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Polyvalent Antigens Stabilize B Cell Antigen Receptor Surface Signaling Microdomains

Rathna Thyagarajan, Nandini Arunkumar, and Wenxia Song

The B cell Ag receptor (BCR) can distinguish subtle differences in Ag structure and trigger differential responses. In this study, we analyzed the effects of Ag valency on the signaling and Ag-targeting functions of the BCR. Although both paucivalent and polyvalent Ags induced the redistribution of the surface BCR into polarized caps, polyvalent Ag-induced BCR caps persisted. Ganglioside G_{M1}, a lipid raft marker, and tyrosine-phosphorylated proteins, but not CD45 and transferrin receptor, were concentrated in BCR caps, suggesting BCR caps as surface-signaling microdomains. Prolonged BCR caps were concomitant with an increase in the level and duration of protein tyrosine phosphorylation and a reduction in BCR internalization and movement to late endosomes/lysosomes. Thus, Ag valency influences B cell responses by modulating the stability of BCR-signaling microdomains and BCR trafficking. The Journal of Immunology, 2003, 170: 6099–6106.

B cells express clone-specific receptors, the B cell Ag receptors (BCR), which sense the presence of Ags. B cell responses are initiated by the engagement of Ags to the BCR. The binding of Ags to the BCR initiates signaling cascades, leading to the expression of genes involved in cell proliferation and differentiation (1–3), and induces the internalization of Ags for processing and presentation, acquiring T cell help (4–6).

Unlike the TCR, the BCR recognizes Ags in their native forms with varying affinity, valency, chemical component, and stereo-chemical structure. BCR engagement can initiate a wide range of responses, including anergy, apoptosis, proliferation, differentiation, or memory B cell generation. The outcome of BCR engagement is influenced by the properties of Ags, differentiation stage of B cells, availability of T cell help, and environment of B cells. In the absence of T cell help, a productive B cell response requires thresholds of Ag concentration, affinity, and valency (7–9). Below these thresholds, MHC class II-restricted T cell help is required. The relationship between Ag affinity and Ag-triggered responses has been extensively studied. Increasing Ag-BCR affinity has been shown to enhance B cell Ag-presenting ability, T cell-independent B cell proliferation, Ab secretion, and IFN-γ secretion in vitro (10, 11). Using transgenic mice carrying Ab genes with a high or low Ag-binding affinity, Shih et al. (12, 13) recently showed that while high and low affinity B cells had the same intrinsic capacity to respond to T-dependent Ags, high affinity B cells generated a 2-fold higher response to T cell-independent Ags than low affinity B cells. When both high and low affinity B cells were cotransferred to recipient mice, only high affinity B cells responded to both T cell-dependent and independent Ags. The effect of Ag valency on B cell responses has also been examined. Increasing Ag valency by conjugating BCR-specific Abs to dextran, Ficoll, Sepharose, or polyacrylamide beads reduced the amount of Ags required for induction of B cell proliferation (14–16). Conversely, increasing Ag valency using biotinylated anti-BCR Abs and avidin or by coating anti-BCR Abs to plastic or erythrocyte surface induced abortive activation of B cells and apoptosis (17–19). Thus, the property of Ags is one of the important factors that regulate B cell responses. However, little is known about how the BCR distinguishes and interprets differences in the structure and configuration of Ags.

The engagement of the BCR by Ags, particularly by multivalent Ags, has been shown to induce the reorganization of the surface BCR. Early studies (20, 21) showed that Ag cross-linking induced the redistribution of the surface BCRs into polarized caps. The formation of BCR caps required multivalent Ags (20, 22). Recent reports (23, 24) showed that Ag cross-linking induced the association of the BCR with detergent-insoluble lipid rafts. Lipid rafts refer to membrane microdomains that are rich in cholesterol and glycosphingolipids (25). The BCR associated with lipid rafts is preferentially phosphorylated by Src family kinases that are either constitutively associated or become associated with lipid rafts upon stimulation (26). Downstream signaling molecules, such as phospholipase Cγ2, are recruited to lipid rafts (27), but phosphatase CD45 is excluded (26). These data support the model that the engagement of the BCR by Ags induces the formation of organized signaling microdomains on the B cell surface, and the lipid rafts provide platforms for BCR-signaling microdomains. A recent report from Batista et al. (28) provides strong evidence for such a model. In their report, it was demonstrated that BCR engagement by Ags tethered on the surface of target cells induced the redistribution of BCR, ganglioside G_{M1}, and tyrosine-phosphorylated proteins to the contact region between B cells and target cells. But CD45, CD22, and SHP1 were excluded from the region. Such surface microdomains are similar to the immunological synapses formed between T cells and APCs in which MHC-restricted interaction is established and regulated (29–31). However, the mechanisms for the formation of BCR-signaling microdomains remain to be elucidated. The factors that regulate BCR-signaling microdomains have not been identified.
The aim of this study is to investigate the role of Ag valency in regulating the functions of BCR. Fab of anti-mouse IgM was used as a monovalent Ag, F(ab')2 of anti-mouse IgM + IgG as a polyclonal Ag, and biotinylated F(ab')2 plus avidin as a polyvalent Ag. These pseudo Ags differ in their binding valences, but the affinity of each individual binding site remains the same. In this study, we report the effect of these pseudo Ags with different valences on the formation of BCR surface-signaling microdomains, BCR-triggered protein tyrosine phosphorylation, and intracellular trafficking of the BCR.

