM8 (20). Probes were prepared by restriction digestion of human Lyn A-pSVL. Probes representing the N terminus (amino acid residues 1–298) and the C terminus (residues 163–512) were purified. The library was plated, and colony lifts were hybridized with either probe. Positive colonies went through secondary and tertiary screening. The nucleotide sequences of two clones, designated N14 (2052 bp) and C18 (2316 bp), were determined by primer walking and DNA sequencing of both strands. The Wisconsin package from the Genetics Computer Group, Inc. was used to assemble and analyze the nucleotide sequences of the isolated clones. N14 contained an open reading frame of Lyn A, beginning with ATG from bp 80 to bp 1616, while clone C18 encoded Lyn B beginning with ATG between bp 236 and bp 1709. The sequence of Lyn A in the coding region was identical to a previously published sequence (21); the sequence of rat Lyn B lacks an "insert" of 21 amino acids found in the A form of the kinase at a position identical to that previously shown for human and murine Lyn (22, 23) but is otherwise identical to Lyn A. Therefore, it differs somewhat from the previously published sequence for rat Lyn B (24).[3]

CHO cells were transiently transfected with the Lyn-pCDM8 plasmids by electroporation, harvested 48–72 h later, and a lysate of the whole cells was prepared using SDS. After separation by PAGE and transfer, Western blotting with anti-human Lyn confirmed that the expressed proteins had the expected the size for Lyn A (56 kDa) and Lyn B (53 kDa) (data not shown).

Yeast Two-hybrid Fusion Constructs—To generate DNA binding domain fusion proteins, the N-terminal (1–58) and C-terminal (201–243) cytoplasmic domains of the rat FcεRIβ were amplified by PCR from the full-length cDNA and cloned into the *Eco*RI/*Bam*HI sites of pAS2-1. The cytoplasmic domain of rat FcεRIγ (residues 27–68), as a PCR fragment, was cloned into *Nco*I/*Bam*HI site of pAS2-1, to generate pAS2-1-γC. To create activation domain fusion proteins, the full-length Lyn A and Lyn B and various deletion mutants were amplified by PCR and cloned into *Bam*HI/*Xho*I sites of pACT (Fig. 1).

Yeast Two-hybrid Co-transformation, Selective Growth, and β-Galactosidase Assays—Plasmid constructs were introduced into yeast cells by lithium acetate, following the protocol provided by CLONTECH. Transformants were plated on synthetic medium containing 5 mM 3-amino-1,2,4-trizole and lacking leucine, tryptophan, and histidine (SD-3) to detect the His phenotype, or synthetic medium lacking leucine and tryptophan (SD-2), to measure transformation efficiency. The β-galactosidase activity of transformants was measured in a filter assay with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as substrate or in a liquid assay with an *o*-nitrophenyl-β-D-galactoside substrate according to the CLONTECH protocol.

Mammalian Transfection Constructs—The 2.3-kilobase pair Lyn B *Xho*I-digested insert was isolated from pCDM8 and subcloned into the *Xho*I site of pZeo. The unique domain construct was generated by PCR amplification using internal sense and antisense primers for the unique domain: (5′-CGGGCGGCTCGATGGGATGTATTAAATCAAAAAGGAAAG-3′, and 5′-CGGCGGCTCGAGCTAGTCCCCTTGCTCCTCTGGATC-3′, respectively).

The final PCR product was digested with *Xho*I and cloned back into the pZeo *Xho*I site. The catalytically inactive Lyn B(K279R)-pZeo construct was prepared using the Altered Sites *in vitro* mutagenesis system from Promega (Madison, WI) as follows. A 2.3-kilobase pair *Xba*I fragment of Lyn B from Lyn-pCDM8 was ligated into the *Xba*I site of pAlter-1. Mutagenesis followed the manufacturer's protocol using the ampicillin repair primer provided in the kit and a Lyn single mutation antisense oligonucleotide: (5′-GCCAGGCTTGAGGGTCCTTACAGCCACTTTTGTGC-3′) to convert TTC (Lys) → TCC (Arg). The mutant Lyn B was digested with *Xho*I and *Bst*BI and ligated back into pZeo.

Transfection of CHO Cells—Using Lipofectin reagent-mediated transfection (Life Technologies, Inc.), pSVL constructs of the α, β, and γ subunits of rat FcεRI along with pSV2neo had been previously introduced into CHO cells, and a clone expressing a high number of receptors was frozen.[4] After thawing, expression of receptors decreased rapidly with time in culture, so we recloned the culture by incubating it with fluorescein-conjugated IgE and sorting on a fluorescence activated cell sorter. The 1% of cells expressing the highest number of receptors were resorted on 96-well plates at 0.5 cell/well. Fifty surviving clones were screened for expression of receptors, by growing the cells to confluence and sensitizing them with ^{125}I-labeled mouse IgE. The washed adherent cells were solubilized with boiling SDS sample buffer and the IgE in the extract quantitated by γ-counting. Of five high expressing clones, one (CHO-B12) proved highly stable and was used for all subsequent studies. CHO-B12 cells were cryopreserved by freezing in 5% dimethyl sulfoxide, 95% growth medium; higher concentrations of dimethyl sulfoxide caused a rapid decline in FcεRI. The cells were electroporated (0.4-cm gap cuvettes, 200 V, 500 microfarads) in the presence of one of several rat Lyn-pZeo constructs or empty pZeo vector, which had been linearized by digestion with Eco57I. To select resistant clones, the medium was supplemented with 250 μg/ml zeocin 72 h post-transfection.

Baculovirus Expression of Human Lyn B—The human Lyn B cDNA (1.5 kilobase pairs) was excised from pSVL by *Xba*I digestion and ligated into the homologous *Nhe*I site of pBlueBac. Sf9 cells were co-transfected with wild type AcMNPV DNA and the Lyn construct to generate recombinant Lyn baculoviruses. Adherent Sf9 cells were infected with plaque-purified baculovirus at a multiplicity of infection of 0.4 and, after 48 h, lysed in 0.1% Nonidet P-40 buffer containing protease and phosphatase inhibitors. Western blotting with anti-Tyr(P) indicated that the Lyn B protein was phosphorylated on tyrosine as it was produced in the insect cells (data not shown).

Stimulation of Cells—CHO cells to be stimulated with antigen were sensitized overnight with ^{125}I-labeled mouse anti-DNP mouse IgE, washed three times in buffer A (150 mM NaCl, 5 mM KCl, 25 mM Pipes, pH 7.2) plus 0.1% (w/v) gelatin and 5.4 mM dextrose, and resuspended at 1×10^7 cells/ml. DNP_6-BSA was added as a 5-fold stock solution to 5×10^6 cells at 37 °C for the times indicated. CHO cells stimulated with IgE oligomers were incubated with the indicated concentrations at 37 °C for the times indicated.

