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Visualization of Syk-Antigen Receptor Interactions Using Green Fluorescent Protein: Differential Roles for Syk and Lyn in the Regulation of Receptor Capping and Internalization

Haiyan Ma,* Thomas M. Yankee,* Jianjie Hu,* David J. Asai,† Marietta L. Harrison,* and Robert L. Geahlen2*

The cross-linking of the B cell Ag receptor (BCR) is coupled to the stimulation of multiple intracellular signal transduction cascades via receptor-associated, protein tyrosine kinases of both the Src and Syk families. To monitor changes in the subcellular distribution of Syk in B cells responding to BCR cross-linking, we expressed in Syk-deficient DT40 B cells a fusion protein consisting of Syk coupled to green fluorescent protein. Treatment of these cells with anti-IgM Abs leads to the recruitment of the kinase from cytoplasmic and nuclear compartments to the site of the cross-linked receptor at the plasma membrane. The Syk-receptor complexes aggregate into membrane patches that redistribute to form a cap at one pole of the cell. Syk is not demonstrably associated with the internalized receptor. Catalytically active Syk promotes and stabilizes the formation of tightly capped BCR complexes at the plasma membrane. Lyn is not required for the recruitment of Syk to the cross-linked receptor, but is required for the internalization of the clustered BCR complexes. In the absence of Lyn, receptor-Syk complexes at the plasma membrane are long lived, and the receptor-mediated activation of the NF-AT transcription factor is enhanced. Thus, Lyn appears to function to negatively regulate aspects of BCR-dependent signaling by stimulating receptor internalization and down-regulation. The Journal of Immunology, 2001, 166: 1507–1516.

The Syk protein-tyrosine kinase is required for the transduction of signals through the B cell Ag receptor (BCR). B cells lines lacking the syk gene are largely nonresponsive to BCR aggregation (1). Likewise, B cell development in Syk-deficient mice is blocked at the pro-B cell to pre-B cell and the immature B cell to mature B cell transitions due to a loss of Ag receptor-dependent signals (2, 3). The participation of Syk in BCR-mediated signaling begins with its physical recruitment to the cross-linked receptor. The Ag-binding component of the BCR is a membrane-bound Ig, and the signal-transducing component is a heterodimer of Ig-α (CD79a) and Ig-β (CD79b) subunits (4, 5). The Syk-receptor interaction is initiated by the phosphorylation of conserved tyrosines present within immunoreceptor tyrosine-based activation motifs (ITAMs) located within the cytoplasmic domains of Ig-α and Ig-β (6, 7). This ITAM phosphorylation creates docking sites for the tandem pair of Syk SH2 domains. Binding to the phosphorylated ITAM and subsequent tyrosine phosphorylation leads to the activation of Syk. Syk then couples the BCR to multiple downstream signaling pathways that are stimulated following B cell activation, including the mobilization of intracellular stores of calcium, activation of the mitogen-activated protein kinase cascade, and generation of phosphatidylinositide 3-phosphates (7–10).

A Src family kinase such as Lyn, which is expressed predominately in hemopoietic cells, is required for the efficient activation of Syk following BCR aggregation. It probably plays two important roles in this regard: the phosphorylation of ITAM tyrosines and the phosphorylation of Syk itself on tyrosines located within the activation loop. This general model for the early events in BCR-mediated signaling is analogous to that proposed for signaling through the TCR and its associated kinases, Lck and ZAP-70 (11, 12). In T cells, Lck is needed for both receptor-ITAM phosphorylation and ZAP-70 phosphorylation and activation. However, several studies have found fundamental differences between ZAP-70 and Syk and their reliance on Src family kinases for activation. For example, Syk, but not ZAP-70, can restore TCR-stimulated signaling to Jurkat T cells deficient in Lck (13, 14). Also, recent genetic studies have shown that, in many contexts, the expression of Lyn has a negative effect on signaling in B cells. For example, certain BCR-stimulated events such as inositol 1,4,5-trisphosphate production and activation of the serine/threonine kinase, Akt/protein kinase B, are actually more robust in DT40 B cells that lack Lyn, the only Src family kinase expressed in these cells at a detectable level (1, 15). Likewise, IgM cross-linking in primary B cells from Lyn knockout mice leads to hyperactivation of the mitogen-activated protein kinase and c-Jun kinase pathways and a hyperproliferative response (16–18).

The recruitment of Syk to the aggregated BCR has not been visualized in intact cells, and the biochemical demonstration of this is difficult due to the association of the aggregated receptor with cytoskeletal components, which hampers the isolation of intact BCR-Syk complexes in high yield. To approach this problem, we generated cDNAs coding for a chimeric molecule of Syk fused with green fluorescent protein (GFP). We then used this construct

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as a tool for visualizing the effects of BCR cross-linking on the re-
distribution of Syk within cells that either express or do not express
endogenous Lyn. In this study we demonstrate that Syk is present in
nuclear and cytoplasmic compartments and is recruited from both
to the site of the aggregated BCR. Syk is associated with receptors ag-
gregated to form both membrane patches and polar caps. The recruit-
ment of Syk to the most efficient when Syk retains catalytic
activity. Syk is not required for the interaction of Syk with the
aggregated Ag receptor. We demonstrate further that Lyn and Syk
differentially regulate the dynamics of receptor internalization. Syk
stabilizes BCR signaling complexes at the plasma membrane, while
Lyn is required for receptor internalization.