Materials and Methods

**Mice, cells, and cell culture**

The B cell lymphoma CH27 is an IgM+, H-2d, and FcγR-IIB1+ cell line. CH27 cells were cultured at 37°C in DMEM supplemented, as described previously (32), and containing 15% FBS.

C57BL/6 mice were purchased from Charles River Breeding Laboratories (Frederick, MD). To obtain splenic B cells, single-cell suspensions of the splenocytes were prepared and then subjected to density-gradient centrifugation in Histopaque-1119 (Sigma-Aldrich, St. Louis, MO) at 2300 × g for 30 min. The lymphocyte-enriched fraction was harvested and washed. The B cells were isolated by specific depletion of T cells using anti-Thy-1.2 Ab and guinea pig complement. The B cells were washed and resuspended in medium.

**Abs and reagents**

Thy-1.2-specific mAb was obtained from BD PharMingen (San Diego, CA). Guinea pig complement and DMEM were purchased from Life Technologies (Grand Island, NY). Fab of goat anti-mouse IgM, Cy3-conjugated Fab of goat anti-mouse IgM + IgG, and avidin and tetramethylrhodamine isothiocyanate-conjugated Fab anti-rat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The 1D4B, a mAb specific for lymosomal-associated membrane glycoprotein-1 (LAMP-1), and TIB219, a mAb specific for mouse transferrin receptor (TIR), were obtained from hybridomas purchased from American Type Culture Collection (Manassas, VA). CD45-specific mAb was from BD Biotechnology (San Diego, CA). FITC-conjugated goat anti-mouse IgG2b was purchased from Southern Technologies (Grand Island, NY). FITC-conjugated F(ab')2 of goat anti-mouse IgM, Cy3-conjugated goat anti- IgM, and splenic B cells was labeled with Cy3-conjugated goat anti-IgM Ab (Cy3-Fab anti-IgM) at 4°C in the absence of cross-linking, a portion of the surface-labeled BCR was moved to the perinuclear area, while some remained on the cell surface, and CTX had a relatively homogenous surface-staining pattern (Fig. 1, A–C and J–L). Cross-linking the BCR with B-anti-Ig and avidin resulted in a polarized distribution of the surface BCR. The surface BCR of CH27 and splenic B cells was labeled with Cy3-conjugated Fab of goat anti-mouse IgM Ab (Cy3-Fab anti-IgM) at 4°C in the presence of 20 µg/ml B-anti-Ig or 20 µg/ml B-anti-Ig plus 20 µg/ml avidin or medium alone for 10 min. Cells were incubated with 20 µg/ml B-anti-Ig, 20 µg/ml B-anti-Ig plus 20 µg/ml avidin, or medium alone for 30 min at 4°C. Cells were washed with DMEM containing 6 mg/ml of BSA (DMEM-BSA) and adhered onto poly(lysine)-coated slides (Sigma-Aldrich) for 40 min at 4°C. Following this, the cells were then incubated at 37°C for varying lengths of time. At the end of the incubation, cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% saponin, and incubated with Ab specific for LAMP-1 (1D4B) and FITC-conjugated goat anti-rat IgG. For detection of phosphotyrosine-containing proteins, cells were incubated with anti-phosphotyrosine mAb (4G10), followed by FITC-conjugated anti-mouse IgG2b secondary Ab. For labeling the surface ganglioside GM1, CD45, or TIR, cells were incubated with FITC-conjugated CTX (FITC-CTX), or Abs specific for CD45 or TIR and their corresponding secondary Abs at 4°C before fixation. Cells were mounted with Gel/Mount (Biomeda, Foster City, CA). Slides were analyzed by laser-scanning confocal microscopy (LSM 510; Zeiss, Oberkochen, Germany) using >100 oil immersion objective. The same settings on the confocal microscope were used for all experimental conditions. Control experiments in which the primary Abs were either omitted or substituted by isotype control Abs showed no significant staining signals.