Solubilization and Immunoprecipitation—After stimulation, the receptors were solubilized in 0.05% Triton X-100 (3). For immunoprecipitation, anti-mouse or anti-rat IgE antibody was prebound to 30 μl of protein A-Sepharose beads overnight in borate-buffered saline, pH 8, containing 0.1% gelatin. The beads were recovered by centrifugation and combined with the lysates ("precleared" with 100 μl of protein A-Sepharose beads overnight) for 2 h. After recentrifugation the immunoprecipitates were washed four times as described previously (3), and the bound proteins released by boiling in SDS sample buffer for 5 min.

Quantitation of Phosphorylation of Receptors—Immunoprecipitated receptors were separated by electrophoresis in SDS on 10% polyacrylamide gels equilibrated with Tricine and the phosphorylated proteins detected with an anti-Tyr(P) antibody and an enhanced chemiluminescent detection system (ECL, Amersham) (25). Autophotographs of Western blots were quantitated by computerized densitometry (Molecular Dynamics, Sunnyvale, CA). Three steps were taken to ensure equal numbers of receptors were being compared in those studies in which cells co-transfected with inactive forms of Lyn were compared with cells that had not been co-transfected. First, the cells were incubated with IgE that had been labeled with ^{125}I and equal numbers of counts were loaded per lane. Second, one lane on each gel was loaded with the same amount of phosphorylated human Lyn B to correct for differences in transfer, antibody staining, washing, etc. Third, the primary anti-Tyr(P) blots were stripped and reprobed with an antibody (JRK) to the β chain of the receptor (26), and densitometric analysis was repeated. The densitometric values from the primary anti-Tyr(P) blots were then corrected for any differences in anti-Tyr(P) staining or loading of receptors. In separate experiments, the linearity of antibody staining (anti-Tyr(P), anti-β) was verified by loading increasing amounts of an appropriate protein extract and quantitating the band intensity.

Quantitation of Lyn—To quantitate the relative amounts of Lyn, whole cell lysates containing either 7×10^4 or 1.6×10^5 cell eq were prepared with SDS for each transfectant. Depending on which molecules had been transfected, the samples were separated on 8% (Lyn B, RK Lyn), 10% (CHO-B12, pZeo), or 4–20% (unique Lyn A) Tris-glycine gels and blotted with an anti-Lyn antibody and an HRP-conjugated anti-rabbit secondary antibody. One (central) lane on each gel was loaded with a fixed amount of human Lyn B (above). The densitometric readings for the bands corresponding to Lyn were normalized relative to the human Lyn B standard.

Quantitation of FcεRI —-CHO cells were suspended at 5×10^6 cells/ml and incubated with 5 μg/ml ^{125}I-labeled IgE for 1 h at 37 °C. Nonspecific binding was evaluated by preincubating the cells with a 10-fold excess of unlabeled IgE for 30 min at 37 °C. Cells were separated

[3] The open reading frame of our Lyn B cDNA is identical to the Lyn A except for the missing 21-residue "insert." The differences in the sequence originally reported (24) probably resulted from sequencing errors or errors introduced during amplification with PCR. Two further clones, a partial Lyn A cDNA and a full-length Lyn B cDNA isolated from an RBL library, confirmed our sequence.

[4] C. Pucillo, unpublished studies.

