Fluorescence resonance energy transfer in living cells reveals dynamic membrane changes in the initiation of B cell signaling

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Edited by Arthur Weiss, University of California School of Medicine, San Francisco, CA, and approved April 10, 2006 (received for review November 11, 2005)

B cell responses are initiated by the clustering of the B cell receptor (BCR) by the binding of multivalent antigens. Clustering leads to phosphorylation of tyrosines in the cytoplasmic domains of the BCR by the inner plasma membrane leaflet-associated Src-family kinase Lyn. At present, little is known about the earliest events after BCR clustering that precede the BCR's phosphorylation by Lyn. Here we use fluorescence resonance energy transfer (FRET) in living cells to detect the interaction of the BCR with a Lyn-based membrane-targeted reporter in the first several seconds after BCR clustering. The results showed that, within seconds of antigen binding, the BCR selectively and transiently associated with the Lyn construct and that this association preceded by several seconds the triggering of Ca^{2+} fluxes and could be prolonged by the engagement of the B cell coreceptor complex, CD19/CD21. Thus, FRET measurements in living B cells revealed highly dynamic and regulated antigen-induced changes in the plasma membrane, allowing association of the BCR with the earliest components of its signaling cascade.

B cell receptor | lipid rafts | initiation of signaling | CD19/CD21 coreceptor complex | Lyn-based reporter

Members of the family of multichain immune recognition receptors (MIRR), which include the B cell antigen receptor (BCR), the T cell antigen receptor (TCR), and the high affinity receptor for IgE expressed on mast cells (FcεR), have no inherent kinase activity but initiate signaling after antigen binding by associating with Src-family kinases that phosphorylate common immune receptor tyrosine activation motifs (ITAMs) in the cytoplasmic domains of the receptor signaling chains (1). Although we now understand the biochemical nature of the signaling cascade triggered by ligand binding to these immune receptors in considerable detail, the critical changes that occur in the monomeric receptors after ligand binding that allow for their association with the Src-family kinases remains only poorly understood. The results of previous studies have provided evidence that the local lipid microenvironments of the immune receptors and the Src-family kinase in the plasma membrane may play an important role in the earliest events in the initiation of immune cell signaling by segregating the antigen receptors from the Src-family kinases in resting cells and facilitating their association after antigen binding, thereby triggering signaling cascades. Indeed, lateral heterogeneities in the membranes of living cells enriched in sphingolipids and cholesterol, often referred to as lipid rafts, have been proposed to function as platforms for signaling in a variety of cell types (2, 3). Lipid rafts are usually operationally defined by their relative detergent insolubility and dependence on cholesterol. By the criteria of detergent insolubility, the members of the MIRR family have been shown in resting cells to be excluded from lipid rafts that contain Src-family kinases and to associate with lipid rafts after ligand binding where they are phosphorylated on ITAMs (4). For the BCR, multivalent antigen binding induced the clustering of the receptors and the association of the clustered receptors with detergent insoluble membranes where the receptors were phosphorylated and signaling initiated. Subsequent studies demonstrated a correlation between

the strength of BCR signaling and the stability of raft association (5, 6). For example, the B cell coreceptor complex CD19/CD21, which when coligated to the BCR by the binding of complement fixed antigens greatly augments BCR signaling, stabilized the association of the BCR with detergent insoluble membranes (7). The ability of the CD19/CD21 complex to stabilize this interaction was shown, in part, to be because of the palmitoylation of the coreceptor induced by the coligation to the BCR (8). Similar correlations were observed for two potent negative regulators of BCR signaling, the $\text{Fc}\gamma\text{RIIB1}$ (9) and the LMP2A protein of EBV (10) that destabilized or blocked BCR-raft association.