Materials and Methods
Plasmids, constructs, and transfections
An Xhol/Hind DNA fragment encoding Syk was cut from EBV-SykMyc (20). Insertion of this fragment into XhoI/Smal sites of the pEGFP-N2 vector (Clon-
tech, Palo Alto, CA) resulted in a fusion of Syk and enhanced GFP (EGFP). The same approach was used to attach the open reading frame of
enhanced GFP (EGFP) to the C-terminus of a catalytically inactive form of
Syk (Syk[K396R]) (20) to generate KDEGFP. Two oligonucleotides (5'-
GCTGTCGAGAAAAATGGTTGAAGAGCTTGAGTCGAGCT-3' and
5'-GCTATCCGACGCTTCTTCTCCAATTTTCTGACAGCT-3'), which encode the nuclear export signal from Xenopus mitogen-activated protein
kinase kinase (21), were synthesized, annealed, and inserted into
an expression vector coding for wild-type murine Syk (SykEGFP). A schematic diagram showing the orien-
tation of the two proteins within the chimera is shown in Fig. 1A.

Results
Expression of SykEGFP in DT40 B cells
To prepare a form of Syk that could be visualized in intact cells, we
constructed a mammalian expression vector with the coding
sequence for a form of GFP with enhanced fluorescent properties
(EGFP) inserted downstream from the cDNA coding for wild-type
murine Syk (SykEGFP). A single protein was detected that migrated at
M, 100,000, the ex-
pected size of the intact fusion protein. Smaller, proteolytic frag-
ments of SykEGFP containing the GFP domain were not detected.

Fluorescence and confocal microscopy
Unstimulated cells were adhered to coverslips precoated with poly-l-lysine
(100 μg/ml; Sigma, St. Louis, MO) for 10 min at room temperature. Cells
stimulated by either goat anti-chicken IgM Abs (Bethyl Laboratories,
Montgomery, TX) or Texas Red-conjugated anti-IgM Abs were adhered to
coverslips 10 min before the end of stimulation. For stimulation at 37°C, Texas Red was
conjugated to goat anti-chicken IgM using the FluoroReporter Texas Red-X
protein labeling kit from Molecular Probes (Eugene, OR). Cells were fixed
in 3.7% paraformaldehyde in PBS for 15 min at room temperature, washed
three times with PBS, stained with 4,6-diamidino-2-phenylindole (Sigma)
and viewed by fluorescence microscopy. Cells undergoing subsequent
staining with anti-human Ig-ß Ab were preincubated with blocking buffer
(3% BSA, 1% goat serum in PBS) for 30 min after incubation with
R-PE-conjugated monoclonal anti-human Ig-ß Ab (Ancell, Bayport, MN)
diluted in the blocking buffer for 1 h. Conventional fluorescence was ex-
amined using an Olympus BH2-RFCa fluorescence microscope with ×60
objective equipped with a Sony DXC-950 3CCD color video camera and
Northern Eclipse 5.0 software from Empix Imaging (Mississauga, Canada).

For real-time observation of live cells, 1 × 106 transfected cells were
adhered to glass coverslips. Immediately after exposure to RPMI medium
containing 20 μg/ml of goat anti-IgM Ab, cells were observed under the
microscope, and a picture was taken as time zero of stimulation. The cells
remained in the presence of anti-IgM Abs and were photographed at timed
intervals.

Promoter-linked luciferase assays
Syk-deficient DT40 cells (1 × 104) were transfected by electroporation with vectors containing cDNAs for the various EGFP- or epitope-tagged
forms of Syk or Syk mutants (20) and an NF-AT-luciferase reporter
plasmid (10 μg) (20). Cells were harvested 36 h following transfection,
plated at a density of 1 × 105 cells/ml, and activated with anti-IgM Abs (10
μg/ml) or a mixture of PMA (50 ng/ml) and ionomycin (1.0 μM) for 6 h
at 37°C. Luciferase activity was determined using the luciferase assay sys-
tem kit (Promege, Madison, WI). Luciferase activity is expressed as a
fraction of that activity observed with activation by PMA plus ionomycin.

Western blot analysis
For the detection of fusion proteins by Western blot analysis, proteins
in extracts from resting or activated cells were separated by SDS-PAGE,
transferred to polyvinylidene fluoride membranes, and probed with
an anti-GFP Ab (Clontech) and an HRP-conjugated secondary Ab. The
Ab-Ab complex was visualized by an enhanced chemiluminescence detec-
tion system. For the detection of epitope-tagged Syk, proteins were first
immunoprecipitated from cell lysates with an anti-Myc mAb and then
probed by Western blotting with anti-Syk Abs (24). The 9E10 anti-Myc
hybridoma cell line was obtained from American Type Culture Collection
(Manassas, VA).