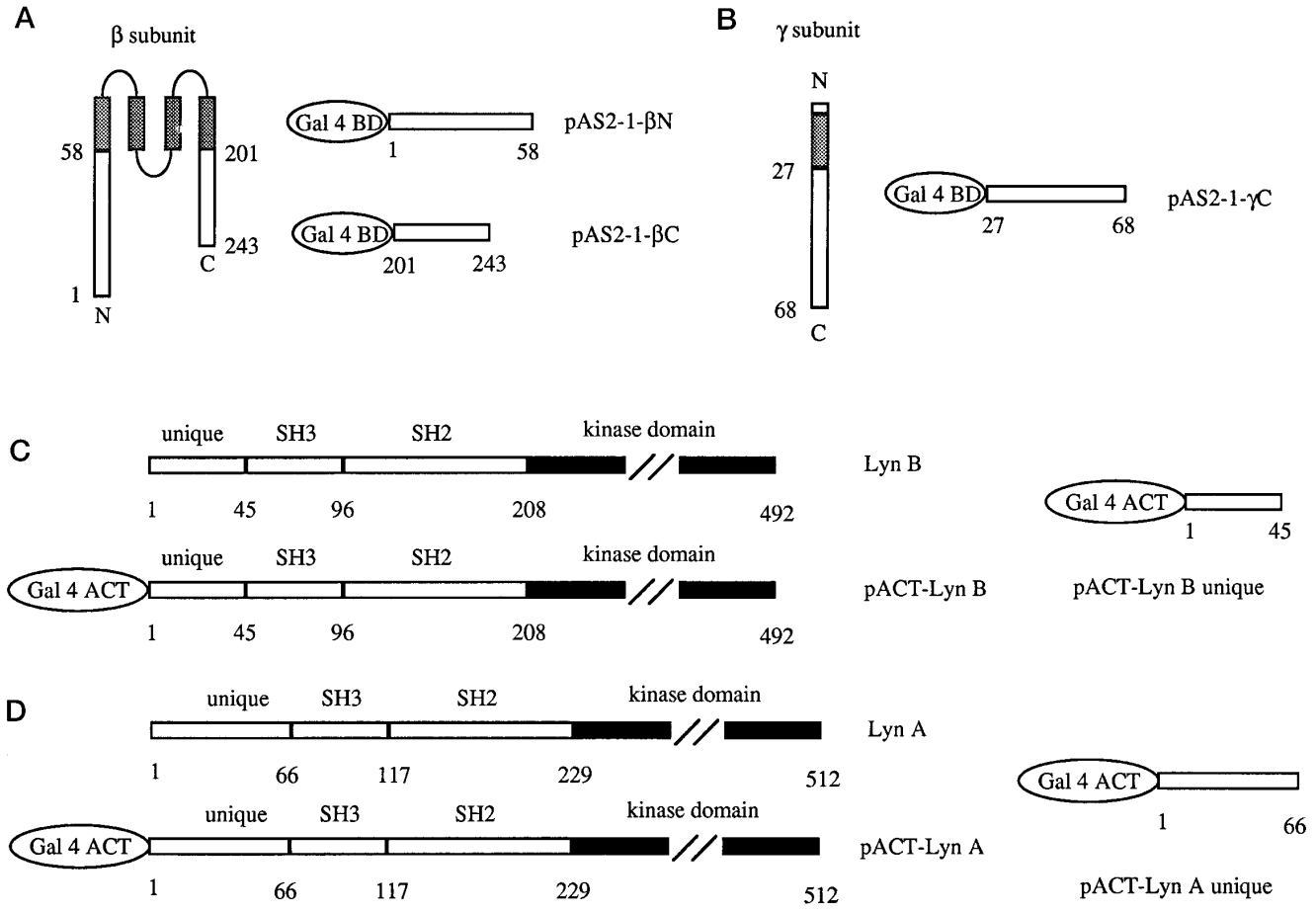

FIG. 1. **Receptor and kinase proteins and the constructs used for the yeast two hybrid experiments.** *A*, the β chain and the Gal4 binding domain (*BD*)-receptor subunit fusion proteins based on the cytoplasmic domains of the subunit. The four transmembrane domains of the subunit are *shaded*. *B*, the γ chain and the Gal4 binding domain-receptor subunit fusion proteins based on the cytoplasmic domain of the subunit. The transmembrane domain is *shaded*. *C*, Lyn B and the Gal4 activation domain (*ACT*)-kinase fusion proteins based on the complete kinase or its unique domain. *D*, Lyn A and the Gal4 activation domain (*ACT*)-kinase fusion proteins based on the complete kinase or its unique domain. Not shown are additional fusion proteins that contained only a portion of the unique domain of Lyn A (pACT-Lyn-1–10, pACT-Lyn-1–27, pACT-Lyn-27–66) or a portion of Lyn A out of the reading frame (pACT-Lyn-27–66-OOF).

from unbound IgE by pelleting through phthalate oil (15, 27).

Subcellular Fractionation—CHO cells were sonicated, and the 140,000 × *g* supernatant (cytosolic fraction) and pellet (membrane fraction) were prepared from the post-nuclear supernatant as described previously (28). Membrane proteins were solubilized in 0.5% Triton X-100, for 30 min at 4 °C. Each subcellular fraction was treated with an equal volume of boiling 2 × SDS sample buffer for 5 min prior to gel electrophoresis.

Other Procedures—Coupled *in vitro* transcription-translation reactions were conducted with [^{35}S]Cys according to the manufacturer's recommendation (T3 TnT® coupled reticulocyte lysate system, Promega).

RESULTS

Yeast Two-hybrid Studies

Initial identification of potentially interacting domains was conducted by co-transforming constructs containing the cytoplasmic domains of the FcεRI fused to the binding domain of the Gal4 transcription factor with constructs containing Lyn or various mutated forms of Lyn fused to the activation domain of Gal4 (Fig. 1).

The nucleotide sequence coding for the N- and C-terminal cytoplasmic domains of the β subunit of the rat IgE receptor, β_N and β_C, were subcloned into pAS2 to generate Gal4 DNA binding domain fusion proteins. Unfortunately, both fusion proteins autonomously activated the reporter genes. This is presumably due to the acidic hemagglutinin epitope located between the Gal4 DNA binding domain and the inserted proteins (29). However, the fusion protein containing the cytoplasmic domain of the γ subunit was not autonomously active. Therefore, we subcloned nucleotide sequences coding for β_N and β_C into the newly developed vector pAS-2-1, which is similar to pAS2, but has the acidic hemagglutinin epitope removed. Neither pAS2-1-β_C or pAS2-1-β_N were autonomously active.

The activities of the His and LacZ reporter genes in CG1945 yeast transformants expressing Lyn and β_N, β_C or γ_C were tested as described (see "Experimental Procedures"). Both the full-length and unique domain of both Lyn A and Lyn B interacted directly with β_C (data not shown). However, the interaction was much weaker than the interaction detected between the p53 and SV40 fusion proteins used as a positive control. Thus, per microgram of DNA, co-transformation with p53 and SV40 resulted in more colonies on His-deficient medium (SD-3) and rapid growth into large colonies. All of the colonies containing p53 and SV40 rapidly turned blue. In contrast, co-transformation with the Lyn and β_C constructs resulted in fewer colonies and slower growth on His-deficient medium and only the large colonies turned blue. No interaction was detected between β_N or γ_C with any forms of Lyn in this assay.

To quantitate the interaction between Lyn and β_C or β_N, we measured the β-galactosidase activity of these co-transformants in yeast strain Y187 in a liquid assay. In addition to the full-length Lyn and the construct containing only the unique domain, we tested a series of Lyn mutants based on

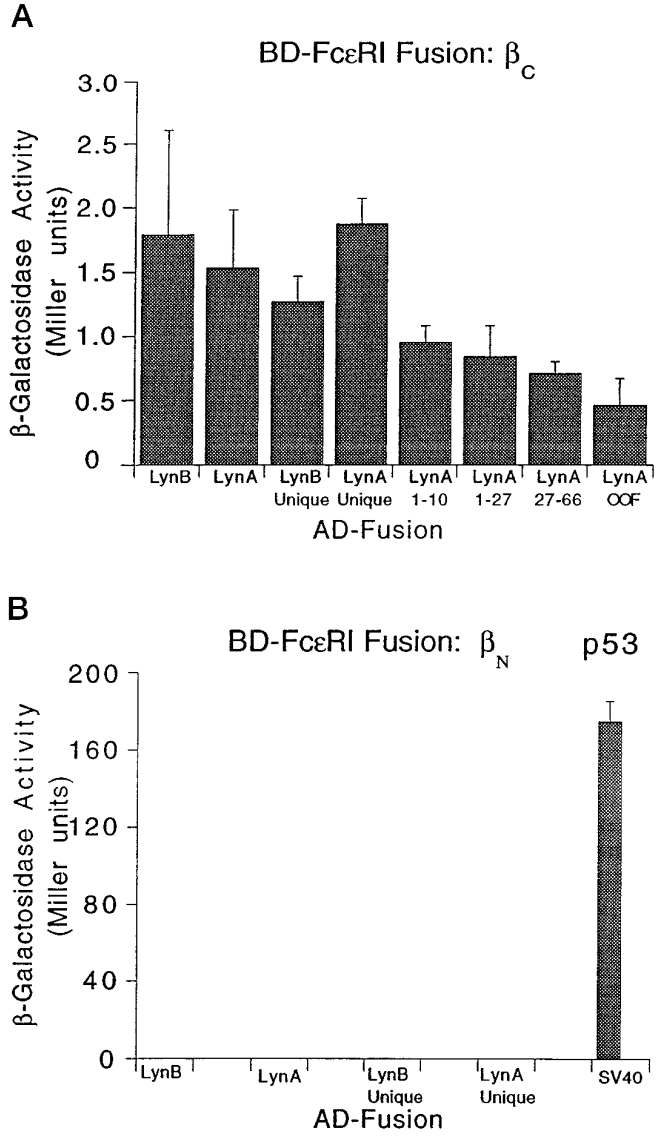

FIG. 2. **Interaction between domains of Lyn and FcεRI as determined by a quantitative yeast two-hybrid assay.** A liquid β-galactosidase assay from the LacZ reporter gene was performed using *o*-nitrophenyl-β-D-galactoside as a substrate and yeast strain Y187 (see "Experimental Procedures"). The values reported represent the average activity ± the standard deviation of three independent transformants. *BD*, binding domain of Gal4; *AD*, activation domain of Gal4. *A*, interaction of the C-terminal cytoplasmic domain of the β subunit with intact Lyn A, intact Lyn B, and various segments of each. *B*, interaction of the N-terminal cytoplasmic domain of the β subunit with intact Lyn A, intact Lyn B ,and the unique domains of each, and interaction of p53 with SV40 (positive control). The activity for the interaction with the N-terminal domain was literally zero (*i.e.* undetectable) and does not simply reflect the change in the ordinate scale.