Although such studies support a role for the lipid microenvironments in B cell signaling, they left open the questions of does antigen binding to the BCR result in membrane changes that induce interactions between the BCR and lipid raft-targeted Src-family kinases in living cells, and, if so, what is the nature of this association? The use of detergent solubility as a criteria for the association of the BCR with lipid raft microdomains is fraught with potential artifacts and cannot provide information about the time course of the earliest events in B cell activation that occur within seconds after receptor clustering. To circumvent these limitations, we took advantage of recently developed, high resolution fluorescence imaging techniques (11–13) that exploit fluorescence resonance energy transfer (FRET) between fluorescent proteins to quantify protein–protein interactions in live cells over the time and length scales necessary to capture the earliest events in the initiation of receptor signaling, such as may occur in the association of the BCR with lipid rafts and Lyn.

Results

Expression of Fluorescently Labeled BCR and Membrane-Targeted Fluorescent Proteins. To investigate the interactions between the BCR and Lyn, which are mediated by Lyn's plasma membrane microenvironment in resting and in antigen-stimulated live cells, B cell lines were generated that expressed BCRs containing a FRET donor protein, cyan fluorescent protein (CFP), in the cytoplasmic domain of the BCR's $\text{Ig}\alpha$ chain ($\text{Ig}\alpha\text{-CFP}$) and a FRET acceptor protein, yellow fluorescent protein (YFP), containing the first 16 residues of the Src-family kinase Lyn (Lyn-YFP), resulting in YFP's myristoylation and palmitoylation and its targeting to the detergent insoluble membranes (14). This Lyn-based construct reports on the earliest lipid-BCR interactions in the association of the BCR and Lyn and eliminates the effects of BCR-Lyn protein–protein interactions. As controls, we characterized the interaction of the BCR with fluorescent proteins directed to the inner leaflet of the plasma membrane by the targeting sequences of two membrane-associated

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BCR, B cell receptor; CFP, cyan fluorescent protein; NFRET, normalized fluorescence resonance energy transfer; YFP, yellow fluorescent protein.

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Fig. 1. Expression and function of Ig α -CFP containing BCRs. (a) CH27 cells stably expressing Ig α -CFP or a control vector were surface biotinylated and lysed, and the lysates were immunoprecipitated (IP) by using Ig α - or IgM-specific antibodies. The immunoprecipitates were subjected to SDS/PAGE and immunoblotting (IB) probing with horseradish peroxidase (HRP)-coupled streptavidin. Shown are the positions of the IgM heavy chains (HC), light chains (LC), Ig α , and Ig α -CFP, identified by probing with Ig μ - or Ig α -specific antibodies. (b) The cell surface expression of the IgM BCR was determined by flow cytometry using a PE-conjugated Ig μ -specific mAb (R6–60.2) or an isotype matched control PE-conjugated mAb (IC) on parental CH27 cells or transfectants expressing Ig α -CFP alone (α C), Ig α -CFP and Lyn-YFP (α CLY), Ig α -CFP and Ger-YFP (α CGY), or Ig α -CFP and Src-YFP (α CSY). (c) The cells described in b were treated with antibodies specific for IgM to crosslink the BCR for 1 min at 37°C (BCR-XL) and lysed, and the lysates were immunoprecipitated (IP) with antibodies specific for green fluorescent protein (GFP) or IgM. The immunoprecipitates were subjected to SDS/PAGE and immunoblotting (IB) probing with either phosphotyrosine (PY)-specific or Ig α -specific antibodies. The bands in the immunoblots corresponding to Ig α -CFP and phospho-Ig α -CFP were quantified by densitometry and expressed as a ratio of PY to total Ig α -CFP (PY:total). Shown are representative results from two different experiments analyzing α C, α CLY and α CSY together, and α CGY separately. (d) CH27 cells expressing Ig α -CFP and Lyn-YFP, Ig α -CFP and Ger-YFP, or Ig α -CFP and Src-YFP were lysed in 1% Triton X-100 containing lysis buffer, and lipid rafts were isolated from the lysates by sucrose density centrifugation as described (5). The fractions from the sucrose density gradient were subjected to SDS/PAGE and immunoblotting probing with rabbit antibodies specific for Ig α to detect Ig α -CFP or with rabbit GFP-specific antibodies to detect Lyn-YFP, Ger-YFP, and Src-YFP, followed in each case by horseradish peroxidase-conjugated goat antibodies specific for rabbit Ig.

proteins that are not involved in the initiation of BCR signaling, namely Src and Rho. Thus, YFPs were expressed with either the first 15 N-terminal residues of Src (Src-YFP) containing only a myristoylation sequence, targeting YFP to detergent soluble membranes (15) or the C-terminal four residues of Rho resulting in YFP's modification by geranylgeranylation (Ger-YFP), targeting YFP to the detergent soluble membranes (14).

