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Polygenic Autoimmune Traits: Lyn, CD22, and SHP-1 Are Limiting Elements of a Biochemical Pathway Regulating BCR Signaling and Selection

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Summary

A B lymphocyte hyperactivity syndrome resembling systemic lupus erythematosus characterizes mice lacking the *src*-family kinase Lyn. Lyn is not required to initiate B cell antigen receptor (BCR) signaling but is an essential inhibitory component. *lyn*$^{-/-}$ B cells have a delayed but increased calcium flux and exaggerated negative selection responses in the presence of antigen and spontaneous hyperactivity in the absence of antigen. As in invertebrates, genetic effects of loci with only one functional allele can be used to analyze signaling networks in mice, demonstrating that negative regulation of the BCR is a complex quantitative trait in which Lyn, the coreceptor CD22, and the tyrosine phosphatase SHP-1 are each limiting elements. The biochemical basis of this complex trait involves a pathway requiring Lyn to phosphorylate CD22 and recruit SHP-1 to the CD22/BCR complex.

Introduction

Susceptibility to complex diseases, such as autoimmune diabetes or systemic lupus erythematosus, is determined by the combined effects of alleles at multiple genetic loci (Drake et al., 1995; Theofilopoulos, 1995b; Vyse and Todd, 1996; Morel et al., 1997). The nature and biochemical effects of allelic variants for polygenic disorders are largely unknown, but the continuous variation in genetic risk from individual to individual represents a complex quantitative trait that might reflect quantitative variation in the protein components of biochemical pathways. Since quantitative traits are subject to natural selection, it may be supposed that the multiple allelic variants that encode them have been maintained by selective advantage. In the immune system, natural selection has to establish a balance between the ability to combat a variety of everchanging infections and the danger of generating life-threatening autoimmune disease. Lymphocyte hyperreactivity is associated with increased susceptibility to autoimmune diseases in laboratory mice (Theofilopoulos, 1995a; Mohan et al., 1997; Morel et al., 1997), although in the wild the same trait might be important for brisk responses to infection. The regulation of antigen receptor signaling is central to balancing immunity and tolerance because it determines both the positive and negative selection of B and T lymphocyte clones (Goodnow, 1996). Therefore, the biochemical pathways that regulate antigen receptor signaling are highly likely to generate quantitative traits and to be the sites of inherited immune modifiers.

The *src* family protein tyrosine kinase Lyn is abundantly expressed in B cells and is thought to be a key initiator of B cell antigen receptor (BCR) signaling (Bolen and Brugge, 1997; DeFranco, 1997; Kurosaki, 1997). A small amount of cellular Lyn is physically associated with the BCR and is activated upon BCR stimulation (Burkhardt et al., 1991; Yamanashi et al., 1991; Campbell and Sefton, 1992). Chicken B lymphoma cells lacking Lyn have delayed and diminished BCR-induced intracellular calcium release, indicating that Lyn has a role in this process (Kurosaki et al., 1994; Takata et al., 1994). Mice homozygous for a disruption of the *lyn* locus (*lyn*$^{-/-}$), however, display normal immature B cell development but have decreased numbers of mature peripheral B cells, greatly elevated serum immunoglobulin (Ig) M and IgA, and production of autoantibodies that cause autoimmune glomerulonephritis reminiscent of systemic lupus erythematosus (Hibbs et al., 1995; Nishizumi et al., 1995; Chan et al., 1997). Surprisingly, antibody responses to antigenic challenge are relatively normal in *lyn*$^{-/-}$ mice, and Lyn-deficient splenic B cells make exaggerated proliferative and extracellular signal-related protein kinase responses after BCR clustering and relatively normal but delayed patterns of antiimmunoglobulin-induced protein phosphorylation (Wang et al., 1996; Chan et al., 1997). These abnormalities contrast sharply with the phenotype of CD45-deficient mice (Cyster et al., 1996) or of Lyn-deficient chicken B lymphoma cells, where BCR signaling and activation are markedly diminished, and raises important questions about the role Lyn serves in B cell signaling and selection.

Here, we examine the function of Lyn in B cell responsiveness and selection to antigen. The data indicate that Lyn is not required to trigger BCR signaling to antigen nor to induce negative selection responses, but it does play an essential and rate-limiting role in an inhibitory

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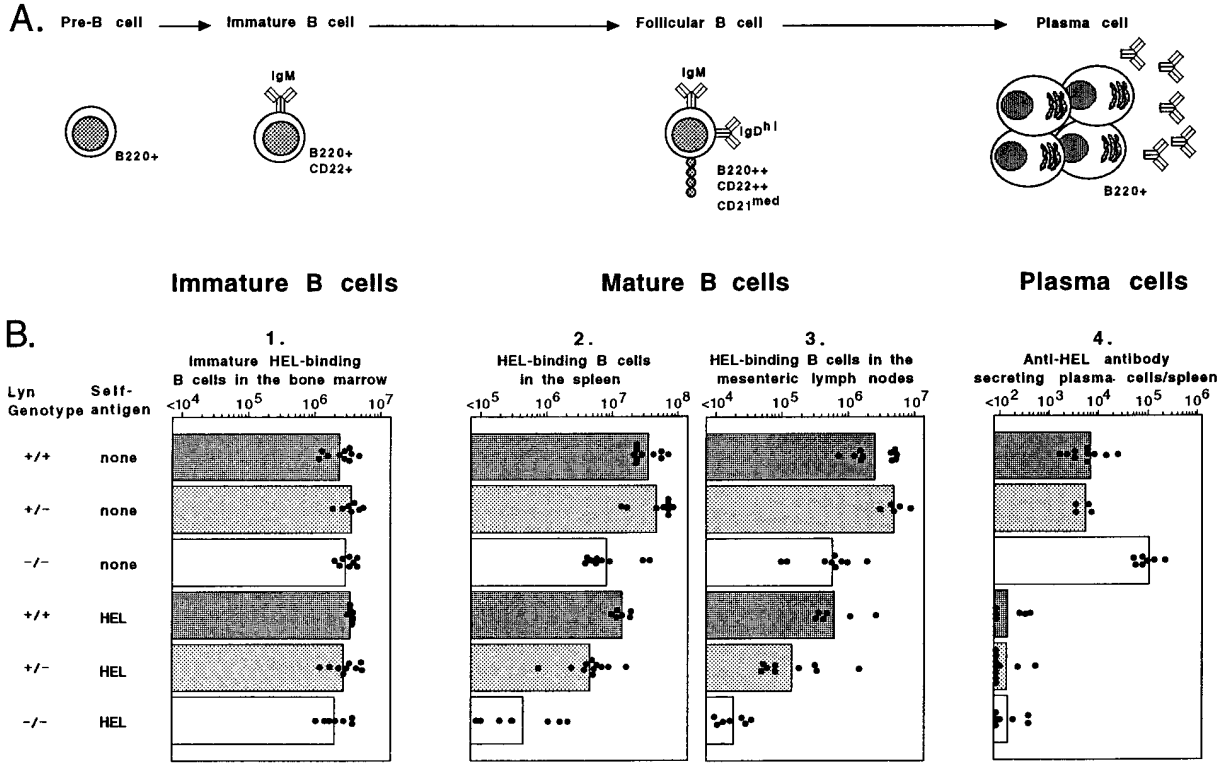

Figure 1. Development of Lyn-Deficient B Cells in the Presence and Absence of Self-Antigen

(A) Stages of B cell differentiation and cell surface markers used to distinguish them.