the results of Pleiman *et al.* (30) and Timson Gauen *et al.* (31). The negative control in this experiment was the 40 amino acid residues (from 27 to 66) of Lyn fused out-of-frame to the Gal4 activation domain (pACT-27–66-OOF). As shown in Fig. 2, the activity of the LacZ reporter gene from co-transformants with the unique domain of either Lyn A or B was as high as that from co-transformants with the full-length form of either Lyn. These values are 3-fold higher than those than from the negative control pACT-27–66-OOF. Consistent with the result from CG1945 strain, the interaction between Lyn and β_C is weaker (on the basis of the β-galactosidase activity, only 1% as strong) than that between p53 and SV40. Co-transformants containing Lyn residues 1–10, 1–27, or 27–66 produced only slightly higher amounts of β-galactosidase than the negative control. Again, no interaction between Lyn and β_N was detected.

Characterization of Transfected FcεRI in CHO Cells

A clone of transfected CHO cells that stably expressed ≈170,000 receptors/cell (CHO-B12) (Table I) was further characterized. When immunoblotted with anti-human Lyn antibody, extracts of these cells, like those of the untransfected CHO cells, show a weakly reactive component at ≈58 kDa, *i.e.* slightly greater than the apparent molecular mass of 53 and 56 kDa observed for rat Lyn (Fig. 3*A*). There was no reactivity with a panel of antibodies to human c-Src, Fyn, or c-Yes (data not shown). Cells from the B12 clone were incubated with anti-DNP-specific mouse IgE and after solubilization with detergent, the bound (unaggregated) receptors were immunoprecipitated with goat anti-mouse IgE. Upon Western blotting with anti-Tyr(P), no evidence for phosphorylation was observed (Fig. 3*B*, *lane 1*). When the cells were incubated with multivalent antigen (DNP-BSA) prior to solubilization, phosphorylation of tyrosines on the β and γ subunits of the transfected receptors was observed (*lane 2*). Disaggregation of the receptors *in vivo* by addition of hapten (DNP-caproic acid) after the exposure to DNP-BSA led to the complete reversal of the antigen-induced phosphorylation of receptor tyrosines within ≤1 min (data not shown).

RBL cells can be stimulated either by aggregating receptor-bound monomeric IgE with antigen or by incubating the cells with preformed dimers of IgE (Fig. 3*B*, *lane 8*). In contrast, incubation of the CHO-B12 cells with dimeric IgE failed to induce detectable phosphorylation of the receptors (Fig. 3*B*, *lane 4*). These results are consistent with a limiting amount of protein-tyrosine kinase being associated with the receptors in these cells (see "Discussion").

Correlation between Total Lyn and Phosphorylation of FcεRI

A series of stable transfectants of the CHO-B12 cells with rat Lyn were isolated. The relative ratios of full-length Lyn/receptor of six clones (A6 through D8) are shown in the upper part of column 5 of Table I. Subcellular fractionation of the transfected cells indicated that the transfected full-length Lyn was expressed as a membrane-associated protein (Fig. 3*A*), as expected for a Src family kinase (32).

The various transfectants were stimulated either with IgE dimers or with monomeric IgE and then antigen, to examine the relationship between the total cellular content of Lyn and the responsiveness of the cells. Care was taken to ensure equal numbers of receptors were being compared (see "Experimental Procedures").

As shown in Fig. 4, there was a good correlation between the amount of Lyn expressed and the amount of receptor tyrosine phosphorylation seen on both the β and γ subunits upon aggregation of the receptors with antigen. Furthermore, all of the cells expressing transfected Lyn now responded to dimers of IgE. More extensive phosphorylation was observed in those cells whose receptors were aggregated with antigen rather than with dimers. However, the stimulation by dimers was more sensitive to the amount of Lyn expressed as can be seen by comparing the slopes of the two response "curves" (Fig. 4).

One clone, A11, in which the relative Lyn/receptor ratio was exceptionally high, showed a significant degree of phosphorylation of the receptors even without stimulation. Western blotting of A11 lysates revealed a phosphorylated component with an apparent molecular mass of 53 kDa (presumably Lyn) but

TABLE I
CHO transfectants

Name	Clone	Insert	FcεRI	Lyn per FcεRI	Lyn (Inact.) per Lyn (End.)
			$\times 10^{-5}$		
CHO-B12[a]	B12	NA[b]	1.7	2.5[c]	NA
Lyn/B12	A6	Lyn B	1.0	1.0	NA
Lyn/B12	A9	Lyn B	0.7	9.3	NA
Lyn/B12	A11	Lyn B	1.3	66	NA
Lyn/B12	D1	Lyn B	1.3	34	NA
Lyn/B12	D7	Lyn B	1.3	3.2	NA
Lyn/B12	D8	Lyn B	1.3	0.94	NA
RK Lyn/B12	RK17	Inact.Lyn B	1.9	7.4	6.0[d]
RK Lyn/B12	RK21	Inact.Lyn B	1.8	4.6	4.2
RK Lyn/B12	RK26	Inact.Lyn B	1.4	24	12
Lyn unique/B12	C6	Unique Lyn A	1.2	5.9	5.0
Lyn unique/B12	U7	Unique Lyn A	1.0	0.83	0.42
Lyn unique/B12	U8	Unique Lyn A	1.7	1.9	0.76
pZeo/B12	Z1	None	0.8	5.2	NA
pZeo/B12	Z2	None	0.8	6.6	NA
pZeo/B12	Z3	None	1.0	5.0	NA
pZeo/B12	Z4	None	0.8	6.8	NA
pZeo/B12	Z5	None	1.5	2.8	NA
pZeo/B12	Z6	None	1.0	6.6	NA

[a] A stable CHO FcεRI transfectant (CHO-B12) was generated by electroporation of FcεRI subunits (α, β, γ). Stable double transfectants, likewise generated by electroporation, were prepared by transfecting various Lyn constructs into CHO-B12 cells and selection with zeocin. Control cells, doubly transfected with FcεRI and empty pZeo vectors, were prepared by the same protocol.

[b] NA, not applicable.

[c] The values shown in this column are strictly relative and were determined as follows. For each transfectant, the normalized densitometric readings of the total Lyn in a fixed number of cell equivalents (see "Experimental Procedures") was divided by the number of FcεRI per cell $\times 10^{-3}$. For example, for the first item in this column the corrected densitometric reading was 430.6. The latter divided by 170 (FcεRI per cell $\times 10^{-3}$) equals 2.5. The quantitation of Lyn was done two to eight times for each transfectant; the enumeration of the FcεRI was done in duplicate.