Localization of SykEGFP in DT40 B cells
To examine the intracellular localization of the fusion protein, we
transfected Syk-deficient DT40 B cells with the SykEGFP expres-
sion plasmid. Cells were then fixed and examined by both fluo-
rescence and confocal microscopy. A composite image is shown in

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Fig. 2. The Syk fusion protein was localized throughout the interior of the cell in both the cytoplasm and nucleus, but was excluded from nucleoli (Fig. 2, A and B). This same distribution was observed for the catalytically inactive KDEGFP (Fig. 2, C and D), indicating that the presence or the absence of catalytic activity did not affect localization of the kinase in resting cells. SykEGFP and KDEGFP appeared in both the nucleus and cytoplasm of cells, whether expressed at abundant levels or in trace amounts.

The localization of a fraction of SykEGFP to the nucleus could be verified by contrasting its appearance in transfected cells with that of a variant of SykEGFP prepared with a short nuclear export signal (NES) added to the carboxyl terminus (SykEGFPNES). SykEGFPNES, when expressed in Syk-deficient cells, was excluded from the nucleus and restricted in its localization to the cytoplasmic compartment (Fig. 2, E and F; the location of the nucleus was verified in all cells by staining with 4,6-diamidino-2-phenylindole; data not shown). The staining pattern observed by fluorescence or confocal microscopy of cells expressing SykEGFPNES was clearly distinct from that observed for cells expressing SykEGFP or KDEGFP. The exclusion of SykEGFPNES from the nucleus could be reversed by treatment of the cells with leptomycin B, a specific inhibitor of NES-mediated nuclear export (23) (data not shown). This result indicated that the peptide that was added to the carboxyl terminus of SykEGFP to form SykEGFPNES was indeed functioning as a nuclear export signal (data not shown). This nuclear-excluded form of Syk was able to restore the BCR-stimulated activation of NF-AT to Syk-deficient cells as efficiently as SykEGFP or Syk(WT) (Fig. 1C).

 Redistribution of SykEGFP to the cross-linked BCR

BCR cross-linking is thought to promote the physical association of SykEGFP with the receptor. To confirm this, we treated Syk-deficient DT40 cells expressing SykEGFP with affinity-purified anti-IgM Abs conjugated with Texas Red dye to allow visualization of the surface IgM component of the BCR by confocal microscopy. In untreated cells, IgM was uniformly distributed around the cell surface (Fig. 1A and B). However, upon BCR cross-linking, IgM was redistributed to form discrete, cross-linked IgM complexes that colocalized efficiently with SykEGFP (Fig. 1C and D). This redistribution of SykEGFP to the cross-linked BCR suggested that SykEGFP was able to associate with the BCR in a manner that facilitated its activation in the context of BCR cross-linking.
the cell surface (Fig. 3C), and there was no obvious colocalization of SykEGFP with the receptor (Fig. 3, A and B). When cells were stimulated by the addition of the fluorescently tagged anti-IgM Abs, the surface IgM first formed membrane patches of variable sizes (Fig. 3F). The patching of cell surface receptors was more easily observed at 25 than at 37°C because the redistribution of the patches into caps occurred more slowly. When the patches of cell surface IgM formed, SykEGFP also began to form aggregates (Fig. 3D). These colocalized with the patched IgM (Fig. 3E), consistent with recruitment of SykEGFP to the clustered receptors. By 20 min at 37°C, the patched receptors had aggregated to form a cap at one pole of the cell (Fig. 3I). SykEGFP was also redistributed to a single cap that colocalized with the capped surface Ig (Fig. 3, G and H). Further incubation of the stimulated cells resulted in the appearance of IgM in small aggregates that were present in the interior of the cell due to internalization of the IgM in endocytic vesicles (Fig. 3L). At this stage, there was no obvious accumulation of SykEGFP at the sites of internalized IgM (Fig. 3J).

To further confirm a colocalization of SykEGFP with components of the BCR complex, we transfected human DG75 B cells with the plasmid expressing SykEGFP and then treated the cells with anti-human IgM Abs. Treated cells were stained using an anti-Ig-β mAb and a tetramethylrhodamine isothiocyanate-conjugated secondary Ab. Human B cells were used for this experiment due to the availability of anti-human Ig-β Abs. A portion of the SykEGFP was also clustered and colocalized with Ig-β (Fig. 3, M and N). In these anti-Ig-β-stimulated DG75 cells, both SykEGFP and Ig-β also colocalized with surface IgM (data not shown). These data are consistent with the recruitment of SykEGFP to the site of the clustered BCR complex.

Several control experiments were conducted to further confirm the receptor-mediated relocalization of SykEGFP into membrane-associated patches and caps. To confirm that the redistribution of SykEGFP occurred in this fashion within individual cells, we examined changes in the localization of SykEGFP following BCR cross-linking in live cells as a function of time. Syk-deficient DT40 cells expressing SykEGFP were treated with anti-IgMAbs at room temperature and then examined by fluorescence microscopy at various time points without fixation to allow events in single cells to be monitored (Fig. 4, A–D). In most cells, SykEGFP initially redistributed to the plasma membrane, moved to one hemisphere of the cell, and eventually formed a single cap at one pole. This was especially prominent in cells expressing lower levels of SykEGFP. The redistribution of the fusion protein was more difficult to visualize in cells with high levels of expression of SykEGFP, suggesting that the membrane-associated binding sites to which SykEGFP was recruited were saturable. SykEGFP translocation to the plasma membrane could also be observed in live, SykEGFP-expressing, DG75 B cells that were then treated with anti-human IgM Abs (data not shown).