(B) Numbers of HEL-binding B cells and anti-HEL IgM^a-secreting plasma cells in lymphoid organs of radiation chimeras constructed in nontransgenic and ML5 HEL-transgenic mice (denoted by self-antigen "none" and "HEL" respectively) reconstituted with *lyn*$^{+/+}$, *lyn*$^{+/-}$, or *lyn*$^{-/-}$ Ig^{HEL}-transgenic bone marrow. Bone marrow numbers represent the total immature B cells in two femurs and two tibias. Data from two separate experiments are combined and mice were analyzed in cohorts between 51 and 117 days after chimera construction. Dots, the number from separate chimeric animals; bars, geometric means. Enzyme-linked immunosorbent assay analysis of serum from these chimeras showed that secreted anti-HEL IgM^a was present at geometric means (standard errors) of 20.93 μg/ml (6.81), 19.94 μg/ml (11.65), and 376.47 μg/ml (88.08) in *lyn*$^{+/+}$, *lyn*$^{+/-}$, and *lyn*$^{-/-}$ mice without sHEL, respectively, and less than 0.4 μg/ml in mice of each genotype expressing sHEL.

pathway that regulates B cell signaling through the phosphorylation of CD22 and recruitment of the Src homology domain (SH)-1–containing tyrosine phosphatase SHP-1. Each of the elements of this pathway is present in limiting amounts, such that heterozygous mutant alleles of *lyn*, *CD22*, and *SHP-1* that lower the functional gene dose by half interact to create a complex quantitative trait of B cell hyperreactivity. These results identify a pathway that tunes lymphocyte responsiveness and susceptibility to autoimmunity and demonstrate that the genetic approaches to analyzing signaling networks pioneered in invertebrates (Simon et al., 1991; Yoon et al., 1995; Verheyen et al., 1996) can also be used in mice.

Results

Development of Lyn-Deficient B Cells in the Presence and Absence of Self-Antigen

To study how Lyn deficiency affects B cell reactivity and selection to antigen, mice heterozygous or homozygous for a targeted disruption of the *lyn* gene (*lyn*$^{+/-}$ and *lyn*$^{-/-}$) (Hibbs et al., 1995) were crossed with Ig^{HEL} (hen egg lysozyme)-transgenic mice carrying immunoglobulin heavy and light chain transgenes encoding IgM^a and IgD^a specific for the well-defined protein antigen HEL (Goodnow et al., 1988). The resulting *lyn*$^{-/-}$ Ig^{HEL}-transgenic mice were of normal appearance, bred normally, and their survival up to 200 days of age was not markedly different from *lyn*$^{+/+}$ Ig^{HEL}-transgenic littermates. The absence of mortality associated with glomerulonephritis implies that the presence of the Ig^{HEL} transgene may have a protective effect against the development of the autoimmune disease, which might coincide with the restricted repertoire of the B cells.

To examine the effect of self-antigen upon the development of the Lyn-deficient B cells, *lyn*$^{+/+}$, *lyn*$^{+/-}$, and *lyn*$^{-/-}$ Ig^{HEL}-transgenic B cells were tracked during maturation in bone marrow chimeric mice that express soluble HEL (sHEL) as a circulating self-antigen in a form and amount sufficient to trigger anergy but not deletion (Figure 1). In control chimeras that lacked HEL antigen, there was a 20-fold increase in the number of plasma cells secreting anti-HEL IgM in the spleen of mice reconstituted with *lyn*$^{-/-}$ bone marrow, compared to *lyn*$^{+/+}$ bone marrow (Figure 1B, panel 4), and an equivalent increase in anti-HEL IgM antibodies was present in the serum (Figure 1). Antigen-independent hypersecretion

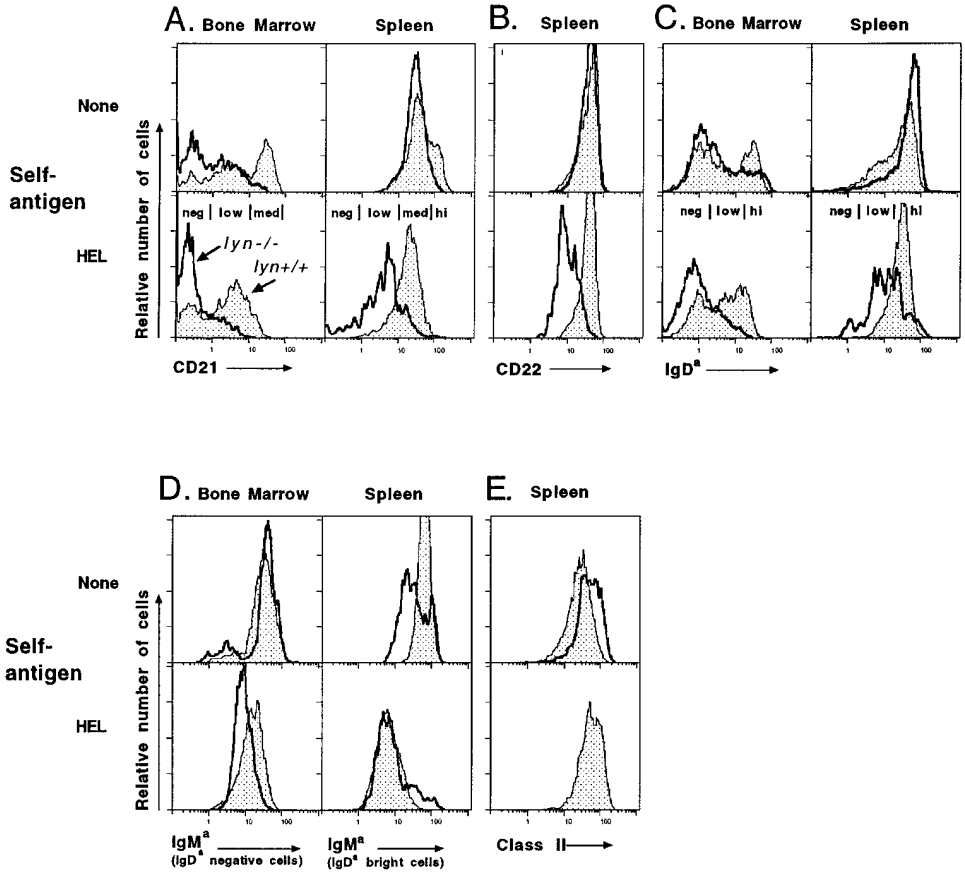

Figure 2. Expression of Different Cell Surface Molecules on Lyn-Deficient B Cells in the Presence and Absence of Self-Antigen

(A-E) Overlaid histograms showing cell surface staining for complement receptor CD21, CD22, IgD, IgM, or MHC class II on $lyn^{+/+}$ Ig^{HEL} B cells and $lyn^{-/-}$ Ig^{HEL} B cells in chimeric mice with no HEL antigen or expressing circulating sHEL (HEL). Cells with different levels of CD21 or IgD expression, corresponding to progressive stages in development, are indicated. Equivalent results were found in a minimum of six chimeric mice from two separate sets of chimeras. There is no histogram for MHC class II expression on $lyn^{-/-}$ Ig^{HEL} B cells in the presence of sHEL because of the negative selection of all splenic B cells in this mouse.

of IgM occurred at a comparable level in unmanipulated $lyn^{-/-}$ Ig^{HEL}-transgenic mice (data not shown). By contrast, in the continuous presence of circulating sHEL antigen (Figure 1), plasma cell formation and anti-HEL antibody secretion (Figure 1B) were completely suppressed regardless of the *lyn* genotype. Thus, active induction of antigen-specific B cell tolerance was intact in Lyn-deficient B cells.

Analysis of the steps in B cell development preceding plasma cells (Figure 1B, panels 1–3) indicated that negative selection to self-antigen was in fact exaggerated by Lyn deficiency. In sHEL-expressing mice, few mature $lyn^{-/-}$ B cells were present in the spleen and lymph nodes despite normal numbers of immature B cells in the bone marrow. Interestingly, $lyn^{+/-}$ B cells showed an intermediate level of deletion in the presence of HEL. The $lyn^{-/-}$ B cells in the bone marrow and spleen of sHEL-expressing mice were primarily immature B cells ($CD21^{neg/low}$, $CD22^{low}$, IgD^{low}) (Figures 2A–2C, unshaded histograms), indicating that sHEL inhibited or aborted maturation of $lyn^{-/-}$ cells rather than inducing anergy as occurred in the $lyn^{+/+}$ B cells. The negative selection response of $lyn^{-/-}$ B cells to sHEL thus resembles the exaggerated response of SHP-1–deficient Ig^{HEL} B cells (Cyster and Goodnow, 1995) and mirrors the normal tolerance response to forms of the antigen that cluster BCRs more avidly, such as membrane HEL (Hartley et al., 1991; Hartley et al., 1993) or a soluble dimeric HEL protein (S. Akkaraju et al., unpublished data).