The gene encoding CFP was cloned into the C terminus of the Ig α chain of the BCR with a 6-aa flexible linker (RDPPAT) between Ig α and CFP, and the construct expressed in the CH27 B cell line that contained an endogenous BCR. Recent studies showed that Ig α -CFP assembles with Ig β - and μ -heavy chains in a 1:1:2 stoichiometry to form a functional cell surface-expressed BCR (13). Immunoprecipitates of the Ig α from transfected cells that were surface biotinylated contained both Ig α -CFP and endogenous Ig α , as well as both the mIg μ -heavy chains and Ig-light chains detected in immunoblots by using streptavidin-horseradish peroxidase (Fig. 1a). Conversely, immunoprecipitates of IgM contained biotin-labeled Ig α -CFP. Quantification of the Ig α -CFP and Ig α indicates that approximately half of the immunoprecipitated BCR contained an Ig α -CFP, and half contained an endogenous Ig α .

The Ig α -CFP transfected cells were cotransfected with one of the three YFP-containing constructs, Lyn-YFP, Src-YFP, or Ger-YFP. As analyzed by flow cytometry, cells coexpressing Ig α -CFP and Lyn-YFP, Ig α -CFP and Ger-YFP, or Ig α -CFP and Src-YFP, or expressing Ig α -CFP alone, showed similar surface expression of the BCR (Fig. 1b). In each case, the expression of the BCR was higher in the transfected cells as compared with untransfected CH27 cells (Fig. 1b), likely because of the expression of the Ig α -CFP increasing the total amount of Ig α available to assemble with Ig μ and Ig β into BCR.

The BCRs in transfected cells that contained Ig α -CFP and either Lyn-YFP, Ger-YFP, or Src-YFP functioned in receptor signaling as

judged by the phosphorylation of Ig α -CFP (Fig. 1c) and endogenous Ig α (data not shown) after BCR crosslinking. The cell lines were treated with Ig μ -specific antibodies to crosslink the BCR for 2 min at 37°C, lysed, and the lysates subjected to immunoprecipitation by using either green fluorescent protein-specific or Ig μ -specific antibodies. The immunoprecipitates were analyzed by immunoblotting, probing with phosphotyrosine-specific or Ig α -specific antibodies. The phosphotyrosine and Ig α immunoblots were quantified, and the ratio of the phosphotyrosine to total Ig α is given (Fig. 1c). Crosslinking the BCR resulted in a 2- to 3-fold increase in the phosphorylation of Ig α -CFP in each cell line, typical of the parent CH27 cell line, indicating that Ig α -CFP functioned in signaling in cells expressing the lipid-anchored YFPs. Similarly, the phosphorylation of the endogenous Ig α increased after BCR crosslinking (data not shown). The cell lines expressing Ig α -CFP and either Lyn-YFP, Ger-YFP, or Src-YFP also internalized a similar percentage of their BCR at similar rates as compared with the parental cell line, CH27, after BCR crosslinking (data not shown). Thus, neither the modified Ig α -CFP nor the membrane-targeted YFPs appeared to interfere with the signaling or internalization functions of the BCR. Analyses of the detergent solubility of the Ig α -CFP construct in CH27 cells confirmed that, in resting cells, the Ig α -CFP was soluble in 1% Triton X-100 (Fig. 1d). Lyn-YFP was present in the detergent insoluble fractions, whereas Ger-YFP and Src-YFP were detergent soluble as observed in ref. 15. A significant portion of Src-YFP was also present in the detergent insoluble fraction under the particular solubilization conditions used here.