Two additional experiments were conducted to establish the specificity of the SykEGFP redistribution. First, the effect of BCR cross-linking on the subcellular distribution of EGFP alone was examined. EGFP was localized diffusely throughout cells that had been transfected with an EGFP expression plasmid (Fig. 4, E and F). Its distribution, however, remained unchanged following BCR aggregation when examined at multiple time points following receptor engagement (shown here for a 30-min treatment at room temperature). Secondly, Syk-deficient DT40 B cells expressing SykEGFP were treated with Con A to examine whether the redistribution of SykEGFP was a general phenomenon that accompanied the clustering and capping of a cell surface receptor other than IgM. Following a 1-h treatment with Con A, Con A receptors were aggregated into a single cap without a concomitant redistribution of SykEGFP (Fig. 4, G and H). Thus, the redistribution of SykEGFP was specific for both the Syk portion of the fusion protein and the cross-linking of surface Ig.

Finally, to determine the fraction of transfected cells that responded to treatment with anti-IgM Abs through the redistribution of SykEGFP, we monitored the changes in SykEGFP localization in response to receptor cross-linking in populations of SykEGFP-expressing cells as a function of time after B cell activation. Cells were classified into one of five categories depending on the distribution of SykEGFP in the cell (Fig. 5A). These categories were: resting, a distribution of SykEGFP throughout the cytoplasm and nucleus; patched (I), patches of SykEGFP appearing at the plasma membrane before capping; capped, SykEGFP appearing in a single

**FIGURE 3.** SykEGFP colocalizes with surface IgM and Ig-β in anti-IgM-activated B cells. A–L, Syk-deficient DT40 cells expressing SykEGFP were stimulated with Texas Red-conjugated anti-IgM Abs for 0 min (A–C), for 10 min at 25°C (D–F), or for 20 min (G–I) or 60 min (J–L) at 37°C. Cells were then fixed and examined by confocal microscopy. Superposed SykEGFP and anti-IgM images are shown in the central panels, with areas of yellow representing colocalization of SykEGFP (green) and IgM (red). Images from D–F were focused on the top surface of cells. Bar, 1 μm. M–O, Human DG75 B cells expressing SykEGFP were stimulated with goat anti-human IgM Abs at 37°C for 30 min. The cells were fixed and processed for immunofluorescence by staining with a mouse anti-human Ig-β Ab. Dual-color images are shown in the central panels, with areas of yellow representing colocalization of SykEGFP (green) and Ig-β (red). Bar = 1 μm.
SykEGFP distribution pattern that characterized the unstimulated cells. By 6 h, 90% of the cells had returned to the diffuse staining pattern. In approximately 50% of these cells, howev-

er, SykEGFP was no longer observed in clusters in the majority of cells and was once again present in a diffuse distribution throughout the cell corresponding to

the resting (no obvious redistribution of SykEGFP from nuclear and cytoplasmic compartments), patched (I) (fluorescence appearing as membrane patches without a major polar redistribution), capped (fluorescence localized at one pole and occupying one-fourth or less of the cell surface), and nuclear excluded (fluorescence diffuse, but excluded from the cytoplasm). The percentage of SykEGFP-expressing cells exhibiting each phenotype was determined. At least 100 cells were scored at each time point. The data shown are representative of three separate experiments.

FIGURE 4. SykEGFP is redistributed to the plasma membrane following cross-linking of the BCR. A–D. A population of living, Syk-deficient DT40 B cells expressing SykEGFP was incubated with anti-IgM Abs for the indicated times at room temperature and examined by fluorescence microscopy after 0 (A), 5 (B), 10 (C), and 15 (D) min of stimulation. Bar = 5 μm. E and F, DT40 cells expressing EGFP alone were examined by fluorescence microscopy after 0 (E) or 30 (F) min of stimulation with anti-IgM Abs at room temperature. G and H, Syk-deficient DT40 cells expressing SykEGFP were stimulated for 60 min with Texas Red-conjugated Con A at 37°C and examined by fluorescence microscopy for the localization of SykEGFP (G) and Con A (H).

FIGURE 5. SykEGFP redistributes to the plasma membrane in the majority of anti-IgM-activated cells. Syk-deficient DT40 B cells expressing SykEGFP were stimulated with anti-IgM Abs for the times indicated. Stimulation was terminated by the incubation of cells with 3.7% paraformaldehyde at room temperature for 15 min. Cells were examined for the autofluorescence of SykEGFP. A, Cellular phenotypes were categorized as resting (no obvious redistribution of SykEGFP from nuclear and cytoplasmic compartments), patched (I) (fluorescence appearing as membrane patches without a major polar redistribution), capped (fluorescence localized at one pole and occupying one-fourth or less of the cell surface), and nuclear excluded (fluorescence diffuse, but excluded from the cytoplasm). B, The percentage of SykEGFP-expressing cells exhibiting each phenotype was determined. At least 100 cells were scored at each time point. The data shown are representative of three separate experiments.