For the quantitative analysis, cells in five images randomly taken from slides of each of three independent experiments were counted for each time point and condition. The number of cells containing polarized BCR caps was counted and expressed as a percentage of the total number of cells in the images.

**Protein tyrosine phosphorylation**

Cells were incubated with B-anti-Ig or B-anti-Ig plus avidin or medium alone for 30 min at 4°C and warmed to 37°C for various times. Reactions were terminated by pelleting the cells at 4°C, followed by lysis of cells in 1% Nonidet P-40 lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, and protease inhibitor cocktail) for 30 min at 4°C. Lysates were subjected to SDS-PAGE and blotted with anti-phosphotyrosine (4G10) and HRP-conjugated goat anti-mouse IgG2b Abs. The blots were then stripped and reblotted with anti-tubulin Abs and an HRP-conjugated secondary Ab as loading controls.

**Internalization assay**

Fab anti-IgM was iodinated by the iodine monochloride method (33). More than 95% of the 125I-labeled Fab (125I-Fab) anti-IgM was precipitated by 10% TCA, indicating little or no free 125I. Cells were incubated with 125I-Fab anti-IgM in DMEM-BSA, in the presence or absence of the Ags with different valences for 60 min at 4°C. Cells were washed in DMEM-BSA to remove unbound ligands, and incubated at 37°C for 0–40 min. At the end of the incubation, cells were centrifuged and incubated in a cell strip solution (20 mM HCl and 150 mM NaCl) at 4°C to remove the 125I-Fab anti-IgM from the cell surface. The radioactivity associated with the cells after the stripping was taken as the fraction of 125I-Fab anti-IgM internalized. The internalization was expressed as a percentage of the total radioactivity initially associated with the cells.

**Results**

**Ag valency and the formation of BCR-signaling microdomains**

Previous studies implicate that lipid rafts play a role in both BCR signaling and trafficking (23, 24). To determine the effect of Ag valency on the spatial relationship between the BCR and lipid rafts, the cellular distribution of the BCR and ganglioside GM1 was analyzed using immunofluorescence microscopy. BCR-specific Abs were used as model Ags. To cross-link the BCR, biotin-conjugated F(ab')2 of goat anti-mouse IgM + IgG (B-anti-Ig) was used, serving as a paucivalent Ag. B-anti-Ig plus avidin was used to mimic a polyvalent Ag. CTX that binds to glycosphingolipid GM1 was used to label the lipid rafts. The surface BCR of CH27 and splenic B cells was labeled with Cy3-conjugated Fab of goat anti-mouse IgM Ab (Cy3-Fab anti-IgM) at 4°C in the presence of 20 µg/ml B-anti-Ig or 20 µg/ml B-anti-Ig plus 20 µg/ml avidin. As previously shown, B-anti-Ig does not compete with Fab-anti-IgM to bind to the BCR (5, 34). After incubation at 37°C for 60 min, cells were cooled to 4°C and incubated with FITC-CTX without permeabilization to label the surface GM1. In the absence of cross-linking, a portion of the surface-labeled BCR moved to the perinuclear area, while some remained on the cell surface, and CTX had a relatively homogenous surface-staining pattern (Fig. 1, A–C and J–L). Cross-linking the BCR with B-anti-Ig alone increased the amount of BCR staining in the perinuclear area, but had no significant effect on the CTX-staining pattern (Fig. 1, D–F and M–O). Treating cells with B-anti-Ig and avidin resulted in a polarized distribution of the surface BCR. BCR staining showed a crescent-like appearance (Fig. 1, G and P). Such a polarized distribution of the surface BCR has been described previously as BCR caps (20, 21). The degree of polarization varied between cells, typically with the BCR being concentrated at one-third to one-half of the cell surface. A small portion of the BCR was detected inside the cells. Significantly, in cells treated with B-anti-Ig and avidin, GM1 staining was preferentially localized to the BCR caps (Fig. 1, G–I and P–R).