[d] The amount of endogenous hamster Lyn (Lyn (End.)) and transfected inactive Lyn (Lyn(Inact.)) expressed per cell was determined by Western blotting of SDS lysates with anti-(human) Lyn. The values shown represent the average of two to eight separate determinations.

no change in overall phosphorylation of tyrosines on other cellular proteins when compared with CHO-B12 lysates (data not shown).

To control for differences in tyrosine phosphorylation that may have arisen due to zeocin resistance alone, CHO-B12 cells were transfected with pZeo vector and resistant colonies isolated and expanded. Upon stimulation with 0.5 μg/ml trimeric IgE from 5 to 30 min, the six zeocin-resistant clones tested showed no significant differences in phosphorylation of the β and γ subunits of the receptor compared with CHO-B12 cells (Fig. 5*A*). In a similar experiment, the responses to varying doses of antigen (25–300 ng/ml) of three zeocin-resistant clones were compared with CHO-B12 cells. A similar dose dependence of phosphorylation of the receptors was observed (Fig. 5*B*). No differences were noted in either the magnitude or pattern of total cellular proteins that became tyrosine phosphorylated. By Western blotting the level of endogenous Lyn was also unchanged. Since the number of FcεRI on the pZeo transfectants varied between 80,000 and 150,000 (clones Z1–Z6, Table I), the degree of phosphorylation was found to be independent of the number of receptors under the conditions used in this study.

Mapping the Site of Lyn-FcεRI Interaction by Competition

The presence in the CHO-B12 cells of an endogenous kinase (presumably Lyn) capable of phosphorylating the receptor, permitted us to probe the site on Lyn interacting with the FcεRI by a competition protocol. We transfected the cells with domains of Lyn that would potentially interact with the receptor but that were themselves catalytically inactive. We compared the responsiveness of such transfectants to aggregation of their FcεRI either to CHO-B12 or to cells co-transfected with the "empty" pZeo vector.

Catalytically Inactive Lyn Kinase—A full-length, catalytically inactive Lyn B kinase was prepared by mutating Lys^{279} to Arg (RK Lyn). As shown in Fig. 6*A*, in a coupled *in vitro* transcription-translation reaction, the wild type Lyn was autophosphorylated[5] whereas the mutant Lyn was not.

Three stable transfectants expressing substantial amounts of the mutant Lyn were isolated and assessed (clones RK17, RK21, and RK26; Table I). The catalytically inactive Lyn was expressed largely or exclusively as a membrane anchored protein (Fig. 3*A*). On a per receptor basis, such stable RK Lyn-FcεRI transfectants showed 20–75% less antigen-induced phosphorylation of receptor tyrosines than cells transfected with the vector alone (Fig. 6*B*). Therefore, a single point mutation converted a construct that stimulated phosphorylation of tyrosines on FcεRI to one that inhibited it (*cf.* Figs. 4 and 6*B*).

Unique Domain of Lyn Kinase—Prompted by our results from the yeast-two hybrid studies, we transfected the unique domain of Lyn A kinase into receptor-containing cells (clones B5, C6, U7, and U8, Table I). The isolated unique domain was also expressed largely or exclusively in a membrane-anchored form (Fig. 3*A*). Figs. 7 and 8 show comparisons between the responses to two different stimuli of the transfectants and CHO-B12 cells not transfected with Lyn. Upon stimulation with multivalent antigen, a partial inhibition of phosphorylation of receptor tyrosines was observed (Fig. 7*A*). A comparison of two clones expressing increasing levels of the unique domain protein showed that increasing amounts of the competing domain led to increasing inhibition (Fig. 7*B*). With a weaker stimulus (IgE trimers), complete inhibition of phosphorylation of the β and γ chains was observed at early time points (Fig. 8*A*) and at low concentrations of stimulant (Fig. 8*B*).

[5] Since the reaction mixture contains Mg^{2+}, ATP, and NaCl in a neutral pH buffer, it can support a kinase reaction.

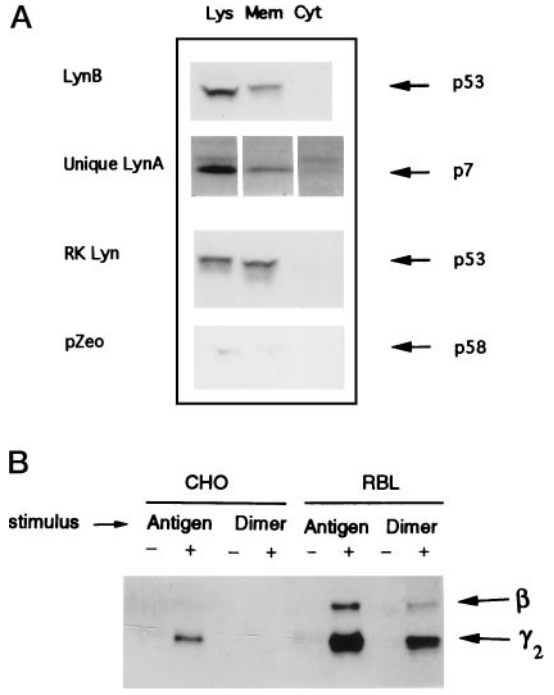

FIG. 3. **Expression, distribution, and activity of Lyn in CHO transfectants and RBL cells.** *A*, expression and membrane association of Lyn in CHO transfectants. CHO cells previously transfected with FcεRI (CHO-B12) were stably co-transfected with either intact Lyn B (clone D1), the unique domain from Lyn A (C6), the catalytically inactive RK mutant of Lyn B (RK26), or the empty pZeo vector (Z5). SDS lysates of intact cells (*Lys*) or of the membrane (*Mem*) or cytosolic (*Cyt*) fractions of sonicated cells were prepared. The proteins from 1.6×10^5 cell eq of each lysate were separated by PAGE and blotted with anti-human Lyn antibody (see "Experimental Procedures"). The apparent molecular mass of the principal component is shown at *right*. *B*, phosphorylation of receptor tyrosines in CHO-B12 and RBL cells. Six million cells were incubated with 5 μg/ml ^{125}I-labeled mouse anti-DNP-specific IgE for 1 h at room temperature. The cells were washed, and duplicate samples were then incubated at 37 °C with (*Antigen* +) or without (*Antigen* −) 100 ng/ml DNP-BSA, for 2 min more. Other samples were incubated for 15 min with either 0.5 μg/ml ^{125}I-labeled monomeric rat IgE (*Dimer* −) or equivalent amounts of chemically cross linked dimers of rat IgE (*Dimer* +). FcεRI were immunoprecipitated from the detergent lysate of the cells with anti-IgE, and the samples were blotted with anti-Tyr(P) (PY-20; see "Experimental Procedures"). Equal numbers of receptors (based on the cpm of bound ^{125}I-IgE) were loaded for each immunoprecipitate. One experiment representative of the two conducted is shown.