polar cap occupying less than one-quarter of the cell surface; patched (II), only a small number of brightly staining clusters of SykEGFP still present at the plasma membrane after dissociation of the tight cap structure; and nuclear excluded, diffuse distribution of SykEGFP throughout the cytoplasm, but excluded from the nucleus. In >98% of unstimulated cells, SykEGFP exhibited a diffuse distribution throughout the cell corresponding to the resting phenotype (Fig. 5B). After 5 min of treatment with anti-IgM Abs at 37°C, SykEGFP was present in membrane patches (58%) or caps (33%) in the majority of the cells. After BCR ligation for 10 min, SykEGFP appeared predominately in cell surface caps (85%). By 1 h poststimulation, most of the SykEGFP was absent from the plasma membrane, except for a few brightly staining clusters (patched, II). These were still associated with the few small clusters of IgM (as determined by staining with Texas Red-conjugated anti-IgM Abs, data not shown) that had not undergone internalization. By 3 h after stimulation, SykEGFP was no longer observed in clusters in the majority of cells and was once again present in a diffuse staining pattern. In approximately 50% of these cells, however, SykEGFP was confined to the cytoplasm and excluded from the nucleus. By 6 h, 90% of the cells had returned to the diffuse SykEGFP distribution pattern that characterized the unstimulated cells.

Lyn is not required for the recruitment of SykEGFP to the BCR

Lyn is thought to be an important kinase acting upstream of Syk to promote its recruitment to the BCR. To determine whether Lyn expression was required for the redistribution of SykEGFP to the cross-linked BCR, we expressed the fusion protein in DT40 B cells in which either the gene for Syk (Syk-deficient) or the genes for both Syk and Lyn (Syk/Lyn-deficient) had been disrupted. In unstimulated cells, the intracellular distribution of SykEGFP in Syk/ Lyn-deficient cells was indistinguishable from that described above for SykEGFP expressed in Syk-deficient cells (data not shown). Following a 10-min treatment with activating Ab at 37°C, SykEGFP was recruited to the plasma membrane in both cell types and appeared in most cells as a single cap at the cell surface (Fig. 6A and C) that colocalized with the capped surface IgM (data not shown). An analysis of multiple transfected cells indicated that the initial cocapping of SykEGFP with the BCR was similar for the Syk-deficient and the Syk/Lyn-deficient cells after 10 min of stimulation (Table I). Interestingly, however, SykEGFP expressed in the Syk/Lyn-deficient cells remained in membrane caps following 60 min of activation in the majority of the cells (Fig. 6D and Table I). These caps persisted for 6 h (the longest time point examined) following addition of the anti-IgM Ab (data not shown). This is in sharp contrast to SykEGFP expressed in Syk-deficient cells, which was present in only a few small clusters at the plasma membrane after 60 min of treatment with anti-IgM Abs (Fig. 6B and Table I) and was absent from the membrane after 6 h of treatment (Fig. 5B).
The "kinase-dead" KDEGFP mutant was used to monitor the receptor-mediated redistribution of the enzyme in the absence of intrinsic catalytic activity. KDEGFP was expressed in both the Syk-deficient and the Syk/Lyn-deficient DT40 B cells, and the effect of receptor cross-linking on its intracellular localization was monitored. In resting cells of both types, KDEGFP was distributed normally throughout the nucleus and cytoplasm (Fig. 2 and data not shown). In the majority of Syk-deficient cells expressing SykEGFP (A–D) or KDEGFP (E–H) were stimulated at 37°C with anti-IgM Abs for 10 min (A, D, E, and G) or 60 min (B, D, F, and H). Cells were then fixed and examined by fluorescence microscopy. Arrowheads indicate examples of SykEGFP present in caps at the cell surface. Arrows indicate examples of SykEGFP or KDEGFP present in small clusters or patches at the cell surface. Bar = 5 μm.

Thus, the presence of SykEGFP in membrane caps is prolonged in the absence of Lyn.

A catalytically inactive Syk can be recruited to the BCR

The “kinase-dead” KDEGFP mutant was used to monitor the receptor-mediated redistribution of the enzyme in the absence of intrinsic catalytic activity. KDEGFP was expressed in both the Syk-deficient and the Syk/Lyn-deficient DT40 B cells, and the effect of receptor cross-linking on its intracellular localization was monitored. In resting cells of both types, KDEGFP was distributed normally throughout the nucleus and cytoplasm (Fig. 2 and data not shown). In the majority of Syk-deficient cells, BCR cross-linking led to the appearance of clusters of KDEGFP at the plasma membrane (Fig. 6E and Table I). However, this redistribution was less robust than that observed in cells expressing the catalytically active SykEGFP. After 10 min of treatment with anti-IgM Abs, 63% of the transfected cells exhibited some degree of KDEGFP redistribution to the plasma membrane. At this time, KDEGFP appeared primarily in clusters of small patches that resembled those observed in Syk-deficient cells expressing SykEGFP that had been treated with anti-IgM Abs for 60 min (Fig. 6B). Most of these small patches of KDEGFP had disappeared by 60 min of treatment with anti-IgM (Fig. 6F). The appearance of clusters of KDEGFP at the plasma membrane was further blunted in cells lacking both Syk and Lyn (Fig. 6G and Table I). In approximately 50% of the cells, no obvious change in the subcellular distribution of KDEGFP could be observed following aggregation of the BCR. In the other 50%, only a relatively limited amount of KDEFGFP appeared in membrane patches (Fig. 6G). These aggregates of KDEGFP at the plasma membrane persisted for at least 60 min following receptor aggregation (Fig. 6H). It is interesting to note that some clustering of KDEGFP can occur in response to BCR cross-linking even in cells lacking Lyn and an active form of Syk.