Next, we analyzed the cellular distribution of tyrosine-phosphorylated proteins, phosphatase CD45, and TIR in response to Ags.
with different valences. CH27 and splenic B cells were incubated with Cy3-Fab anti-IgM at 4°C to label the surface BCR, cross-linked with either B-anti-Ig or B-anti-Ig plus avidin, and chased at 37°C for 60 min. Tyrosine-phosphorylated proteins were stained by anti-phosphotyrosine Ab after fixation and permeabilization. The surface CD45 and TfR were labeled with specific Abs at 4°C without permeabilization. In the absence of cross-linking Ags, tyrosine-phosphorylated proteins were nearly undetectable, particularly in the splenic B cells (Fig. 2, B and K). Both cross-linking Ags increased the staining level of phosphotyrosine (Fig. 2, E, H, N, and Q). In cells treated with B-anti-Ig alone, phosphotyrosine staining primarily colocalized with the BCR in the perinuclear location (Fig. 2, D–F and M–O). In cells treated with B-anti-Ig and avidin, tyrosine-phosphorylated proteins primarily colocalized with the BCR in the polarized BCR caps (Fig. 2, G–I and P–R). In contrast, CD45 and TIR staining appeared evenly distributed around the cell surface (Fig. 3). Neither the paucivalent nor polyvalent Ag had any significant effect on the surface distribution of CD45 and TIR (Fig. 3). Different from CTX and tyrosine-phosphorylated proteins, CD45 and TfR did not concentrate in the polarized BCR caps.

Parallel experiments were conducted using cells treated with avidin alone or unrelated biotinylated Abs. The results of these experiments were similar to those seen in untreated cells (data not shown), indicating that without BCR-specific Abs, avidin alone

FIGURE 1. Effect of Ag valency on the cellular distribution of BCR and ganglioside G M1. CH27 (A–I) and mouse splenic B cells (J–R) were incubated with Cy3-Fab anti-IgM alone (−XL) or in the presence of 20 μg/ml B-anti-Ig (+XL) or 20 μg/ml B-anti-Ig plus 20 μg/ml avidin (+dXL) for 40 min at 4°C. After incubating at 37°C for 60 min, the cells were cooled for 10 min at 4°C and stained with FITC-CTX for 40 min at 4°C. Cells were then fixed and mounted. Images were acquired in the middle of the cells using a confocal fluorescence microscope. Bar, 10 μm.

FIGURE 2. Effect of Ag valency on the cellular distribution of BCR and tyrosine-phosphorylated proteins. CH27 cells (A–I) and mouse splenic B cells (J–R) were incubated with Cy3-Fab anti-IgM alone (−XL) or in the presence of 20 μg/ml of B-anti-Ig (+XL) or 20 μg/ml of B-anti-Ig plus 20 μg/ml avidin (+dXL) for 40 min at 4°C. Cells were washed and incubated at 37°C for 60 min. After fixation and permeabilization, the cells were incubated with anti-phosphotyrosine mAb (4G10), followed by FITC-conjugated goat anti-mouse IgG2b Ab (Y–P). Images were acquired in the middle of the cells using a confocal fluorescence microscope. Bar, 10 μm.

FIGURE 3. Effect of Ag valency on the cellular distribution of the BCR, CD45, and TIR. Splenic B cells (A–F) and CH27 (G–L) were incubated with FITC-Fab anti-IgM in the presence of 20 μg/ml B-anti-Ig (XL) or 20 μg/ml B-anti-Ig plus 20 μg/ml avidin (dXL) for 40 min at 4°C. After incubating at 37°C for 60 min, the cells were cooled for 10 min, and stained with mAbs specific for CD45 (A–F) or TIR (G–L) and secondary Abs at 4°C. Cells were then fixed, mounted, and observed under a confocal fluorescence microscope. Bar, 10 μm.
does not affect the cellular distribution of the BCR, ganglioside GM1, tyrosine-phosphorylated proteins, CD45, and TIR.

Taken together, polyvalent Ags induce the colocalization of the BCR with lipid raft maker ganglioside GM1 and tyrosine-phosphorylated proteins in the polarized caps, suggesting that BCR caps function as surface-signaling microdomains.