In mice lacking HEL, mature $lyn^{-/-}$ B cells ($CD21^{med}$, $CD22^{high}$, IgD^{high}) (Figure 1A) developed but were present in smaller numbers in the spleen and lymph node (Figure 1B, panels 2 and 3), and few mature cells recirculated back to the bone marrow (Figure 2A, open histogram). Mature $CD22^{high}$ IgD^{high} $lyn^{-/-}$ B cells expressed 4- to 5-fold lower surface IgM, 2-fold lower levels of CD21, and twice as much major histocompatibility complex class II (MHC class II) as on mature $lyn^{+/+}$ B cells in the absence of antigen (Figures 2A–2E). These antigen-independent changes in cell number and phenotype parallel the changes in SHP-1–deficient Ig^{HEL} B cells in the absence of antigen (Cyster and Goodnow, 1995) and mirror the effects of B cell anergy normally brought about in $lyn^{+/+}$ cells by chronic binding of sHEL antigen (Figures 1 and 2, shaded lower panels). It is possible that the IgM^{low} class II^{high} phenotype of mature $lyn^{-/-}$ B cells reflects selection of rare variant cells from the immature B cell pool, rather than an effect of exaggerated signaling on

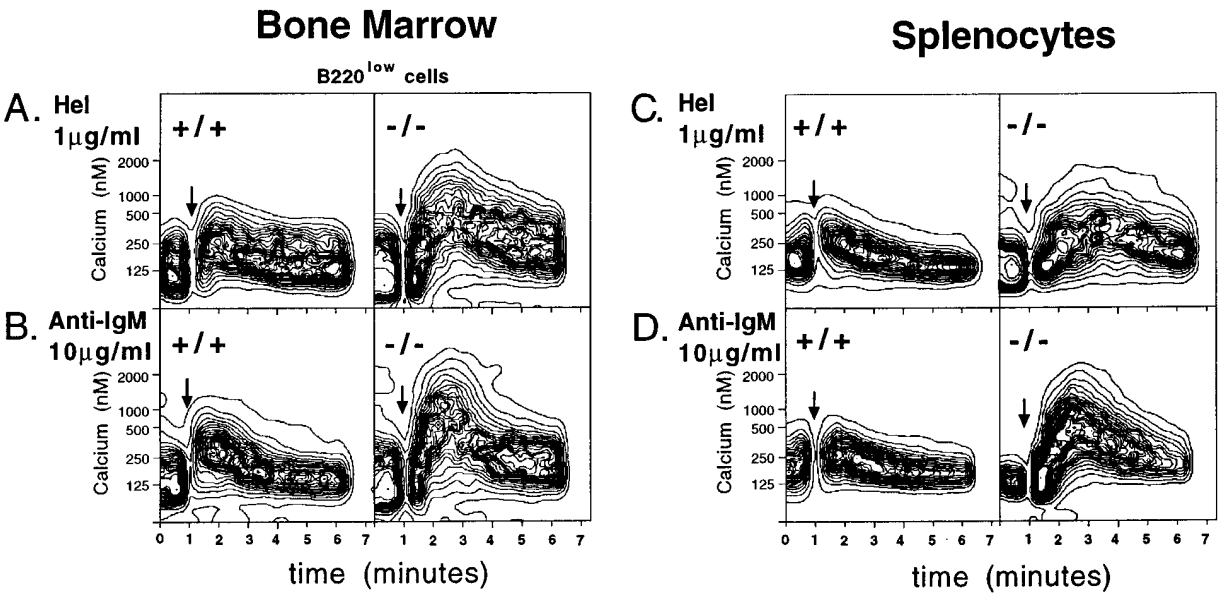

Figure 3. Exaggerated Intracellular Calcium Response to Antigen or Anti-Receptor Antibody in Lyn-Deficient B Cells

Flow cytometry of indo-1 loaded cells was used to monitor intracellular calcium levels as a function of time after stimulation in immature bone marrow B cells or mature splenic B cells expressing *lyn* ($^{+/+}$) or lacking *lyn* ($^{-/-}$). Arrows, time of addition of HEL antigen or polyclonal goat anti-mouse IgM. All measurements were gated on B220^{+} cells. (A-B) immature B cells were selectively gated as B220^{low}. Note that the peak calcium elevation occurs later in *lyn*$^{-/-}$ cells than in *lyn*$^{+/+}$ cells, but calcium reaches much higher concentrations and is elevated for longer periods of time. Equivalent results were obtained from four separate experiments.

all of the cells. Selection seems unlikely because cells with this phenotype are not detected in the immature pool, and the small numbers of mature *lyn*$^{-/-}$ B cells and their apparent poor survival in the periphery would seem to provide little scope for selection.

Unlike nontransgenic *lyn*$^{-/-}$ mice, no peritoneal B1 lineage cells were detected in *lyn*$^{-/-}$ Ig^{HEL}-transgenic mice (data not shown), indicating that the HEL-specific BCR remains nonpermissive to B1 cell formation in the absence of Lyn. This result contrasts with SHP-1 deficiency, which allows peritoneal B1 cells expressing the HEL-specific BCR to accumulate (Cyster and Goodnow, 1995) and indicates that IgM hypersecretion due to Lyn deficiency can be dissociated from B1 cell accumulation.

Construction of mixed chimeras with *lyn*$^{-/-}$ and *lyn*$^{+/+}$ bone marrow, using methods described previously (Cyster and Goodnow, 1995), confirmed that the exaggerated negative selection in the presence of sHEL antigen and the developmental changes and antibody hypersecretion in the absence of antigen were due to Lyn deficiency in the B cells themselves and not secondary to disregulation of macrophages or other cell types in Lyn-deficient mice (data not shown).

Evidence for Exaggerated BCR Signaling in Lyn-Deficient Cells

The exaggerated negative selection to self-antigen, together with the spontaneous hypersecretion of antibody, down-regulation of IgM, and elevated MHC class II on mature cells in the absence of antigen suggested that the BCRs on *lyn*$^{-/-}$ cells might be hyperactive. To test for the presence of exaggerated BCR signaling in Lyn-deficient mice, indo-1–loaded B cells were stimulated with sHEL or anti-IgM and the changes in intracellular calcium measured by flow cytometry. Immature B cells from the bone marrow as well as mature cells from the spleen were examined in case compensatory processes in mature cells such as IgM down-regulation might have obscured signaling differences. In both developmental subsets, acute exposure to sHEL or anti-IgM induced a higher peak intracellular calcium concentration and a more sustained rise of calcium in *lyn*$^{-/-}$ cells than in *lyn*$^{+/+}$ controls (Figures 3A–3D). The effects of Lyn deficiency were evident at receptor-saturating concentrations of HEL and at lower concentrations and were B cell autonomous, as indicated by analysis of calcium responses in cells from mixed chimeric animals (data not shown). In addition, *lyn*$^{-/-}$ B cells showed a consistent delay in the initial phase of the calcium response (Figures 3A–3D). These results imply that Lyn plays a role in the initiation of calcium mobilization as well as in its inhibition.