Syk and Lyn differentially regulate receptor clustering and internalization

Differences in the appearance of the clustered, fluorescently tagged Syk molecules in cells lacking catalytically active Syk or Lyn suggested that these kinases might play important roles in modulating BCR dynamics following receptor aggregation. As a consequence, the fate of aggregated BCR complexes in DT40 cells treated with anti-IgM Abs for various periods of time was monitored using the fluorescently tagged anti-IgM Ab. In wild-type DT40 cells, which express normal levels of endogenous Lyn and Syk, treatment with anti-IgM Abs led to the formation of surface IgM aggregates, which had redistributed into caps in the majority of cells by 10 min (Figs. 7B and 8). Only 12% of the cells exhibited internalized receptors at this time. By 60 min in the majority of cells the receptors had been internalized (Fig. 8). By fluorescence microscopy, internalized IgM appeared as a mass of punctate spots seen when focusing on the interior of the cell (Fig. 7C).

In DT40 cells lacking Lyn, treatment with anti-IgM Abs led to a rapid clustering and redistribution of surface IgM into tightly formed polar caps (Fig. 7, D and E). These capped receptors persisted at the cell surface and failed to become internalized even after 60 min of treatment with anti-IgM Abs (Fig. 8). The caps appeared in immunofluorescence images as bright clusters of stained receptors seen by focussing on the surfaces of the cells (Fig. 7F). These results are consistent with a requirement for Lyn for receptor internalization.

In cells lacking Syk, treatment with anti-IgM Abs also led to receptor clustering and the movement of clustered receptors to a single pole of the cell (Fig. 7G). However, few cells formed tightly capped receptors, and receptor internalization occurred rapidly, with 48% of the cells exhibiting internalized receptors within 10 min of treatment with anti-IgM Abs (Fig. 8). By 60 min, nearly all cells had internalized receptors (Fig. 8), which appeared within the cell as small, punctate clusters (Fig. 7I). Thus, Syk appears to be necessary for the stable appearance of tightly capped receptor complexes at the cell surface.

DT40 cells lacking both Syk and Lyn demonstrated a composite of the defects observed in the single kinase-deficient lines. In these cells, receptors were also aggregated by treatment with anti-IgM Abs (Fig. 7, J and K). The clustered BCR complexes redistributed to one hemisphere of the cell, but failed to form tightly clustered caps. These clustered Igs remained on the cell surface for an extended period of time, with no receptor internalization observed by 60 min following receptor aggregation (Figs. 7I and 8).

Similar results were observed using an FITC-conjugated goat anti-chicken IgM Ab. The fluorescence of the extracellular fluorescein can be quenched selectively by a reduction in the pH of the medium. In a typical experiment, FACS analysis of Lyn-expressing cells or Lyn-deficient cells treated on ice (conditions under
which internalization does not occur) with the fluorescein-conjugated Ab indicated that 95 and 96%, respectively, of the fluorescence could be quenched after acidification of the medium to pH 4.0. The fluorescence of Lyn-expressing cells treated with labeled Ab at 37°C for 30 min was quenched by only 32% due to internalization of a majority of the receptors. In contrast, the fluorescence of Lyn-deficient cells treated with Ab at 37°C for 30 min was quenched by 92%, indicating that the receptors remained at the cell surface.

A role for Syk in the formation of tightly capped receptors could be confirmed by examining a mixed population of Syk/Lyn-deficient cells and Syk/Lyn-deficient cells expressing SykEGFP. Following treatment with Texas Red-conjugated anti-IgM Abs, tightly capped receptor complexes appeared only in those cells expressing SykEGFP. Tightly capped receptors were not observed in cells that did not express the tagged fusion protein (Fig. 7, M and N). The appearance of these tightly capped receptors required Syk catalytic activity, since they also did not form in anti-IgM-treated cells that expressed the catalytically inactive KDEGFP chimera (25). The nuclear localization activity of SykEGFP and ZAP-70 GFP raises the intriguing possibility that Syk family kinases have unrecognized nuclear functions that are independent of their roles as mediators of proximal Ag receptor signals. At least one Syk function, coupling the BCR to the activation of NF-AT, is elevated in Lyn-negative B cells (23). The enhanced signaling in Lyn-deficient cells was also observed in cells transfected with the expression plasmid coding for SykEGFP in place of Syk(WT) (data not shown).