**Ag valency and the time course of signaling microdomain formation**

To follow the time course of the BCR redistribution, CH27 and splenic B cells were incubated with Cy3-Fab anti-IgM at 4°C in the presence of either B-anti-Ig or B-anti-Ig plus avidin and chased at 37°C for varying lengths of time. After the chase, the cells were labeled for surface GM1 (Fig. 4A) or cellular tyrosine-phosphorylated proteins (Fig. 4B). Before the incubation at 37°C, the BCR and CTX had a relatively homogenous surface-staining pattern in CH27 and splenic B cells treated either with the paucivalent or polyvalent Ag (Fig. 4A, a–d). The phosphotyrosine staining was detected both on the surface and the cytoplasm of cells (Fig. 4B, a–d). Upon warming to 37°C, the BCR gradually moved from the cell surface to the perinuclear location in cells treated with B-anti-Ig alone. The staining pattern of ganglioside GM1 was largely unchanged and remained relatively homogenous on the cell surface (Fig. 4A, e, g, i, k, m, and o). Besides the surface and cytoplasmic staining, the tyrosine-phosphorylated proteins appeared as punctate staining and colocalized with the BCR at the cell periphery at 10 and 30 min (Fig. 4B, e, g, i, and k) and in the perinuclear location at 60 min (Fig. 4B, m and o). Polarized BCR caps were also detected in cells treated with B-anti-Ig alone, particularly in the splenic B cells after 10- and 30-min incubation at 37°C. In cells that showed the polarized BCR caps, a majority of the GM1 (Fig. 4A, e, g, i, and k) and phosphotyrosine (Fig. 4B, e, g, i, and k) staining was concentrated in the same location corresponding to the polarized BCR caps. In cells treated with B-anti-Ig and avidin, the surface-labeled BCR, GM1 (Fig. 4A, f and h), and tyrosine-phosphorylated proteins (Fig. 4B, f and h) concentrated to one pole of the cells as early as 10 min of warming at 37°C. Although the majority of the BCR remained in BCR caps, the amount of the BCR moving into cells increased over time (Fig. 4A, j, n, l, and p). Significantly, the GM1 (Fig. 4A, j, l, n, and p) and tyrosine-phosphorylated proteins (Fig. 4B, j, l, n, and p) remained together with the BCR in the polarized caps for at least 60 min, the longest time point tested.

Using these immunofluorescence images, the percentage of cells containing polarized BCR caps was quantified (Fig. 4C). When treated with B-anti-Ig and avidin, 43% of CH27 cells and 63% of splenic B cells showed BCR caps at 10 min (Fig. 4C). The percentages of cells containing BCR caps increased with time and reached 72% in both CH27 and splenic B cells by 60 min. When treated with B-anti-Ig alone, the BCR was polarized into caps in ~40% of the splenic B cells, and only 9% of CH27 cells at 10 min (Fig. 4C). The percentage of CH27 cells containing BCR caps remained low, and the percentage of splenic B cells containing BCR caps decreased with time. By 60 min, the BCR remained in the caps in ~14% of the splenic B cells, and ~7% of the CH27 cells (Fig. 4C).

Thus, both paucivalent and polyvalent Ags induce the polarized distribution of the surface BCR and colocalization of the BCR with GM1 and tyrosine-phosphorylated proteins. However, the BCR caps induced by the polyvalent Ag persist on the cell surface much longer than those induced by the paucivalent Ag.

**FIGURE 4.** Time course of the formation of BCR-signaling microdomains. A and B. Experiments were conducted, as described in Figs. 1 and 2, except that after the surface labeling and Ag treatment, cells were incubated at 37°C for times indicated. Bar, 10 μm. C. Shown is a quantitative analysis of cells containing the polarized BCR caps. Five images were randomly taken from slides of three independent experiments. The number of cells with polarized BCR caps was counted for each time point and condition, and expressed as a percentage of the total number of cells in the images. Shown are the averages (±SD) of three independent experiments.

**Ag valency and BCR-triggered protein tyrosine phosphorylation**

Ag engagement of the BCR leads to a rapid tyrosine phosphorylation of cytoplasmic and membrane proteins, which is one of the early events in BCR signaling. To study the effect of Ag valency on the BCR-induced protein tyrosine phosphorylation, CH27 cells and splenic B cells were treated with the Ags with different valences for 30 min at 4°C, followed by incubation at 37°C for various times. Cell lysates were prepared, analyzed by SDS-PAGE...
and Western blot, and probed for phosphotyrosine-containing proteins.

When CH27 cells were incubated with increasing concentrations (0–40 μg/ml) of B-anti-Ig at 37°C for 2 min, the number and intensity of tyrosine-phosphorylated proteins gradually increased (Fig. 5A). The maximal phosphorylation intensity was observed at 40 μg/ml Ag concentration. Thus, BCR-triggered protein tyrosine phosphorylation exhibits a dose dependency on Ag concentration. These data also showed that at a concentration of 20 μg/ml of B-anti-Ig that was used in this study, BCR cross-linking was not yet saturated.