Genetic Interaction between Lyn and SHP-1

The exaggerated BCR signaling and selection in *lyn*$^{-/-}$ Ig^{HEL} B cells described above represents an almost exact phenocopy of the changes brought about in Ig^{HEL} B cells by the homozygous loss of function mutation in the SHP-1 tyrosine phosphatase motheaten viable (*me*^{v}) (Cyster and Goodnow, 1995). This similarity raised the question of whether the two proteins could be operating in a single pathway. To address this possibility, we employed an approach that has been very useful for analyzing signaling networks in *Drosophila melanogaster* and *Caenorhabditis elegans* (Simon et al., 1991; Yoon et al., 1995; Verheyen et al., 1996) by looking for a genetic interaction between heterozygous mutants of Lyn and SHP-1 where the dosage of functional genes was halved. First, we observed that B cells in heterozygous mice

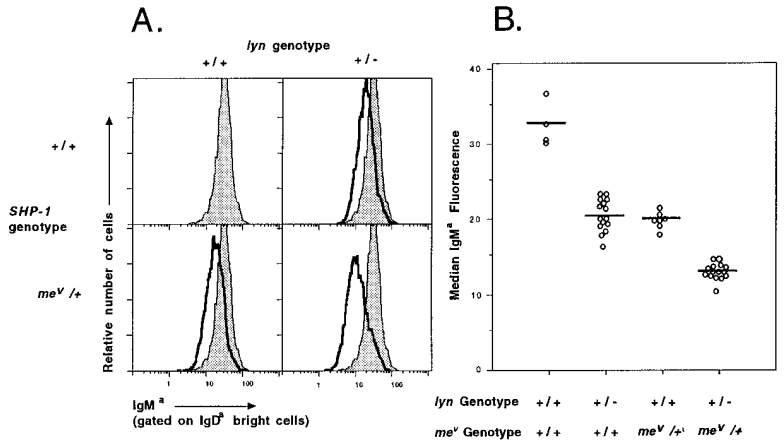

Figure 4. Genetic Interaction between Heterozygous Alleles of *lyn* and *me^v* Affects the Density of IgM on the B Cell Surface

A) Overlaid histograms showing IgM^a expression on mature (IgD^a bright) Ig^{HEL} spleen B cells of the indicated genotypes: WT (shaded) versus *lyn*$^{+/-}$*me*$^{v+/+}$, *lyn*$^{+/+}$*me*$^{v+}$, *lyn*$^{+/-}$*me*$^{v/+}$ (unshaded, thick lines). Results are representative of more than ten separate experiments. (B) Median IgM^a expression on mature IgD^a bright Ig^{HEL} B cells in the blood of mice with the indicated genotypes. Dots, values from individual animals from a single experiment; bars, means for each genotype. Animals were bled and samples stained for IgM^a, B220, and IgD^a and analyzed by three-color FACS analysis, before gating on IgD^a bright cells (upper 25th percentile of IgD^a). Two-way analysis of variance (ANOVA) showed that IgM expression was very significantly affected by heterozygosity at *lyn* ($F = 254.43$, 1 degree of freedom [df], $p < 0.0001$) or *me^v* locus ($F = 221.229$, 1 df, $p < 0.0001$) as well as by interaction between the two ($F = 16.887$, 1 df, $p = 0.0002$).

with a single functional *lyn* or *SHP-1* allele (*lyn*$^{+/-}$ or *me*$^{v/+}$) exhibited a degree of spontaneous IgM down-regulation that was intermediate between wild-type (WT) and *lyn*$^{-/-}$ or *me*^{v}/*me*^{v} cells (Figure 4). This implied that a partial reduction in the function of either protein was detected within the B cells and resulted in a compensatory adjustment of surface IgM density. By intercrossing heterozygotes for the *me*^{v} mutation and the *lyn* allele, double heterozygous Ig^{HEL} mice with partial deficiency in both proteins were produced. Simultaneous heterozygosity at both *lyn* and *me*^{v} loci caused B cells to adjust their surface IgM density to a lower level than heterozygosity at either locus alone (Figures 4A and 4B), indicating a genetic interaction (ANOVA, $p = 0.0002$).

Apart from the spontaneous antigen-independent adjustment of IgM surface density, B cells from the single and compound heterozygotes appeared to tolerate partial deficiency of Lyn and SHP-1 and were phenotypically similar to WT cells. There was no reduction in numbers of mature B cells nor increased MHC class II in the absence of antigen and no hypersecretion of antibody (data not shown). The antigen-induced calcium response was similar to WT in the single and double heterozygotes, with the exception of a subtle increase of rate and amplitude in *me*$^{v/+}$ B cells.

Lyn Is Required for Constitutive and Antigen-Induced Phosphorylation of CD22 and Recruitment of SHP-1

The genetic interaction between alleles encoding Lyn and SHP-1 indicates that these proteins regulate the BCR through the same or parallel biochemical pathways. A possible link between Lyn and SHP-1 is the coreceptor CD22, because SHP-1 is recruited to phosphorylated tyrosine-based inhibition motifs (ITIMs) on CD22 after BCR clustering (Campbell and Klinman, 1995; Doody et al., 1995; Law et al., 1996; reviewed by Cyster and Goodnow, 1997) and has been reported to be immunoprecipitated in a complex with both CD22 and Lyn (Tuscano et al., 1996). Moreover, 0.2%–2% of surface IgM receptor is constitutively associated with CD22, (Leprince et al., 1993; Peaker and Neuberger, 1993) and B cells from CD22-deficient (*cd22*$^{-/-}$) mice also have spontaneous down-regulation of IgM and exaggerated calcium responses to BCR clustering (O'Keefe et al., 1996; Otipoby et al., 1996; Sato et al., 1996; Nitschke et al., 1997). This raises the possibility that Lyn-induced phosphorylation of CD22 governs the recruitment of SHP-1 to BCR-associated CD22. To test this hypothesis, *lyn*$^{-/-}$ and *lyn*$^{+/+}$ Ig^{HEL} B cells were stimulated with HEL in vitro and cell lysates examined by immunoprecipitation and Western blotting (Figure 5A). In *lyn*$^{+/+}$ B cells, antigen or anti-IgM induced a marked increase in tyrosine phosphorylation of CD22 and increased binding of SHP-1 to CD22 (Figure 5A, i). By contrast, little increase in CD22 tyrosine phosphorylation or SHP-1 binding to CD22 was induced by these stimuli in *lyn*$^{-/-}$ cells. Antigen-induced phosphorylation of CD79α/β and Syk was only modestly reduced in *lyn*$^{-/-}$ B cells (Figure 5A, ii-iii), in agreement with other recent findings (Chan et al., 1997). Within the sensitivity of these biochemical assays, no difference could be detected between *lyn*$^{+/-}$ and *lyn*$^{+/+}$ Ig^{HEL} B cells (data not shown).

A basal level of CD22 phosphorylation and SHP-1 association with CD22 exists in unstimulated Ig^{HEL} B cells in the absence of antigen (Figures 5A and 5B), as does a basal phosphorylation of CD79α/β and Syk (Figure 5A and data not shown). *lyn*$^{-/-}$ B cells retained normal basal phosphorylation of CD79α/β and Syk, but basal phosphorylation of CD22 and SHP-1 association was markedly reduced relative to *lyn*$^{+/+}$ cells (Figure 5B).

Genetic Interaction between Lyn, SHP-1, and CD22

To test the functional significance of the biochemical pathway involving Lyn, CD22, and SHP-1 in the regulation of the BCR, we asked whether partial deficiency in CD22 could exaggerate the effects of partial Lyn and SHP-1 deficiencies. Mice heterozygous for a targeted deletion of CD22 (cd22$^{+/-}$) (Otipoby et al., 1996) were mated with *me*^{v}*/+ and lyn*$^{+/-}$ Ig^{HEL}-transgenic mice to generate sibships with fully WT or heterozygous mutant alleles at each of the three loci. Several animals from each of the eight resulting genotypes were examined, and radiation chimeras were established using one set of bone marrows to extend these observations and study negative selection to self-antigen.