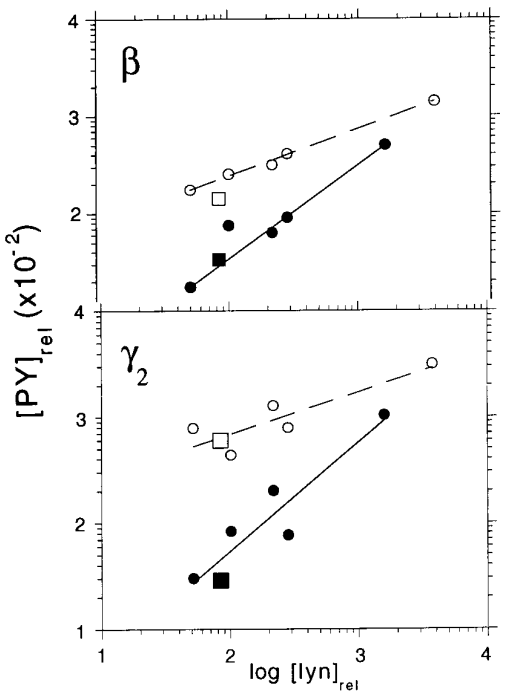

FIG. 4. **Phosphorylation of tyrosine in FcεRI from Lyn B/FcεRI transfectants.** The FcεRI on CHO cells transfected only with FcεRI (CHO-B12; *squares*) or co-transfected with Lyn B (clones A11, D1, D7, A6, A9, and D8; *circles*) were aggregated. The receptors were solubilized, immunoprecipitated with anti-IgE, separated by PAGE, and Western-blotted with PY-20 as described. *Open symbols*, cells sensitized with monomeric anti-DNP IgE and activated with 50 ng/ml DNP-BSA for 2 min. *Filled symbols*, cells reacted with 0.5 μg/ml dimeric IgE for 15 min. Each data point represents a *different* transfectant. *Ordinate* ($[PY]_{rel}$), The densitometric values for β and γ were corrected for variations in anti-Tyr(P) blotting and receptor loading (see "Experimental Procedures"). The values for each receptor chain obtained with the resting (unstimulated) cells have been subtracted from the values shown. The relative concentrations of Lyn shown on the *abscissas* ($[lyn]_{rel}$) were estimated (see "Experimental Procedures") from 7×10^4 cell eq of SDS lysates prepared from the transfected cells. The autophotographs were scanned by computing densitometry. The data represent one of two such experiments performed. The equation used to generate the regression lines is $Y = (a \times X^b)$, and the correlation coefficients were 0.9945 (β-dimer), 0.9972 (β-antigen), 0.9902 (γ-dimer), and 0.9669 (γ-antigen). In this figure, the same transfectants were studied with each stimulus, with the exception of the transfectants expressing the highest level of Lyn (points furthest to the *right*) where different transfectants were stimulated with antigen and with dimers, respectively.

DISCUSSION

Interactions in the Yeast Two-hybrid System—Several groups have studied the interaction between FcεRI and Lyn kinase by a variety of techniques. Consistent with previous findings (7–10), the results from our studies in the yeast two-hybrid system indicate a direct interaction between the kinase and the C-terminal cytoplasmic extension of the receptor's β chain. No interaction was detected between Lyn and β_N or γ_C. As judged by the relative activity of a reporter gene, the interaction is very weak (Fig. 2). This is consistent with the difficulty in demonstrating co-immunoprecipitation of Lyn with unphosphorylated FcεRI in the absence of chemical cross-linking (4). Our results extend those of previous workers in showing that the Lyn A and Lyn B behave equivalently (5). This result is also consistent with our previous finding that the two forms of Lyn become equivalently attached to the receptor after chemical cross-linking (4). Furthermore, we demonstrated that the unique domain alone interacts with the receptor as effectively as the full-length kinase, but the weakness of the interaction makes problematic any attempt to define the site of interaction more narrowly by this method (Fig. 2).

It is conceivable that in this experimental system the receptor component is phosphorylated, but this seems unlikely because in the natural setting, dephosphorylation of the receptor is strongly favored over phosphorylation in the absence of aggregation (28, 33). Therefore, the interactions we observed probably mimic the constitutive association of Lyn with the receptor rather than the interaction of recruited Lyn with the phosphorylated receptor (3, 4).

CHO Transfection Studies: Quantitative Aspects of FcεRI Aggregation-induced Phosphorylation of Tyrosine—The results with the cells transfected with active Lyn provide strong evidence that the amount of Lyn available to the receptor determines the capacity of the system to initiate signaling. The results with the cells transfected with catalytically inactive forms of Lyn (below) provide strong evidence that an equilibrium exists between receptor-associated and non-receptor-associated Lyn. The molecular mechanism we currently envision predicts that the capacity of small aggregates of receptors to initiate a response will be particularly sensitive to the amount of Lyn per receptor (3).[2] The slopes of the lines in Fig. 4 indicate that, indeed, cells stimulated with dimers of IgE are more

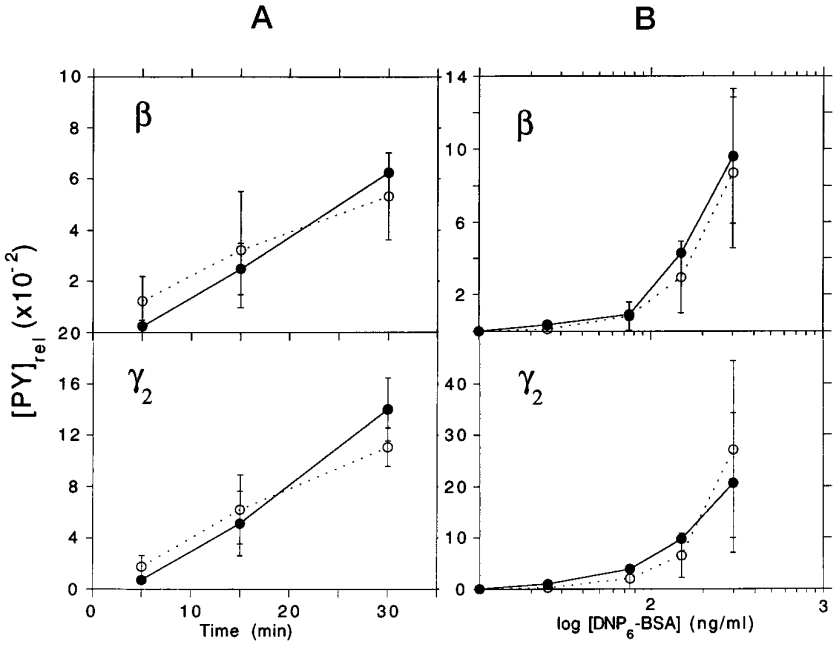

FIG. 5. **Phosphorylation of tyrosines on FcεRI in pZeo/FcεRI transfectants.** *A*, time course of phosphorylation induced by addition of 0.5 μg/ml trimeric IgE to CHO cells transfected only with FcεRI (CHO-B12) (*filled circles*) or with both FcεRI and empty pZeo (*open circles*). Phosphorylation of receptor tyrosines was determined by Western blotting of anti-IgE immunoprecipitates separated by PAGE. The data are the average of duplicate determinations from one transfectant (CHO-B12) and six different co-transfectants (Clones Z1-Z6, Table I). The number of FcεRI per cell and the ratios of transfected Lyn per FcεRI for each clone are described in Table I. The *error bars* represent the range between samples. The densitometric values from specimens of cells incubated with 0.5 μg/ml monomeric IgE for 15 min were subtracted. *B*, antigen dose dependence of phosphorylation. Transfectants containing FcεRI only (*filled circles*) or pZeo and FcεRI (*open circles*) were triggered with increasing doses of antigen for 2 min. The data represent the average of three different pZeo/B12 clones (Z1, Z5, Z6) compared with a single clone containing FcεRI only (CHO-B12), from three separate experiments. The *error bars* show the standard deviations.