Next, we compared the protein tyrosine phosphorylation induced by the paucivalent and polyvalent Ags. In the absence of cross-linking, a higher number of faint bands was detected in CH27 cells than in the splenic B cells (Fig. 5, B and C, lane 1). This reflects a higher constitutive level of tyrosine phosphorylation in CH27 cells. Upon cross-linking with 20 μg/ml of B-anti-Ig alone, followed by incubation at 37°C, the intensity and number of tyrosine-phosphorylated proteins increased in both CH27 and splenic B cells. The maximal levels of protein tyrosine phosphorylation were reached at 2 min (Fig. 5, B and C, lane 2). After 2 min, the phosphorylation levels gradually decreased and nearly returned to basal levels by 20 min (Fig. 5, B and C, lanes 3–5). In both CH27 and splenic B cells, cross-linking the BCR with B-anti-Ig and avidin induced a much higher level of protein tyrosine phosphorylation than those observed with B-anti-Ig alone (Fig. 5, B and C). Similar to cells treated with B-anti-Ig, the maximal levels of tyrosine phosphorylation in cells treated with B-anti-Ig and avidin were reached at 2 min, but the intensity of the phosphorylated proteins was much higher (Fig. 5, B and C, lanes 2 and 6). Tyrosine phosphorylation was attenuated with time (Fig. 5, B and C, lanes 6–9), but at 20 min, the level, particularly in the splenic B cells (Fig. 5, B and C, lane 9), was still significantly higher than the unstimulated state (Fig. 5, B and C, lane 1). These results indicate that increases in valences of Ags that cross-link the BCR enhance the overall level and duration of protein tyrosine phosphorylation.

**Ag valency and the intracellular trafficking of the BCR**

To test whether Ag binding influences the kinetics of BCR internalization, CH27 cells were incubated at 4°C with 125I-Fab anti-IgM in the presence or absence of varying concentrations of B-anti-Ig. Cells were washed and warmed to 37°C for varying intervals of time. The released, surface, and internal fractions were collected, and their radioactivity was measured. The internalized fraction was expressed as a percentage of the total radioactivity initially associated with the cells. In the absence of cross-linking, maximal internalization, representing ~16% of the 125I-Fab anti-IgM initially bound to the cells, was reached in ~40 min (Fig. 6A). With increasing concentrations of the Ag, the rates of uptake exhibited

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**FIGURE 5.** Protein tyrosine phosphorylation in response to Ags with different valences. A, CH27 cells were incubated with varying concentrations of B-anti-Ig for 30 min at 4°C and then warmed up to 37°C for 2 min. B and C, CH27 (B) and splenic B cells (C) were incubated with 20 μg/ml of B-anti-Ig (+XL), 20 μg/ml of B-anti-Ig plus 20 μg/ml of avidin (+dXL), or medium alone (−XL) for 30 min at 4°C and then warmed up to 37°C for varying lengths of time. After each incubation, cells were cooled to 4°C, immediately pelleted, and lysed. Cell lysates were subjected to SDS-PAGE and Western blotting. The blots were probed with anti-phosphotyrosine mAb (4G10) and HRP-conjugated secondary Ab (top panels). The blots were then stripped and reprobed with anti-tubulin Ab for loading controls (bottom panels).

**FIGURE 6.** Effect of Ag valency on BCR internalization. CH27 (A and B) and splenic B cells (C) were incubated at 4°C with 125I-Fab anti-IgM in the presence or absence (−XL) of varying concentration of B-anti-Ig (A), or 20 μg/ml of B-anti-Ig (+XL), or 20 μg/ml of B-anti-Ig plus 20 μg/ml of avidin (+dXL) (B and C). Cells were washed and warmed to 37°C for varying lengths of time. The released, surface, and internal fractions were collected, and their radioactivity was measured. The internalized fraction was expressed as a percentage of the total radioactivity initially associated with the cells. The released, surface, and internal fractions were collected, and their radioactivity was measured. The internalized fraction was expressed as a percentage of the total radioactivity initially associated with the cells. In the absence of cross-linking, maximal internalization, representing ~16% of the 125I-Fab anti-IgM initially bound to the cells, was reached in ~40 min (Fig. 6A). With increasing concentrations of the Ag, the rates of uptake exhibited
a gradual increase from ~16 to 29%. The maximal uptake of 29% was reached in 20 min when cells were cross-linked with 20 μg/ml of anti-Ig (Fig. 6A). Even though the increase is relatively small, it is significant.