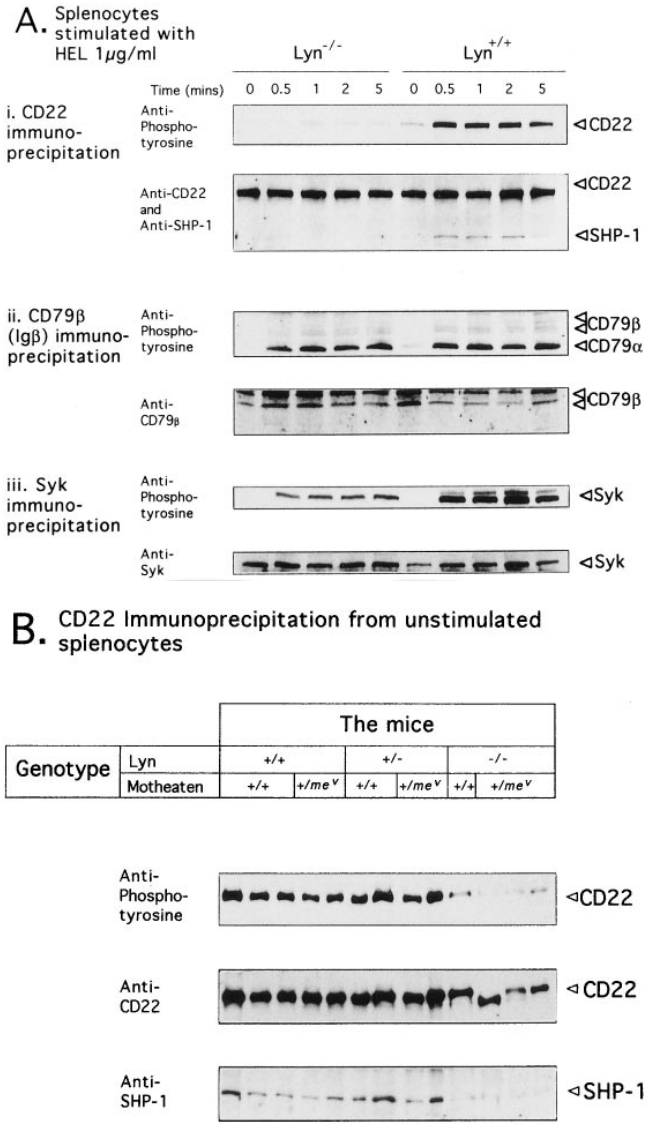

Figure 5. Lyn Is Required for Basal and Antigen-Induced Phosphorylation of CD22 and Recruitment of SHP-1

(A.i–iii) Western blots showing results of serial immunoprecipitation of CD22, CD79β, and Syk from *lyn*$^{-/-}$ and *lyn*$^{+/+}$ Ig^{HEL} spleen B cells. Cells were stimulated with media alone (time = 0) or HEL (1 μg/ml) for periods of 30 s to 5 min. Panels show induced tyrosine phosphorylation by probing the immunoprecipitates for anti-phosphotyrosine, relative to total immunoprecipitated protein measured by reprobing the same blot with antisera to CD22, SHP1, CD79β, or Syk. (i) The level of SHP-1 association with phosphorylated CD22 is demonstrated by probing the CD22 immunoprecipitates with an anti-SHP-1 antibody. Results are representative of four experiments using HEL as stimulant and four experiments using anti-IgM as stimulant.

(B) Western blot of anti-CD22 immunoprecipitates from unstimulated Ig^{HEL} spleen B cells of the indicated genotypes. Each lane represents splenic cells from a separate mouse. Levels of CD22 tyrosine phosphorylation, CD22 loading, and SHP-1 association with CD22 are shown. The size difference between phosphorylated and unphosphorylated forms of CD22 in B cells from one of the *lyn*$^{-/-}$*me*$^{v/+}$ mice (lane 11) is of unknown significance.

Mature B cells in *cd22*$^{+/-}$ Ig^{HEL} mice expressed half of the normal cell surface density of CD22 (data not shown). In the absence of antigen, mature B cells from *cd22*$^{+/-}$ mice adjusted their IgM receptors to a lower cell surface density (Figure 6) but exhibited no other functional abnormality. When the partial deficiency of CD22 was combined with partial deficiency for Lyn or SHP-1, the B cells further adjusted cell surface IgM to lower levels that varied with the combination of alleles inherited. This effect was most extreme in the compound *cd22*$^{+/-}$ *lyn*$^{+/-}$*me*$^{v/+}$ triple heterozygote, which expressed 15% of WT receptor levels (Figures 6A and 6B). Spontaneous adjustment of surface receptor density was thus a continuous, quantitative trait upon which single alleles at three loci had a partial and additive effect. Each of the three proteins was limiting based on the effects of reducing gene dosage to a single functional allele, in the order *me*^{v}>*lyn*>*cd22*, and there was locus heterogeneity because different combinations of alleles generated similar levels of modulation, e.g., *lyn*$^{+/-}$*cd22*$^{+/-}$ heterozygote having the same effect as *me*$^{v/+}$ heterozygosity (Figure 6).

The variation in IgM receptor density contrasted with the expression of developmental abnormalities that varied less continuously as a function of gene dosage, and were presumably triggered by more discrete signaling thresholds. These more qualitative B cell phenotypes became most evident in the *cd22*$^{+/-}$*lyn*$^{+/-}$*me*$^{v/+}$ triple heterozygotes. In the absence of self-antigen, partial deficiency in all of the three proteins caused no decrease in the number of mature B cells, but the cells expressed lower levels of CD21 (Figure 7A) and increased MHC class II (Figure 7B), which in 3 of 5 mice was comparable to the phenotype of *lyn*$^{-/-}$ or *me*^{v}/*me*^{v} B cells in the absence of antigen (Figure 2 and Cyster and Goodnow, 1995). A greater than 15-fold increase in serum concentration of HEL-binding IgM was present in 2 of 4 triple heterozyogous mice, but not in any single or double heterozygous animals (data not shown). In the presence of sHEL, negative selection was exaggerated in a cumulative manner, as indicated by the decrease in number of splenic B cells in both *cd22*$^{+/-}$*lyn*$^{+/-}$*me*$^{v/+}$ triple and *cd22*$^{+/-}$*lyn*$^{+/-}$ and *cd22*$^{+/-}$*me*$^{v/+}$ double heterozygotes

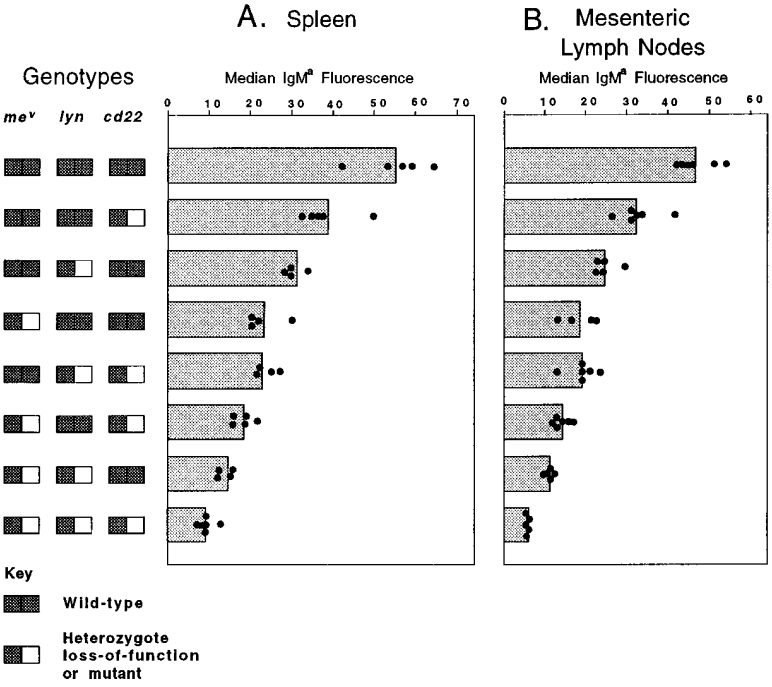

Figure 6. Genetic Interaction between lyn, me^v, and *cd22* Loci Defines a Quantitative Trait

(A-B) Median IgM^a levels on IgD^a bright Ig^{HEL} B cells (gated on the upper 25% of IgD^a-positive cells) from spleen and mesenteric lymph node. At least two separate mice were analyzed for each genotype. Further data was generated by analyzing radiation chimeras that had been reconstituted with bone marrow of the different genotypes, and mice of different genotypes were analyzed simultaneously. Chimeras were examined at 9–10 weeks after reconstitution and other mice at 6–8 weeks of age. Dots, individual mice; bars, arithmetic means.

(Figure 7C). Negative selection was most exaggerated in *cd22*$^{+/-}$*lyn*$^{+/-}$*me*$^{v/+}$ triple heterozygous B cells because the spleen and bone marrow B cells that remained were arrested at the CD21^{low} immature stage (Figure 7D) and very few reached the lymph nodes (Figure 7D), similar to the *lyn*$^{-/-}$ mice (Figure 1B).