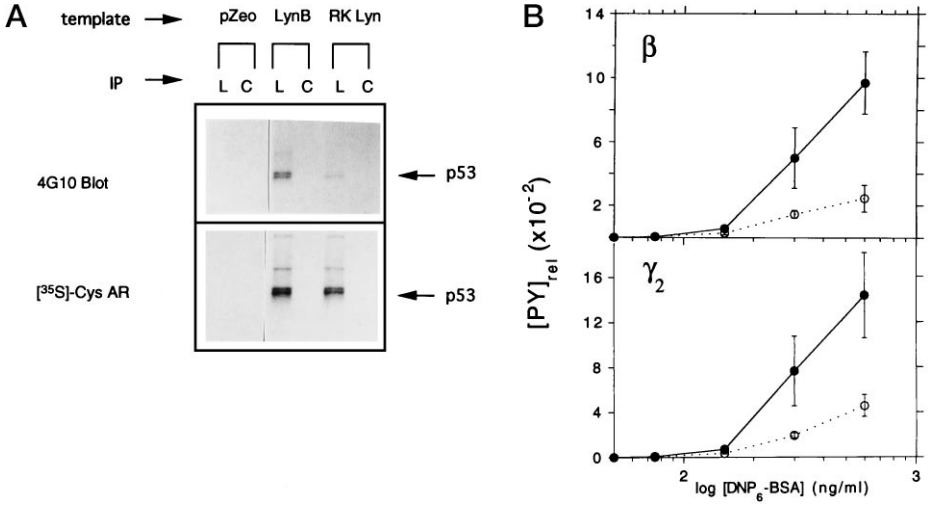

FIG. 6. **Assay of catalytically inactive Lyn B and its effect on the phosphorylation of tyrosines in FcεRI in RK Lyn/FcεRI co-transfectants.** *A*, autophosphorylation of wild type Lyn B and catalytically inactive Lyn B (RK Lyn). *In vitro* transcription-translation reactions containing [^{35}S]Cys were performed using RK Lyn B, Lyn B, or pZeo vector templates, and T3 polymerase as described under "Experimental Procedures." The reaction mixtures were incubated with anti-Lyn sera (*L*) or control (preimmune rabbit) sera (*C*) and the immunoprecipitated proteins (*IP*) resolved by PAGE. The *upper panel* shows the autophotograph of a blot with the 4G10 anti-Tyr(P); the *lower panel* represents the autoradiogram (*AR*) of the same gel. *B*, antigen dose dependence of phosphorylation. Cells co-transfected with catalytically inactive Lyn (RK Lyn) (*open circles*) or vector alone (*filled circles*) were sensitized with mouse anti-DNP IgE and stimulated for 2 min with increasing doses of antigen (DNP-BSA). The data for the cells transfected with catalytically inactive Lyn are the averages of two clones tested separately in duplicate (RK17, RK26); for those transfected with vector alone, the data are from a single clone (Z5) tested in duplicate. One experiment representative of three conducted is shown. *Error bars* show the standard deviations for the RK Lyn clones and the range for the pZeo clones.

sensitive to the concentration of Lyn than those stimulated with antigen.

Mapping of Sites of Interaction by Competition for Binding to the FcεRI—Catalytically inactive Lyn (RK Lyn) consistently inhibited signaling by the receptor compared with control cells (Fig. 6*B*). The effect was even more dramatic using trimers of IgE in cells transfected with the unique domain, and complete inhibition was detected at early time points (Fig. 8*A*). Again, this is consistent with the prediction that small aggregates would be more sensitive to the ratio of active Lyn:receptor. The inhibitory effect of the catalytically inactive Lyn indicates that the interaction between Lyn and the receptor is not dependent on an intact catalytic site on the kinase.

Notably, the unique domain alone was about as effective as

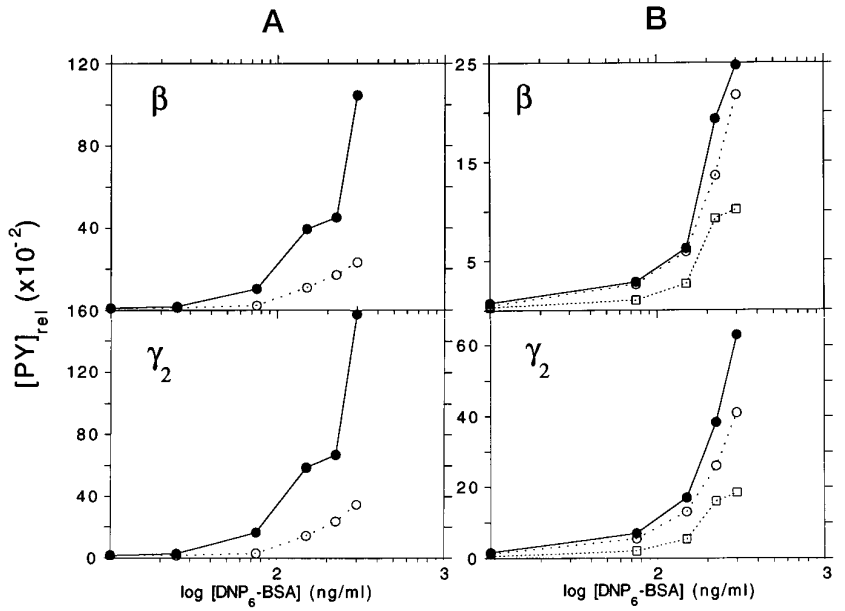

FIG. 7. **Inhibition of antigen-induced phosphorylation of tyrosines in cells co-transfected with FcεRI and the unique domain of Lyn A.** FcεRI transfectants of CHO cells were co-transfected with vector alone (pZeo/B12) or with the unique domain of Lyn A. The phosphotyrosines on immunoprecipitates of the receptors were quantitated by Western blotting as before, after stimulating the IgE-sensitized cells with antigen. *A*, comparison between (CHO-B12) (*filled circles*) and clone C6 (*open circles*) transfected with the unique domain of Lyn A. One experiment, representative of three conducted, is shown. *B*, comparison between pZeo 5 (*filled circles*) and cells transfected with the unique domain of Lyn A, and expressing a low amount (clone U7, *open circles*) or higher amount (U8, *open squares*) of the unique domain of Lyn A .

