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

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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 domain) 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, pCDMS, and pI2e, as well as a baculovirus MAXBAC expression kit from Invitrogen (Carlsbad, CA); polyacrylamide gels used for electrophoresis (PAGE) from NOVEX (San Diego, CA); the antibodies (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 (KY-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 125I using chloramine T (16). Goat anti-mouse IgE was purchased from ICN (Costa Mesa, CA); rabbit anti-rat IgG 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% CO2 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 AF000530.1

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2 The abbreviation used are: FcεRI, high affinity receptor for IgE; bp, base pair(s); BSA, bovine serum albumin; CHO, Chinese hamster ovary; DNP, diinitrophenyl; 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.

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strant 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. Cloning of positive plaques was performed through screening with 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).

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 size for Lyn A (56 kDa) and Lyn B (53 kDa) (data not shown). After digestion 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).

**Stimulation of Cells**—CHO cells to be stimulated with antigen were sensitized overnight with 125I-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 0.5 mm dextrose, and resuspended at 1 × 10^9 cells/ml. DNP-BSA was added as a 5-fold stock solution to 5 × 10^9 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 0.5% 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 centrifugation 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% acrylamide gels equilibrated with Tricine and the phosphorylated proteins detected with an anti-Tyr(P) antibody and an enhanced chemiluminescence 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 were pretreated with the lysates ("precleared" with 100 μl of protein A-Sepharose beads overnight) for 2 h. After centrifugation 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 FcR Association**—CHO cells were suspended at 5 × 10^9 cells/ml and incubated with 5 μg/ml 125I-labeled mouse 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 by gradient centrifugation, and their radioactivity was quantitated by liquid scintillation counting. Of five high expressing 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 XhoI digestion and ligated into the homologous Nhel 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 shown in the inset blot (not shown).
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 [35S]Cys according to the manufacturer’s recommendation (T3 Taq® 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 subunit to the yeast two hybrid domain (BD)–receptor subunit fusion proteins based on the cytoplasmic domains of the subunit. The four transmembrane domains of the subunit are shaded. **A**, the β chain and the Gal4 binding domain (BD)–receptor subunit fusion proteins based on the cytoplasmic domains 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).

The nucleotide sequence coding for the N- and C-terminal cytoplasmic domains of the β subunit of the rat IgE receptor, βN and βC, 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 βN and βC into the newly developed vector pAS-2-1, which is similar to pAS2, but has the acidic hemagglutinin epitope removed. Neither pAS2-1-βC or pAS2-1-βN were autonomously active.

The activities of the His and LacZ reporter genes in CG1945 yeast transformants expressing Lyn and βN, βC or γC were tested as described (see “Experimental Procedures”). The full-length and unique domain of both Lyn A and Lyn B interacted directly with βC (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 Lyn and βC 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 βC constructs resulted in fewer colonies and slower growth on His-deficient medium and only the large colonies turned blue. No interaction was detected between βN or γC with any forms of Lyn in this assay.

To quantitate the interaction between Lyn and βC or βN, 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

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**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).
produced only slightly higher amounts of β-galactosidase than the negative control. Again, no interaction between Lyn and \( \beta_N \) was detected.

**Characterization of Transfected FcεRI in CHO Cells**

A clone of transfected CHO cells that stably expressed \( \sim 170,000 \) 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 \( \sim 58 \) kDa, i.e. slightly greater than the apparent molecular mass of 53 and 56 kDa observed for rat Lyn (Fig. 3A). 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. 3A, lane 1). When the cells were incubated with multivalent antigen (DNP-BSA) prior to solubilization, phosphorylation of tyrosines on the \( \beta \) and \( \gamma \) 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 \( \leq 1 \) 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. 3B, lane 8). In contrast, incubation of the CHO-B12 cells with dimeric IgE failed to induce detectable phosphorylation of the receptors (Fig. 3B, 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. 3A), 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 \( \beta \) and \( \gamma \) 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
Lyn-FcεRI Association

TABLE I

| CHO transfectants | Name          | Clone | Insert | FcεRI | Lyn per FcεRI | Lyn (Inact.) per Lyn (End.) |
|-------------------|---------------|-------|--------|-------|---------------|-----------------------------|
|                   | CHO-B12       | B12   | NA     | ×10^-5 | 1.7           | 2.5                         |
| Lyn/B12           | A6            | Lyn B | 1.0    | 1.0   | NA            | NA                          |
| Lyn/B12           | A9            | Lyn B | 0.7    | 9.3   | NA            | NA                          |
| Lyn/B12           | A11           | Lyn B | 1.3    | 66    | NA            | NA                          |
| Lyn/B12           | D1            | Lyn B | 1.3    | 34    | NA            | NA                          |
| Lyn/B12           | D7            | Lyn B | 1.3    | 3.2   | NA            | NA                          |
| Lyn/B12           | D8            | Lyn B | 1.3    | 0.94  | NA            | 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^d         |
| 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            | NA                          |
| pZeo/B12          | Z2            | None   | 0.8    | 6.6   | NA            | NA                          |
| pZeo/B12          | Z3            | None   | 1.0    | 5.0   | NA            | NA                          |
| pZeo/B12          | Z4            | None   | 0.8    | 6.8   | NA            | NA                          |
| pZeo/B12          | Z5            | None   | 1.5    | 2.8   | NA            | NA                          |
| pZeo/B12          | Z6            | None   | 1.0    | 6.6   | NA            | 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 × 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 × 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 zoein 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. 5A). 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. 5B). 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 tyrosine phosphorylated. By Western blotting the level of magnitude or pattern of total cellular proteins that became

Catalytically Inactive Lyn Kinase—A full-length, catalytically inactive Lyn B kinase was prepared by mutating Lyn^5279 to Arg (RK Lyn). As shown in Fig. 6A, in a coupled in vitro transcription-translation reaction, the wild type Lyn was autophosphorylated 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. 3A). 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. 6B). Therefore, a single point mutation converted a construct that stimulated phosphorylation of tyrosines on FcεRI to one that inhibited it (cf. Figs. 4 and 6B).

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. 3A). 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. 7A). 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. 7B). With a weaker stimulus (IgE trimers), complete inhibition of phosphorylation of the β and γ chains was observed at early time points (Fig. 8A) and at low concentrations of stimulant (Fig. 8B).

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 FcRI (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 × 10⁶ 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 +). FcRI 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 FcRI from Lyn B/FcRI transfectants. The FcRI on CHO cells transfected only with FcRI (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 ([P]Y_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 × 10⁴ cell eq of SDS lysates prepared from the transfected cells. The autoradiographs 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 × X²), 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 FcRI 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 βN or γC. 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 FcRI 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 FcRI 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...
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. 6B). 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. 8A). 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
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 $b$ 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 Ig$\alpha$ (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 targeting of Lyn and the β subunit (8). Timson Gauen et al. have studied the targeting of p59Fyn 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. Gly2, Cys3, Lys7, and Lys9, were required for both interactions. It should be noted however, that in their analysis, the interaction of p59Fyn with the ε chain might well have been influenced by the interaction of p59Fyn 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 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. Their experiment is in effect the mirror image to the ones we describe.

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