Discussion

The above results establish that Lyn kinase is not required to initiate BCR signal transduction in B lymphocytes, but it is one of three molecules that are essential for regulation of BCR signaling and sensitive to small decreases in activity. The exaggerated calcium and negative selection responses of *lyn*$^{-/-}$ Ig^{HEL} B cells to antigen (Figures 1–3) are a phenocopy of the hyperresponse in Ig^{HEL} B cells homozygous for the poorly active *me*^{v} allele of SHP-1 (Cyster and Goodnow, 1995). Likewise, the hyperactivity in the absence of antigen, the spontaneous down-regulation of surface IgM on *lyn*$^{-/-}$ B cells, the diminished number of recirculating mature B cells, and increased MHC class II, mirrors the antigen-independent changes in *me*^{v}/*me*^{v} Ig^{HEL} B cells. The biochemical and genetic data in Figures 4–7 place Lyn and SHP-1 in a common pathway with Lyn kinase required to phosphorylate the BCR coreceptor protein CD22 and recruit SHP-1. Binding of SHP-1 via its SH2 domains to phosphorylated ITIMs of CD22 is known to activate the phosphatase (Doody et al., 1995), and crosslinking CD22 to the BCR can suppress extracellular signal-related protein kinase 2, c-Jun N-terminal kinase, and p38 activation (Tooze et al., 1997). The immediate downstream targets of SHP-1 are unresolved, but its action diminishes calcium flux as well as the mitogen-activated protein (MAP) kinases (above) and opposes responses such as the adjustment of surface IgM density and class II developmental arrest and deletion, proliferation, and plasma cell differentiation.

All three known elements of the regulatory pathway are limiting, since the presence of a single functional copy of *lyn*, *cd22*, or *me*^{v} caused compensatory adjustment of surface IgM receptor density. Combinations of these partial deficiencies had cumulative effects on IgM receptor density and had threshold effects on other responses to BCR signaling, such as class II induction and negative selection. The sensitivity to gene dosage and genetic interactions among the members of the pathway provide a clear example of polygenic control of both continuous (quantitative) and threshold (qualitative) traits and has implications for polygenic susceptibility to autoimmunity, for genetic analysis of signaling networks in mice, and for combination drug therapy approaches to immune modulation.

Compensation for Lyn Deficiency in the Initiation of BCR Signaling

The BCR appears to be in an equilibrium between unphosphorylated and phosphorylated states that is normally tuned by balancing BCR density, *src*-family and Syk kinases, and signal-enhancing coreceptor docking sites (e.g., CD19/CD21) against phosphatases (e.g., SHP-1) and inhibitory coreceptor docking sites (e.g., CD22 and FcγRIIB1). A basal level of tyrosine phosphorylation of CD79α/β, Syk kinase, CD22, and CD19 exists in naive Ig^{HEL} B cells in the absence of antigen (Figure 5 and data not shown), and this is rapidly increased when antigens cluster and increase the local density of BCRs or when tyrosine phosphatases are inhibited by pervanadate treatment (Wienands et al., 1996; B. T. Weintraub and C. C. G., unpublished data). In primary B cells, Lyn kinase is not essential for the basal or antigen-induced phosphorylation of CD79α/β or Syk, although it appears to

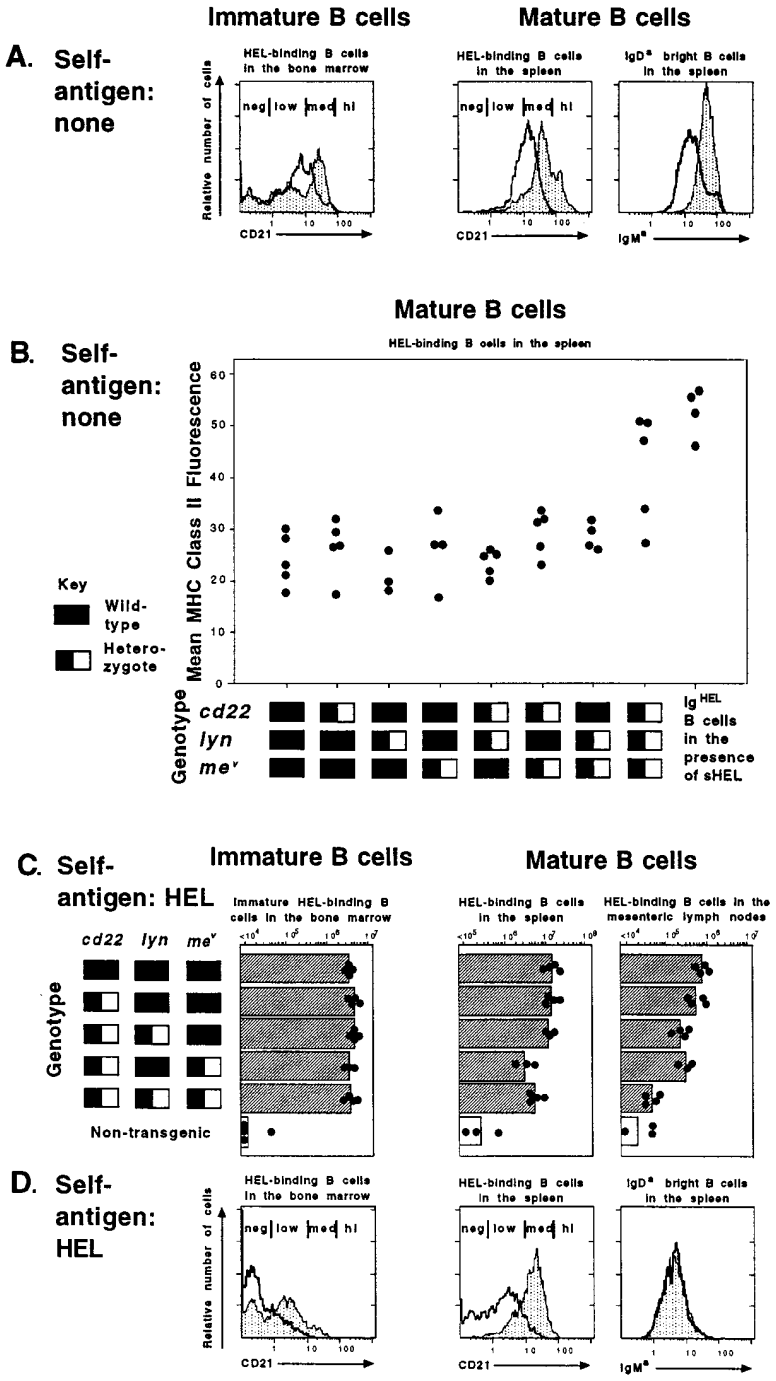

Figure 7. B Cell Hyperresponsiveness in Triple Heterozygote Mice

(A) Cell surface expression of CD21 and IgM^a on WT (shaded) and *lyn*$^{+/-}$*cd22*$^{+/-}$*me*^{v}/+ triple heterozygote (dark line) Ig^{HEL} B cells in the bone marrow and spleen of mice lacking sHEL self-antigen. Examples are representative of at least four mice in each group.

(B) Mean class II expression on Ig^{HEL} B cells from mice of different genotypes lacking the sHEL self-antigen, compared to each other and to class II expression on anergic WT Ig^{HEL} B cells exposed to sHEL. Dots, individual mice.

(C) The number of HEL-binding B cells of different genotypes in bone marrow, spleen, and lymph node of mice expressing sHEL antigen. With the exception of the nontransgenic B6/J staining controls, the mice were radiation chimeras analyzed at 9–10 weeks after reconstitution. Immature HEL-binding B cells were enumerated from two femurs and tibias. Dots, individual mice; bars, geometric means.

(D) Cell surface expression of CD21 and IgM^a on WT (shaded) and *lyn*$^{+/-}$*cd22*$^{+/-}$*me*$^{v/+}$ triple heterozygote (dark line) Ig^{HEL} B cells in the bone marrow and spleen of mice with circulating sHEL self-antigen. Examples are representative of 4 mice in each group.

assist the initial antigen-induced signaling because phosphorylation of CD79α/β or Syk was diminished, and elevation of calcium occurred more slowly in HEL-stimulated *lyn*$^{-/-}$ Ig^{HEL} cells (Figures 3 and 5). It is likely that the other *src* kinases expressed in primary B cells, such as Fyn and Blk, compensate for Lyn in promoting these signaling steps because HEL-induced calcium and extracellular signal-related protein kinase signaling and negative selection responses are diminished in Ig^{HEL} B cells lacking the *src*-family activator CD45 (Cyster et al., 1996). In CD45-deficient B cells, decreased activity of all the *src* kinase members would be expected to preclude compensation amongst them.