Measurement of FRET in Resting and Activated Cells. FRET between CFP and YFP was measured in cells coexpressing Ig α -CFP and Lyn-YFP. Current evidence indicates that lipid raft microdomains are too small to be optically resolved (3), and, consistent with these

observations in unactivated cells, the Ig α -CFP and Lyn-YFP fluorescence appears to be distributed relatively evenly over the plasma membrane by confocal microscopy (Fig. 2*a Left*). Indeed, a pixel-by-pixel analysis of the CFP and YFP fluorescence of the plasma membranes in these confocal images showed that YFP and CFP are nearly completely colocalized (Fig. 2*a Right*). Moreover, crosslinking the BCR did not change the distribution of Ig α -CFP and Lyn-YFP that, at 30 s after crosslinking, remained evenly distributed and colocalized over the plasma membrane in the confocal images (Fig. 2*a*).

FRET between Igα-CFP and Lyn-YFP was quantified by sensitized acceptor emission in resting cells and, with time, after BCR crosslinking as described (13, 16). To do so, a Zeiss Meta 510 spectral laser scanning confocal microscope was used to collect fluorescence from three channels, namely CFP (CFP excitation and CFP emission), FRET (CFP excitation and YFP emission) and YFP (YFP excitation and YFP emission) from a region of interest selected as the plasma membrane of the cells. Because the detection of FRET is known to be sensitive to the ratio of donor and acceptor fluorophores (12), only cells expressing CFP:YFP in $\approx 1:1$ ratios were analyzed. Control experiments showed that, after correction for crosstalk between channels (16), no signal was detected in the FRET channel in cells expressing only the individual CFP or YFP or a construct containing CFP and YFP separated by a 232-aa

spacer representing residues 272–501 of the TRAF2 protein (CFP-T2DN-YFP) previously shown to prohibit FRET between CFP and YFP (17) (Fig. 5, which is published as supporting information on the PNAS web site). Cells expressing Ig α -CFP and Lyn-YFP were treated with a Ig μ -specific rat mAb and F(ab')₂ of goat antibodies specific for rat Ig to crosslink the BCR at time 0, and confocal images were acquired, corrected for crosstalk between channels, analyzed as described (13), and displayed as FRET ratio images (Fc/D) (12) (Fig. 2*b*). Little FRET was detected between Ig α -CFP and Lyn-YFP at time 0. The FRET increased dramatically 10 s after BCR crosslinking, peaked at 20–30 s, and decreased thereafter, such that, by 60 s after BCR crosslinking, little FRET between Ig α -CFP and Lyn-YFP was detected. The FRET between Ig α -CFP and Lyn-YFP was quantified and expressed as normalized fluorescence energy transfer (NFRET) values (Fig. 2*c*). The FRET between Ig α -CFP and Lyn-YFP increased immediately after the addition (at time 0) of the Ig-specific antibodies to crosslink the BCR and gradually diminished with time. The same pattern of FRET was observed when the fluorescent proteins were switched, such that Ig α contained the FRET acceptor YFP, and the donor CFP was targeted to the insoluble membranes by the first 16 aa of Lyn (Fig. 1*c*). Further analysis of the data showed that there was no relationship between either the acceptor intensity or the donor/acceptor ratio, and NFRET measured over the plasma membrane,

Fig. 3. A summary of the percent increase in FRET in cells after a variety of treatments. The maximum percent increase in FRET occurring between 10 and 50 s in individual cells expressing Ig α -CFP and Lyn-YFP (α CLY) ($\bullet$), Ig α -CFP and Ger-YFP (α CGY) ($\blacksquare$), or Ig α -CFP and Src-YFP (α CSY) ($\square$) after BCR crosslinking alone (BCR), BCR/CD19/CD21 coligation (BCR + CD19) (∇), or BCR crosslinking after pretreatment with PP2 ($\circ$) or latrunculin ($\blacktriangledown$) is given. The mean of the distribution is given by bars. The *P* values from *t* tests comparing the FRET values obtained from cells for each experimental condition with FRET values obtained from α CLY cells treated to crosslink the BCR alone are given.