**Discussion**

The expression of proteins fused to GFP provides a useful, non-invasive methodology for monitoring changes in protein localization as a function of time and stimulus. We have adapted this methodology to the investigation of Syk and Syk-receptor interactions in B cells. The monitoring of cells expressing SykEGFP as a function of time following BCR cross-linking has provided the first visualization of the changes that Syk undergoes in its intracellular localization during B cell activation. This approach has also uncovered several interesting roles for BCR-associated kinases in regulating receptor dynamics.

In unstimulated cells, SykEGFP is localized to both cytoplasmic and nuclear compartments, but is excluded from the nucleoli (Fig. 2). In preliminary studies not illustrated here, we have been able also to demonstrate a similar distribution of endogenous Syk in human DG75 B cells by indirect immunofluorescence. This subcellular distribution is consistent with the previously reported localization of a ZAP-70 GFP chimera (25). The nuclear localization of SykEGFP and ZAP-70 GFP raise the intriguing possibility that Syk family kinases have unrecognized nuclear functions that are independent of their roles as mediators of proximal Ag receptor signals. At least one Syk function, coupling the BCR to the activation of NF-AT, is independent of its nuclear localization, since the attachment of a nuclear exclusion signal to the carboxyl terminus of SykEGFP does not reduce its activity in this assay (Fig. 1C). The large size of the SykEGFP chimera (~100 kDa) precludes the possibility that it diffuses freely into and out of the nucleus through nuclear pores, and there is no evidence that the observed nuclear fluorescence of SykEGFP-expressing cells is a consequence of proteolysis of the fusion protein, as we could not detect the formation of smaller m.w. species of protein containing the EGFP tag (Fig. 1B). Thus, the fusion protein must interact directly or indirectly through associated proteins with components of nuclear transport systems. The transport of SykEGFP into and out of the nucleus is likely to be a dynamic process. Upon BCR
cross-linking, it appears that SykEGFP in both nuclear and cytoplasmic compartments is eventually recruited to the plasma membrane. It is possible that downstream signal transduction pathways stimulated by the initial cross-linking of the BCR function actively to enhance the nuclear export of Syk. Then, once Ag receptor complexes have been internalized, the reappearance of SykEGFP in the nucleus requires several hours. This prolonged exclusion of the kinase from the nucleus occurs in cells expressing SykEGFP, but not in those expressing the catalytically inactive KDEGFP, which cannot reconstitute receptor-mediated signaling.

Phosphorylation of the ITAMs of the BCR complex that leads to the recruitment of Syk is an activity normally attributed to members of the Src family of protein tyrosine kinases, of which Lyn is the only member expressed at any significant level in DT40 cells. In fact, a catalytically inactive form of SykEGFP (KDEFGP) can be recruited to the cross-linked BCR in cells expressing Lyn and lacking Syk (Fig. 6E). This process appears relatively inefficient compared with the recruitment of a catalytically active form of SykEGFP, due in part to the rapid rate at which the receptors become internalized in the absence of an active Syk kinase (Figs. 7, G–I, and 8). However, as long as Syk retains intrinsic catalytic activity, Lyn is not required for its recruitment to the receptor, since this process is not compromised in Lyn-deficient cells (Fig. 6C and D). The most straightforward explanation for this observation is that Syk itself can catalyze the phosphorylation of receptor ITAMs. The ability of Syk to catalyze the phosphorylation of Ig-α in immune complexes (26) and to phosphorylate ITAM tyrosines in Lck-deficient Jurkat T cells (27) and CD45-deficient B cells (28) has been suggested by previous studies. This apparent lack of a requirement for a Src family kinase for the recruitment of Syk to the BCR probably explains why receptor engagement in Lyn-deficient DT40 cells is still coupled to inositol 1,4,5-trisphosphate production, calcium mobilization, and the activation of Akt/protein kinase B (1, 15).

FIGURE 7. Syk and Lyn regulate different aspects of receptor clustering and internalization. A–L, Wild-type (A–C), Lyn-deficient (D–F), Syk-deficient (G–I), or Syk- and Lyn-deficient (J–L) DT40 cells were incubated with Texas Red-conjugated goat anti-chicken IgM (20 μg/ml) at 37°C for 5 (A, D, G and J), 10 (B, E, H and K) or 60 (C, F, I and L) min. Stimulated cells were adhered to polylysine-coated coverslips, fixed and examined by fluorescence microscopy. All photographs were made by superimposing the fluorescence image with the visual light image. Bar = 5 μm. M–P, Syk- and Lyn-deficient DT40 cells were transiently transfected with a plasmid coding for SykEGFP (M and N) or KDEGFP (O and P), stimulated with Texas Red-conjugated goat anti-chicken IgM (20 μg/ml) at 37°C for 10 min, fixed and examined for fluorescence of the Texas Red dye (red) and SykEGFP or KDEFGFP (green). Arrows indicate examples of the colocalization of clustered receptors and fluorescently tagged Syk.