The conclusion that other *src* kinases compensate for Lyn in promoting certain BCR signaling pathways is consistent with analyses of *lyn*$^{-/-}$ DT40 chicken B cell lymphoma cells. Lyn is the sole *src* kinase expressed in DT40 cells, and BCR signaling is delayed and diminished when these cells lack a functional *lyn* gene, much as in *cd45*$^{-/-}$ B cells (Kurosaki et al., 1994; Takata et al., 1994). Transfection of *lyn*$^{-/-}$ DT40 cells with Lck or Fyn kinases restores BCR signaling and results in a delayed but

exaggerated calcium response comparable to the *lyn*$^{-/-}$ primary B cells described here. In chicken DT40 cells, Syk does appear to promote low-level BCR signaling without the involvement of Lyn or other src kinases. *Src* kinases cannot compensate for Syk, because BCR-induced calcium flux is completely abolished in *syk*$^{-/-}$ DT40 cells (Kurosaki et al., 1994) or *syk*$^{-/-}$ Ig^{HEL} bone marrow B cells (R.J.C. et al., unpublished data). Interestingly, Syk does not appear to be a limiting component in BCR signaling, because *syk*$^{+/-}$ Ig^{HEL} B cells with half of the normal amount of this protein are not detectably different from WT in any of the sensitive assays described here.

Quantitative and Qualitative Effects of Deficiency in the Lyn/CD22/SHP-1 Pathway

In contrast to the initiation and propagation of BCR signaling, other tyrosine kinases cannot compensate for Lyn in establishing the basal or induced phosphorylation of CD22 and recruitment of SHP-1, as these were greatly reduced in *lyn*$^{-/-}$ cells. A range of second messengers and responses to antigen are exaggerated or occur spontaneously when the Lyn/CD22/SHP-1 feedback pathway is crippled to differing degrees. Calcium responses to antigen are exaggerated comparably in *lyn*$^{-/-}$ and *me*^{v}/*me*^{v} B cells, although the delay to peak calcium seen with Lyn-deficiency does not occur in SHP-1 deficient cells (Cyster and Goodnow, 1995), consistent with a positive role for Lyn in the initial phase of the calcium response. The calcium response following BCR clustering is also exaggerated in *cd22*$^{-/-}$ B cells (O'Keefe et al., 1996; Otipoby et al., 1996; Nitschke et al., 1997). The antigen-induced calcium response nevertheless does appear to be well-buffered against partial disruption of the Lyn/CD22/SHP-1 feedback pathway, because little exaggeration in the calcium flux could be detected in single or compound heterozygous mutants (data not shown). Compensatory processes such as the decrease in IgM and CD21 on the partially deficient cells (Figure 7) may provide this buffering, although they appear insufficient to offset complete disruption of the Lyn/CD22/SHP-1 pathway.

Consistent with the role of a buffer, the adjustment of surface IgM density is a continuous trait that is sensitive to a range of subthreshold perturbations. Thus, low concentrations of sHEL antigen that are below the threshold for inducing anergy or class II up-regulation nevertheless cause a 50%–80% decrease in surface IgM on mature cells that are antigen dose-dependent (Goodnow et al., 1989; Akkaraju et al., 1997). In the absence of HEL antigen, surface IgM also decreases in a gene dose-dependent manner depending on the combination of loss of function mutations in the Lyn/CD22/SHP-1 feedback pathway (Figure 6). Overexpression of CD19, which increases BCR signaling, also causes a dose-dependent downward adjustment of surface IgM on mature B cells (Sato et al., 1997), whereas partial or complete deficiency of CD45 diminishes BCR signaling and IgM modulation to differing degrees (Cyster et al., 1996). The adjustment of surface IgM density presumably functions as a compensatory mechanism to help the cell tolerate chronic stimulation, much as occurs for many other cell surface receptors. In the case of anergic B cells, the mechanism for adjusting surface IgM levels is achieved by inhibiting the transport of newly assembled BCRs from the endoplasmic reticulum (Bell and Goodnow, 1994).

Other downstream responses to BCR signaling exhibited varying sensitivities to defects in the Lyn/CD22/SHP-1 feedback pathway. Up-regulation of class II, which is normally induced by acute or chronic antigen stimulation, occurs spontaneously in B cells with homozygous mutations in *lyn* or *SHP-1* and in the triple heterozygote but has not been observed in B cells where the feedback pathway was crippled at only two of the three elements. Negative selection to self-antigen by developmental arrest and clonal abortion normally requires the binding of HEL that is either a multivalent membrane array (Hartley et al., 1991) or a secreted covalent dimer (S. Akkaraju et al., unpublished data), implying that a higher threshold of BCR clustering is required for this response than for induction of anergy. Secreted HEL monomer is nevertheless able to trigger clonal abortion when the feedback pathway is fully crippled by Lyn or SHP-1 homozygous mutations, or to a lesser degree when the pathway is partially crippled in the triple heterozygous mutant. Heterozygous Lyn deficiency on its own also allowed this response to be triggered by sHEL, although the effect was less than the triple heterozygous deficit.

Although the variation in class II expression and antibody hypersecretion amongst triple heterozygote mice could be due to genetic heterogeneity at other loci in the backcrossed mice, it is more likely to be due to the incomplete penetrance of effects at *lyn*, *cd22*, and *me*^{v} loci. Incomplete penetrance due to age, stochastic, or environmental effects is an important feature of polygenic disease and for any single disease-predisposing genotype is manifest as a ratio of affected and unaffected individuals.

Immunoglobulin Hypersecretion and Autoimmunity Associated with Lyn Deficiency

IgM-secreting plasma cells are spontaneously produced at a low level in the absence of HEL antigen in Ig^{HEL}-transgenic mice and even in homozygous *Rag2*$^{-/-}$ Ig^{HEL} mice where no helper T cells are present. This antigen-independent formation of plasma cells is greatly exaggerated by the homozygous *lyn* or *SHP-1* mutations and was observed in two of four triple heterozygous mice but not in single or double heterozygous mutants. It is conceivable that basal (antigen-independent) BCR clustering and signaling, or occasional clustering of CD19/CD21 coreceptors by complement C3d on particular cells, normally promotes differentiation into plasma cells at a low rate and that this is greatly exaggerated by the defects in the inhibitory feedback pathway created by Lyn/CD22/SHP-1 deficiency. The greatly increased plasma cell formation is reminiscent of B cells in mice lacking the transcription factor Ets-1 (Bories et al., 1995). BCR-induced elevation of calcium results in phosphorylation and inactivation of Ets-1 (Fisher et al., 1991), raising the possibility that exaggerated signaling to the calcium/Ets-1 pathway in Lyn/CD22/SHP-1 deficiency might account for IgM hypersecretion.

The hypersecretion of immunoglobulins, autoantibodies, and glomerulonephritis that occurs in *lyn*$^{-/-}$ or *me*^{v}/*me*^{v} mice with a normal BCR repertoire (Hibbs et al., 1995; Nishizumi et al., 1995; Chan et al., 1997) may reflect a similar elevation of spontaneous plasma cell differentiation. Because negative selection of immature B cells to circulating self-antigens is shown here to be intact and even exaggerated, the autoantibodies secreted in *lyn*$^{-/-}$ mice may arise from low-affinity autoreactive B cells that bind self-antigens below the threshold BCR occupancy needed to trigger negative selection. Under these circumstances, autoimmune disease might result as much from nonspecific B cell hyperactivity causing high antibody titres and immune complexes as from preferential production of autoantibodies. B cell hyperactivity with increased MHC class II expression and hypersecretion of immunoglobulins is also a feature of at least two as yet unidentified murine lupus susceptibility genes, *Sle3* and *Sle4* (Mohan et al., 1997; Morel et al., 1997). These susceptibility genes interact with loci that affect other processes leading to autoantibody production, which might include the release of antigen from tissues. It is possible that self-reactive B cells lacking Lyn could be driven further into an exaggerated plasma cell response by the intermittent release of sequestered autoantigens that they have not encountered during development, for example, on the surface of apoptotic cells.