indicating that the FRET is not because of ratio-dependent random bumping of the donor and acceptor fluorescent proteins (Fig. 5). Moreover, a pixel-by-pixel analysis of the plasma membrane showed no relationship between the acceptor intensity or the donor/acceptor ratio and NFRET at either 0 or 30 s after BCR crosslinking (Fig. 6, which is published as supporting information on the PNAS web site).

To determine the selectivity of the association of the BCR Ig α -CFP with raft-targeted Lyn-YFP, FRET was measured between the Ig α -CFP and Ger-YFP or Src-YFP (Fig. 2*d*). The cells analyzed expressed CFP and YFP in $\approx$ 1:1 ratios, and the levels of expression of Ig α -CFP and Ger-YFP or Src-YFP were similar to the levels of expression of Ig α -CFP and Lyn-YFP in cells expressing Ig α -CFP and Lyn-YFP. No FRET was detected between Ig α -CFP and Ger-YFP in resting cells before the addition of antibodies to crosslink the BCR or in cells after BCR crosslinking (Fig. 2*d*). The percent increase in FRET efficiencies for a large number of cells imaged after BCR crosslinking are plotted and show that a significant portion of cells coexpressing Ig α -CFP and Lyn-YFP showed FRET as compared with cells coexpressing Ig α -CFP and Src-YFP or Ig α -CFP and Ger-YFP (Fig. 3). These results provide evidence that the interaction of Ig α -CFP with the lipid raft targeted Lyn-YFP is selective and not a result of random bumping of the clustered BCR with membrane lipids.

To correlate the rapid association of the BCR's Ig α -CFP with Lyn-YFP with cell activation triggered by crosslinking the BCR, Ca²⁺ fluxes were imaged in cells loaded with the Ca²⁺ dye Fluo-4 with time after BCR crosslinking (18). Because of spectral overlap of the Ca²⁺ indicator and the fluorescent proteins, Ca²⁺ fluxes were measured in cells expressing Ig α -CFP and Lyn-YFP cells but not simultaneously with FRET measurements. A Ca²⁺ flux was triggered 30 s after the addition of the BCR crosslinking reagent to the cells, which peaked at 40 s and returned to background levels by 50 s (Fig. 2*e*). Thus, in these cell lines, the association of the BCR Ig α -CFP with Lyn-YFP preceded the BCR-triggered Ca²⁺ flux by 20 s. The same pattern of Ca²⁺ flux was observed in the entire population measured by flow cytometry (data not shown). As compared with primary B cells, the Ca²⁺ flux in CH27 cells is characteristically slightly delayed and of shorter duration.

FRET Between Ig α -CFP and Lyn-YFP Is Prolonged by B Cell Coreceptor Engagement. To determine whether the coligation of the BCR and the CD19/CD21 complex that prolongs signaling influenced the

Fig. 4. The association of the BCR with raft lipids is prolonged by coligating the BCR and the CD19/CD21 coreceptor and requires Src-family kinase activity. (a) NFRET in cells expressing Ig α -CFP and Lyn-YFP with time after the addition of antibodies to crosslink the BCR at time 0 from 2c (Upper) or to coligate the BCR and the CD19/CD21 complex at time 0 s (Lower). Each point represents the mean $\pm$ SD for 19 cells for BCR crosslinking alone and 20 cells for BCR/CD19/CD21 coligation from at least three independent experiments. (b) Time course of NFRET in cells expressing Ig α -CFP and Lyn-YFP untreated from 2c or pretreated with PP2 (50 μ M) for 1 h (Upper) or with latrunculin (10 μ M) for 30 min (Lower) at 37°C. Each point represents the mean $\pm$ SD for 19 untreated cells, 17 PP2-treated cells, and 12 latrunculin-treated cells from at least three independent experiments.