To assess the relationship between Ag valency and BCR internalization, CH27 and splenic B cells were incubated at 4°C with 125I-Fab anti-IgM in the presence or absence of 20 μg/ml of B-anti-Ig or 20 μg/ml of B-anti-Ig plus 20 μg/ml of avidin. Cells were washed and warmed to 37°C for varying lengths of time. In CH27 cells treated with B-anti-Ig and avidin, the amount and rate of internalization were reduced and comparable to internalization in the absence of BCR cross-linking (Fig. 6B). Maximal uptake of 18% was reached at 40 min in contrast to an uptake of 29% in cells treated with B-anti-Ig (Fig. 6B). In splenic B cells, in the absence of cross-linking of the BCR, the maximal uptake was as low as 10% (Fig. 6C). Cross-linking the BCR with B-anti-Ig alone increased the internalization to 38%. Cross-linking the BCR with B-anti-Ig plus avidin reduced the internalization to 23%. This indicates that the paucivalent Ag increases BCR internalization rate in a dose-dependent manner, and the polyvalent Ag reduces this increase.

The movement of the BCR from the cell surface to late endosomes/lysosomes was analyzed using immunofluorescence microscopy. The BCR on the surface of CH27 cells was labeled with Cy3-Fab anti-IgM in the presence of the Ags with different valencies and chased at 37°C for various times. The cells were labeled with anti-LAMP-1 Ab to mark the late endosomes/lysosomes. Similar to the results shown in Fig. 4, A and B, before the incubation at 37°C, a relatively homogenous surface-staining pattern of the BCR was observed (Fig. 7, A and B) in cells either treated with B-anti-Ig alone or B-anti-Ig plus avidin. Upon warming to 37°C, the BCR in cells treated with B-anti-Ig appeared as punctate staining around the cell periphery (Fig. 7C), and then moved to the center of the cells (Fig. 7E). At 60 min, the majority of the BCR clustered in the perinuclear area and colocalized with LAMP-1 (Fig. 7G). Cross-linking of the BCR with B-anti-Ig and avidin resulted in the redistribution of the BCR to polarized caps and significantly reduced the colocalization of the BCR with LAMP-1 (Fig. 7, F, H, and J). Although BCR caps persisted, the colocalization of the BCR with LAMP-1 in polyvalent Ag-treated cells increased over time (Fig. 7, F, H, and J).

Taken together, cross-linking the BCR with paucivalent Ags increases the internalization and movement of BCR to late endosomes/lysosomes. In contrast, cross-linking of the BCR with polyvalent Ags reduces the internalization and movement of the BCR from the cell surface to late endosomes/lysosomes.

**Discussion**

Considered together, the results presented in this study suggest that although both paucivalent and polyvalent Ags induce the formation of BCR surface-signaling microdomains, increases in Ag valencies prolong the lifetime of the surface-signaling microdomains, which leads to a higher magnitude and longer duration of protein tyrosine phosphorylation.

It has long been observed that the binding of polyvalent Ags to the BCR induces the reorganization of the surface BCRs into segregated surface domains, called BCR caps (20, 21). However, the significance of BCR caps in B cell activation is not well understood. In this study, we showed that cross-linking the BCR with a polyvalent Ag not only induced the formation of polarized BCR caps, but also led to the colocalization of the lipid raft marker, ganglioside G<sub>441</sub>, and cellular tyrosine-phosphorylated proteins with the BCR, thereby suggesting that BCR caps are signaling microdomains on the B cell surface. Our time course study following the cellular distribution of surface-labeled BCR showed that BCR caps were formed within 10 min in cells treated with both paucivalent and polyvalent Ags. For paucivalent Ag-treated cells, the maximal number of cells showing BCR caps was observed within 10 min, and the number of cells containing BCR caps decreased as internalization increased with time. Compared with paucivalent Ag-treated cells, the number of polyvalent Ag-treated cells that contained BCR caps was much higher. Furthermore, the polarized BCR caps and the cocapping of surface G<sub>441</sub> and tyrosine-phosphorylated proteins with the BCR persisted much longer in cells treated with the polyvalent Ag than in cells treated with the paucivalent Ag. Significantly, the persistence of the polarized BCR caps is correlated with a higher level and longer duration of protein tyrosine phosphorylation. This suggests that multivalent Ags with high or low binding valence can induce the formation of BCR surface-signaling microdomains. The surface-signaling microdomains induced by Ags with relatively low valences are transient, followed by BCR internalization, while Ags with high valences stabilize the BCR-signaling microdomains. The relationship between Ag valency and the lifetime of BCR-signaling microdomains implicates that modulating the stability of the BCR-signaling microdomains is a mechanism by which Ags regulate the functions of BCR.