Polygenic Control of Disease Susceptibility

The Lyn/CD22/SHP-1 negative feedback pathway illustrates that an accumulation of 2-fold differences in key signaling molecules in mice creates quantitative traits and qualitative changes in cell behavior. Different combinations of *lyn*, *cd22*, and *SHP-1 me*^{v} alleles generate a spectrum of BCR signaling that is reflected in quantitative compensatory IgM modulation. When the cumulative genetic defects in the Lyn/CD22/SHP-1 pathway exceed a certain threshold, as in the triple heterozygous mutant mice, overt B cell hyperactivity occurs. This gene dosage sensitivity has several implications. First, it indicates that the powerful genetic enhancer/suppressor approaches used to understand signaling networks in *Drosophila* or *C. elegans* (Simon et al., 1991; Yoon et al., 1995; Verheyen et al., 1996) should also be feasible in mice. Second, cumulative effects of multiple weak alleles in the manner described here may characterise genetic predisposition to many complex diseases such as autoimmune diabetes and systemic lupus erythematosus. In these diseases, multiple loci contribute to the relative risk of disease, and locus heterogeneity exists such that different combination of alleles can have equivalent effects on the risk of disease (Morel et al., 1994; Drake et al., 1995; Theofilopoulos, 1995b; Vyse and Todd, 1996; Morel et al., 1997). It is interesting that one of the intervals associated with B cell hyperactivity and autoimmune glomerulonephritis in the NZB/W mouse model of systemic lupus, *Sle3*, contains the CD22 locus (Morel et al., 1997). Because *lyn*, *cd22*, and *SHP-1* encode limiting steps in the B cell response to antigen, any genetic variants arising at these loci could generate a powerful mechanism for their own natural selection. For these reasons, it will be interesting to discover if alleles relevant to human autoimmune susceptibility exist within this or related regulatory pathways. Third, the physiological significance of dosage sensitivity at each step in this signaling pathway may be that supply of each protein can be tightly regulated by processes that activate, inhibit, or sequester these molecules, as has been proposed for CD22. Finally, the data here indicate that it should be possible to inhibit signaling pathways with combinations of drugs that individually reduce the activity of different targets by small increments. This pharmacological approach, combined with an understanding of the genetic interactions between limiting elements in signaling pathways and the behaviour of common polymorphic alleles, should enable combination therapies of high specificity that correct qualitative immune defects by quantitative adjustment.

Experimental Procedures

Mice

C57BL/6 MD4 immunoglobulin-transgenic mice were mated with homozygote *lyn*$^{-/-}$ mice (Hibbs et al., 1995), heterozygote C57BL/6 *me*$^{v/+}$ mice (Jackson Laboratory), or homozygote *cd22*$^{-/-}$ mice (Otipoby et al., 1996). The targeted lyn allele had been incompletely backcrossed from the 129/Sv strain onto C57BL/6, and so to minimize the overall effect of other potentially modifying genes from the 129/Sv, a series of breeding pairs were established with intercrossing *lyn*$^{+/-}$ heterozygotes. Mice from two or more of these intercrosses were used in each experiment to control for any effects of strain background. The same strategy was used for crosses with the disrupted *cd22* allele. Heterozygote immunoglobulin-transgenic progeny were mated with heterozygote nontransgenic partners in a series of intercrosses. MD4 encodes an anti-HEL immunoglobulin with both IgM and IgD isotypes and C57BL/6 ML5 HEL-transgenic mice encode soluble HEL under the metallothionien promoter at a concentration of 1 nM in serum (Goodnow et al., 1988) (Induced Mutant Repository, Jackson Laboratory). The mice were genotyped using a series of polymerase chain reaction assays that are available on request.

Radiation Chimeras, Flow Cytometry, and Antibody Measurements

Radiation chimeras were reconstituted as previously described (Cyster and Goodnow, 1995). As far as possible, chimeras of different genotype were analyzed as complete cohorts, and no differences were observed between mice that had been reconstituted for different periods, which were not less than 7 or greater than 17 weeks.

A three-color fluorescence-activated cell sorter (FACS) analysis was performed on a FACScan with FACS desk software (Beckman Center Shared FACS facility). Surface marker staining and calcium analysis was performed as previously described (Goodnow et al., 1988; Mason et al., 1992; Hartley et al., 1993; Cyster and Goodnow, 1995; Cyster et al., 1996).

Serum anti-HEL IgM^{a} and IgD^{a} was measured by enzyme-linked immunosorbent assay as previously described (Cyster and Goodnow, 1995), and anti-HEL IgMa–secreting cells were enumerated in spleen by spot enzyme-linked immunosorbent assay as described (Rathmell and Goodnow, 1994).

Cell Lysates and Western Blot Analysis

Splenocytes for stimulation were isolated at room temperature, washed, and resuspended in 5% fetal calf serum, RPMI, and 10 mM HEPES. Prior to stimulation, both cells and stimulant were prewarmed for 5 min at 37°C, and reactions were initiated by combining them in a ratio of 3:1, which gave instant mixing. Stimuli were media alone for the full time course in the case of all unstimulated samples,

HEL (final concentration in media 1 μg/ml), and polyclonal goat anti-mouse IgM (final concentration 10 μg/ml; Jackson Immunochemicals). Reactions were terminated by transferring cells into an equal volume of ice-cold 2× Nonidet P-40 (NP-40) lysis buffer (composition of 1X NP-40 lysis buffer: 1% NP-40, 50 mM Tris [pH 8.0], 150 mM sodium chloride, 5 mM EDTA, 20 mM sodium fluoride, 20 mM β-glycerol phosphate, 5 mM iodoacetamide, 1 mM $NaVO_4$, 2.5 mM PMSF, and 40 μg/ml each aprotinin and leupeptin). After 15 min on ice, the samples were centrifuged at 14,000 × g and 4°C for 15 min and the supernatants transferred to ice-cold streptavidin–agarose (Gibco-BRL) that had been preblocked with 1% bovine serum albumin and washed in 1× lysis buffer. After 15 min incubation with gentle rotation, the samples were centrifuged and the supernatant transferred to anti-CD22-biotin (Pharmingen) that had been prebound in the presence of 1% bovine serum albumin to strepavidin–agarose (Gibco-BRL) and washed several times in 1× NP-40 lysis buffer. Prebinding the anti-CD22 biotin to the agarose was critical to the yield of CD22 in immunprecipitation. Immunoprecipitation of CD22 from the lysates proceeded with gentle rotation of the samples for 1 hr in the cold. Agarose samples were then washed three times in ice-cold 1× NP-40 lysis buffer (without inhibitors) with gentle centrifugation and careful fine-needle aspiration of supernatants. Finally, agarose pellets were suspended in SDS–polyacrylamide gel electrophoresis sample buffer and boiled for 5 min. Serial immunoprecipitations of first IgB and then Syk followed that of CD22 using rabbit polyclonal antisera to IgB (prepared by JGC) and Syk (a gift from A. DeFranco). At each cycle, the residual supernatant was preincubated with protein G– or protein A–agarose (Gibco-BRL), incubated with anti-serum for 1–3 hr, and then added once again to protein G– or protein A–agarose for 1 hr with mixing and finally the agarose pellet washed and resuspended in SDS–polyacrylamide gel electrophoresis sample buffer. Great care was taken not to cross contaminate samples and all steps were performed at 4°C. Immunoprecipitated samples were resolved on 8%–12% SDS–polyacrylamide gel electrophoresis and immunoblotted with antiphosphotyrosine (Mab 4G10, Upstate Biotechnology) and anti-SHP-1 (a gift from M. Thomas), and rabbit polyclonal anti-CD22 (a gift from P. Crocker), anti-Igβ, and anti-Syk. Detection was by enhanced chemiluminescence (Amersham).

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