association of the Ig α -CFP with Lyn-YFP, FRET between Ig α -CFP and Lyn-YFP was determined after the coligation of the BCR and the CD19/CD21 complex by the addition of mAbs specific for IgM and CD19 followed by secondary crosslinking by antibodies specific for mouse Ig. The addition of the coligating antibodies induced immediate FRET between Ig α -CFP and Lyn-YFP (Fig. 4*a*). In contrast to the FRET in cells in which the BCR alone was crosslinked, which drops precipitously after peaking at 20 s, the FRET after coligation persists for 5 min, indicating a more stable interaction of the BCR with raft lipids. A comparison of the results of FRET analyses for a large number of B cells in which the BCR and the CD19/CD21 complex were crosslinked showed a significantly larger increase in FRET as compared with cells in which the BCR alone was crosslinked (Fig. 3). The level of FRET between Ig α -YFP and Lyn-CFP in cells that responded to BCR and CD19/CD21 coligation were consistently lower than that in cells that responded to BCR crosslinking alone (Fig. 3*a* and Fig. 7, which is published as supporting information on the PNAS web site). This observation suggests that BCR crosslinking alone may only activate B cells in which the BCR is already activated to some extent and associated with raft lipids.

FRET Between Ig α -CFP and Lyn-YFP Is Dependent on Src-Family Kinase Activity but Independent of the Actin Cytoskeleton. Two of the earliest events in antigen-induced BCR signaling include the phosphorylation of the BCR by the Src-family kinase Lyn and the association of the BCR with the actin cytoskeleton (19). To determine whether the BCR crosslinking-induced FRET between Ig α -CFP and Lyn-YFP is dependent on Src-family kinase activity or on an intact actin cytoskeleton, cells were pretreated with the Src-family kinase inhibitor, PP2 (20), or with latrunculin (21) to disrupt the actin cytoskeleton before BCR crosslinking and FRET measurements (Fig. 4*b*). PP2 treatment had a dramatic effect, completely blocking BCR crosslinking-induced FRET between the Ig α -CFP and Lyn-YFP. In contrast, latrunculin had little effect on

construct was generated by inserting the BsrG1 fragment of the CFP-YFP fusion construct into pECFP-C1 [kindly provided by A. Grammer (National Institutes of Health, Bethesda, MD)] into the BsrG1 site of Lyn-YFP in pcDNA3.1(+). Retroviral infections of CH27 cells were performed by spin infection after collecting viral particles produced from the PT67 packaging cells transiently expressing the plasmids of interest. Stable cell lines were selected for Ig α -CFP expression by several rounds of FACS of transfectants that were then subcloned for high expression of Ig α -CFP. The clones with the highest expression of Ig α -CFP (α C) were infected with plasmids expressing lipid modified YFPs Lyn-YFP or Src-YFP or transfected by nucleofection with Ger-YFP (Amara, Gaithersburg, MD). Several clones expressing $\approx$ 1:1 ratio of CFP to YFP were obtained from infections of each lipid-modified YFP plasmid. A similar strategy was followed to generate cell lines expressing Ig α -YFP and Lyn-CFP. Separately, cells were infected with plasmids expressing lipid modified CFPs or YFPs to generate control cell lines for FRET experiments (26). The Lyn-CFP-YFP fusion plasmid was introduced into Daudi, a human B cell line, by electroporation (26), and cells stably expressing Lyn-CFP-YFP were selected by FACS.

Surface Biotinylation, Immunoprecipitation, Immunoblotting, and Flow Cytometry. To detect BCR expressed on the cell surface, cells (1×10^8) were biotin-labeled with 200 μ g/ml of sulfo-LC-biotin in sodium bicarbonate buffer at pH 8.3 (20 mM NaHCO₃/150 mM NaCl) at 4°C and lysed. The lysates were immunoprecipitated by using rabbit antibodies specific for Ig α or goat antibodies specific for mouse IgG+IgM and analyzed by immunoblotting as described (5). The expression levels of IgM on the surfaces of transfectants was quantified by using PE-conjugated rat mAb specific for mouse IgM (R6-60.2, BD Clontech) by flow cytometry using a FACScan (Becton Dickinson).