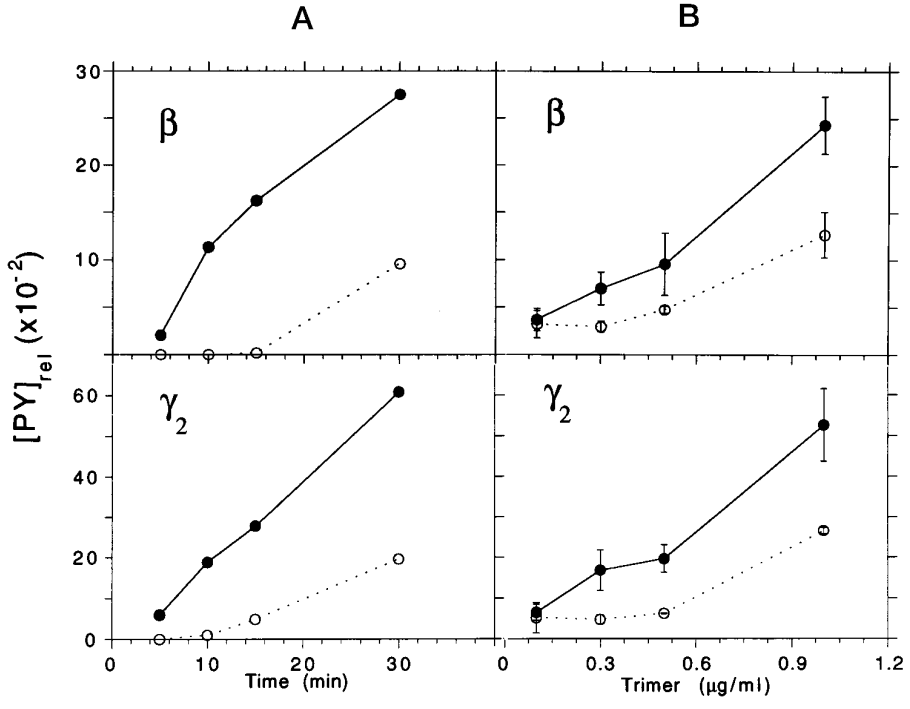

FIG. 8. **Inhibition of trimer-induced phosphorylation of tyrosines in FcεRI by transfected unique domain of Lyn A.** Clone C6 (Lyn A unique) (*open circles*) cells and CHO-B12 cells (*filled circles*) were stimulated with trimers of IgE as described in the legend to Fig. 5*A*. *A*, cells were incubated for varying lengths of time with 0.5 μg/ml trimeric IgE. One experiment, representative of four, is shown. *B*, the same clones as in *A* were stimulated for 15 min with varying doses of trimeric IgE. The data shown are the averages of two separate experiments, and the *error bars* represent the range between samples.

the catalytically inactive Lyn in inhibiting the interaction between the receptor and the wild type endogenous Src family kinase in the CHO cells. This was not necessarily predictable for the following reason. We previously demonstrated that after aggregation, an initial phosphorylation of the receptor by the constitutively bound Lyn kinase is required for the recruitment of additional molecules of Lyn to the activated receptor (3). Because direct studies *in vitro* have shown that the SH2 domain of Lyn can interact with the phosphorylated ITAM of the β subunit (10), it is reasonable to think that the recruitment occurs through the interaction of the SH2 of the Lyn kinase with the phosphorylated receptor. Therefore, high expression of the catalytically inactive Lyn, which retains its SH2 domain, might have affected the level of phosphorylation differently than the unique domain alone. For example, in addition to blocking constitutive association of the endogenous kinase, it might have prevented the recruitment of further kinase to the phosphorylated receptors. Alternatively, the inactive Lyn might have protected the phosphotyrosine(s) from dephosphorylation (34). Because these would lead to opposing effects on the level of phosphorylation, we cannot rule out the possibility that fortuitously the two effects quantitatively canceled each other out. A more likely explanation is that the SH2 region of Lyn does not play a major role in regulating the level of phosphorylation of the receptor and may not be the basis of the recruitment to the phosphorylated receptors.

It appears likely that the principal interaction the competition experiments are assessing is the constitutive interaction between Lyn and the receptor. For example, *in vitro* experiments on the antigen receptor of B lymphocytes have demonstrated an interaction of the unique domain of Lyn and the related Src family kinase Fyn with the unphosphorylated but not the phosphorylated ITAMs from Igα (35). Earlier studies on the association of Fyn with the subunits of the CD3 complex of

the T-cell receptor (36) and of Lck with CD4 (37) have implicated the analogous region in those kinases. Resh *et al.* have noted the highly homologous sequences within the first 10 residues of the Src family kinases and have presented evidence that this region, which she dubbed SH4, is critical for the targeting of the kinases to membranes (32). Citing unpublished data, Lin *et al.* implicate the same region in the interaction of Lyn and the β subunit (8). Timson Gauen *et al.* have studied the targeting of $p59^{fyn}$ to membranes and its interaction with chimeric constructs of the T cell receptor CD3 ε-chain using mutational analysis (38). They concluded that four residues within the SH4 region, *i.e.* Gly^2, Cys^3, Lys^7, and Lys^9, were required for both interactions. It should be noted however, that in their analysis, the interaction of $p59^{fyn}$ with the ε chain might well have been influenced by the interaction of $p59^{fyn}$ with the plasma membrane. Lyn shares these critical N-terminal residues with Fyn, and it is likely, therefore, that this region plays a homologous role in this kinase's interaction with the FcεRI.

Alternative constructs of Lyn could be used to probe further the nature of this interaction, but such studies would have to be very extensive to obtain any more insight than the present studies provide. Furthermore, such additional studies could only provide rather indirect evidence about which structures in Lyn are important. Rather than pursuing such intermediate results, what the field really needs is structural information at the atomic level of resolution, and we are turning our experimental strategy in that direction. More detailed analyses must also control for the possibility that these interactions may be occurring in the context of specialized membrane domains (39).

As already noted, our experimental findings are consistent with the prediction of the current model that the ability of small aggregates to initiate a response should be particularly sensitive to the fraction of receptors constitutively associated with kinase. No such enhanced sensitivity is predicted for the recruitment of further molecules of kinase to the phosphorylated receptors. The interpretation that it is the constitutive interaction that is affected is also consistent with one of the findings reported by Wilson *et al.* (9). They observed that a chimeric construct bearing the β_c domain when transfected into RBL cells failed to become phosphorylated but inhibited both base-line and aggregation-induced phosphorylation of the endogenous FcεRI. This result likely reflects competition by the transfected β chain for limiting amounts of constitutively associated kinase. Thus their experiment is in effect the mirror image to the ones we describe.

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