FIGURE 8. Syk and Lyn have different effects on receptor internalization. Wild-type (A), Lyn-deficient (B), Syk-deficient (C), or Syk- and Lyn-deficient (D) DT40 cells were incubated with Texas Red-conjugated goat anti-chicken IgM Abs (20 μg/ml) at 37°C for 5, 10, or 60 min as indicated. Cells were fixed, examined by fluorescence microscopy, and scored for the appearance of cell surface IgM as patched ( ), capped (see Fig. 5; ), or internalized ( ). The data represent one of three similar trials with >100 cells scored at each time point.
A, Tyrosine-phosphorylated proteins in cell lysates prepared from wild-type, Syk-deficient, and Syk/Lyn-deficient DT40 B cells incubated with anti-IgM Abs for 0 (lanes 1, 4, and 7), 3 (lanes 2, 5, and 8) or 10 (lanes 3, 6, and 9) min were identified by Western blot analyses with anti-phosphotyrosine Abs. The migration positions of m.w. standards are indicated. B, DT40 cells lacking Syk (Syk−) or both Syk and Lyn (Syk−/Lyn−) were transiently transfected with both the NF-AT-luciferase reporter plasmid and an expression plasmid for Syk(WT). Transfected cells either remained unactivated (□) or were activated by treatment with anti-IgM Abs (●). Relative luciferase activity is reported as described in Fig. 1B. The values represent the mean and S.E.s of 33 (Syk−) or 25 (Syk−/Lyn−) separate analyses. C, Lysates from cells representing a typical experiment from panel B were analyzed for relative levels of Syk(WT) expression by immunoprecipitation and Western blot analysis. The migration position of Syk(WT) is indicated.

FIGURE 9. Syk-dependent activation of NF-AT is elevated in Lyn-deficient DT40 B cells. A, Tyrosine-phosphorylated proteins in cell lysates prepared from wild-type, Syk-deficient, and Syk/Lyn-deficient DT40 B cells following receptor aggregation (Fig. 9A). We think it is likely that the appearance of KDEGFP at the site of cross-linked BCR complexes reflects the aggregation of small amounts of KDEGFP preassociated with the unligated BCR. A low level of Syk associated with the BCR was reported previously in unstimulated B cells (29). This preassociated Syk could conceivably initiate receptor phosphorylation and the recruitment of additional Syk molecules to the cross-linked receptor, bypassing the requirement for a member of the Src family in the early events of B cell signaling.

The presence or the absence of Syk and Lyn in DT40 B cells has major effects on the dynamics of receptor aggregation and internalization. The treatment of B cells with anti-IgM Abs leads first to the appearance of the BCR and its signaling component Ig-α (and associated Ig-β) in surface patches. The formation of these receptor-containing patches and their initial redistribution to one hemisphere of the cell are independent of the expression of either Syk or Lyn. If Syk is present, it is being actively recruited to the sites of the aggregated receptors during formation of the BCR-containing membrane patches. These patches, with time, redistribute into a single cap. The appearance of these caps is strongly influenced by the presence or the absence of Syk within the clustered BCR complex. If Syk is absent, the receptor caps are large, relatively diffuse, and internalized rapidly if Lyn is present in the cells. If Lyn is also absent, the receptor clusters that form are broad and cover one-quarter to one-half of the cell surface, where they remain for an extended period of time, indicating that Lyn is important for receptor internalization. In the presence of Syk, the BCR redistributes into tightly clustered caps regardless of whether Lyn is expressed. This effect requires the expression of a catalytically active form of Syk. If Lyn is also expressed, the receptors in these caps are internalized, although at a slower rate than in Syk-deficient cells. In the presence of Syk, but in the absence of Lyn, the tightly clustered receptors remain on the cell surface for an extended period of time (at least 6 h). Thus, Syk is required for the formation of tightly clustered caps of aggregated Ag receptors, and Lyn is required for receptor internalization.

A role for Lyn in receptor internalization is consistent with several studies that have used pharmacologic tyrosine kinase inhibitors to block BCR endocytosis (30–32). This suggests that one role for Lyn may be the down-regulation of activated receptors, since, in its absence, the Syk-BCR complexes that form following receptor cross-linking remain at the cell surface for a prolonged period of time (Figs. 6D and 7F). Thus, the activation of Lyn-deficient cells leads to a slow, but prolonged, increase in cytosolic free calcium (1) and an enhanced activation of NF-AT (Fig. 9B). It is possible that reduced receptor internalization may contribute to the hyper-responsiveness to IgM cross-linking that is observed in B cells from Lyn-deficient mice, which also demonstrate a delayed, but prolonged, calcium response (17), but this has not been determined.

The capping of surface IgM is an energy-dependent process that is mediated by the contractile activity of the actomyosin peripheral cytoskeleton and is inhibited by the treatment of cells with cytochalasin D, which disrupts actin microfilaments. A requirement for Syk has been demonstrated previously for the increased assembly of cortical actin in DT40 cells following the cross-linking of expressed Fcγ subunits (33). Thus, Syk might influence the clustering of receptors by regulating aspects of the actin assembly process important for the formation of the tightly clustered and capped receptor complexes. How this affects the overall process of receptor internalization and subsequent trafficking is not known. However, it is known that dominant-negative mutants of Syk inhibit Ag presentation (34), which requires the proper uptake and delivery of BCR-Ag complexes to MHC II-containing endosomes, the assembly of which is inhibited by tyrosine kinase inhibitors (35). Thus, both Lyn and Syk play important roles in regulating Ag receptor dynamics in B cells.

References