Imaging and Image Processing. The acquisition of fluorescence images was described in ref. 13. For inhibitor studies, 50 μ M PP₂ or 10 μ M latrunculin B (Calbiochem) were added 1 or 0.5 h before stimulation, respectively. To achieve abrupt changes in ligand concentration, 200 μ l of 200% concentrated stimulating antibodies were added to the chamber containing 200 μ l of buffer between the second and third scan. CFP and YFP FRET time lapse images were acquired by using sequential excitation with 458 nm for simultaneous collection of CFP emission (475–525 nm) and FRET emission (>525 nm) followed by excitation with 514 nm for collection of YFP emission (>530 nm). Laser switching was performed after each line of the image so that individual pixels had <10 ms between the two scans. Images were collected every 10 s for 2–5 min. Images were acquired as 12-bit images of 512 $\times$ 512 pixel sizes. To increase

the signal to noise ratio, four time-scan averages and a 1.6- μ s pixel dwelling time were used. Intracellular Ca²⁺ fluo-4 images were acquired by exciting at 488 nm and collecting emissions at >505 nm. All resulting images were horizontally realigned and corrected for lateral inhomogeneities in illumination by using Zeiss LSM software. No vertical disparities between channels were found. Regions of interest were designed to cover the whole plasma membrane in equatorial section of cells. For FRET ratio (FR_D) or NFRET imaging, images from the CFP, FRET, and YFP channels were background-subtracted from the plasma membrane regions of nonfluorescent cells.

FRET Analysis. The techniques for FRET determination from sensitized acceptor emission have been described in detail by others (16, 27) and in our previously published report (13). Intermolecular FRET efficiencies in the time lapse experiments were imaged by FRET ratio (FR_D) (12) or NFRET (28, 29) by using a FRET macro of Zeiss LSM software (version 1.5 for release 3.2 add-on; Zeiss). Briefly, correction factors were determined from single CFP or YFP positive control cells: β (F/D) from Ig α -CFP single positive cells, and γ (F/A) and δ (D/F) from Lyn-YFP single positive cells, where F, D, and A are fluorescence intensities of the donor when excited under wavelength of the donor that of the acceptor when excited under excitation of donor (FRET), and that of the acceptor when excited under excitation wavelength of the acceptor, respectively. β , γ , and δ depended on the image acquisition settings and were determined independently for each experiment. (In our setting, the range of each correction factor was $0.5 < \beta < 0.7$, $0.3 < \gamma < 0.5$, $0.01 < \delta < 0.03$, and $0.95 < 1 - \beta\delta < 0.99$). From the original images, CFP images were further corrected for bleed-through components consisting of fractions of actual acceptor fluorescence at 458 nm excitation by image processing using the following equation: $D_{\text{cor}} = D - \delta F / 1 - \beta\delta$. The corrected three-channel images were used for quantitative FRET analysis. FR_D or NFRET images were processed on a pixel-by-pixel basis by using the following equations: $\text{FR}_D = (F - \beta D_{\text{cor}} - \gamma A) / D_{\text{cor}}$ or $\text{NFRET} = (F - \beta D_{\text{cor}} - \gamma A) / (D_{\text{cor}} \times A)^{1/2}$ and then smoothed. To minimize the influence of the ratio, stable clones having $\approx$ 1:1 ratio of CFP to YFP were used, as determined by the fluorescence values of YFP and CFP after bleaching YFP from the cells expressing Lyn-CFP-YFP fusion construct (positive FRET control). For estimation of FRET at each time point, the regions of interest covering the entire plasma membrane were chosen from the corrected and smoothed images, and NFRET values were calculated as above equation on a pixel-by-pixel basis by using a macro of the Zeiss LSM software.

This research was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

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