Ag-induced BCR clusters that we reported in this work appear to be similar to BCR synapses recently described by Batista et al. (28). Their study showed that B cell interaction with Ags attached to a target cell surface led to the concentration of the surface BCR in the contact region between the B cell and the target cell. The BCR along with CTX, phosphotyrosine-containing proteins, actin
filaments, and phospholipase Cy2, were found to accumulate in the region of the synapse, while the phosphatases SHP1 and CD45 were specifically excluded. Similarly, our data showed that polyvalent Ags induced co-clustering of the BCR, CTX, and tyrosine-phosphorylated proteins, but not CD45. The term immunological synapse was first introduced to describe the organized signaling domain formed between a T cell and an APC (29–31). In T cell immunological synapses, the TCR is located in the center, surrounded by a ring of adhesion molecules (30). The formation of T cell immunological synapses has been shown to be directly correlated to the binding affinity of TCR ligands and TCR-initiated T cell proliferation (30). The results presented in this study show that the stability of BCR-surface signaling microdomains is correlated to the binding valency of Ags and BCR-triggered protein tyrosine phosphorylation. This provides further evidence supporting that the formation of such surface-signaling microdomains is an active and dynamic mechanism by which immune recognition receptors regulate their functions in response to different antigenic ligands.

Different from the immunological synapses reported previously, the surface BCR clusters reported in this study are induced by soluble polyvalent Ags, but not Ags tethered on the cell surface. This extends the previous studies by demonstrating that soluble polyvalent Ags can induce the formation of stable surface-signal- ing microdomains similar to the immunological synapses. However, the surface-signaling microdomains induced by soluble Ags may be different from those formed in the contact region between two cells, considering the ability of the BCR to distinguish Ags of different forms. Indeed, we found that unlike the synapses formed in the cell-cell contact regions in which CD45 is excluded, CD45 was neither preferentially concentrated in nor excluded from the polarized BCR caps induced by soluble Ags. Further studies are required to characterize BCR-signal microdomains induced by soluble Ags and compare them with those induced by Ags tethered on the cell surface.

How Ags regulate the stability of BCR-signal microdomains remains to be elucidated. In this study, we found that the increase in BCR residency in the surface-signaling microdomains and BCR-triggered protein phosphorylation was associated with a decrease in BCR internalization. This is consistent with our previous observation that an inhibition of BCR internalization slowed down the attenuation of BCR-triggered protein tyrosine phosphorylation (34). These results suggest that inhibition of internalization is one of the mechanisms for stabilizing surface-signal microdomains. In addition to internalization, the stability of BCR-signal- ing microdomains is likely to be regulated by multiple interrelated factors. Syk has been shown to play a role in the formation of tightly capped BCR complexes on the cell surface (35), probably by regulating the assembly of actin filaments. Pure and Tardelli (36) reported that capping of the BCR in cells treated with the tyrosine kinase inhibitors genistein or tyrphostin was incomplete and retarded, which suggests a link between BCR capping and signaling. Therefore, the actin cytoskeleton, signaling components that are recruited to the signaling microdomain, and activation of coreceptors could all influence the stability of BCR-signal microdomains.

The results from this study show that Ags with different valences differentially regulate the intracellular trafficking of the BCR. Ags with a relatively low valence increase the rate of BCR internalization and its movement to late endosomes/lysosomes. This increase has been shown to depend on the activation of BCR-signaling cascades (35–37). Interestingly, Ags with a relatively high valence enhance BCR-triggered protein phosphorylation, but have a reduced ability to stimulate BCR internalization and its movement to late endosomes/lysosomes. The reduced internalization is probably the result of large sizes of Ags, which become unmanageable for the endocytosis machinery. Additionally, the actin cytoskeleton that has been shown to accumulate under BCR caps (38) could prevent the endocytosis machinery proteins from entering the signaling microdomains or inhibit membrane deformation required for endocytosis. Ag valency may also influence the intracellular trafficking pathway of the BCR. However, we found that even though BCR caps persisted in polyvalent Ag-treated cells, the colocalization of the BCR with LAMP-1 increased over time, suggesting that the BCR in the stable signaling microdomains eventually moves to late endosomes/lysosomes. How the BCR switches from the signaling mode into internalization mode is an interesting question. Delineation of the mechanism for this transition will greatly increase our understanding of the cross talks between BCR signaling and Ag-targeting pathways.

Consistent with our previous reports (5, 6), paucivalent Ags speed up the movement of the BCR from the plasma membrane to LAMP-1 compartments. Interestingly, in the paucivalent Ag-treated cells, the BCR colocalized with tyrosine-phosphorylated proteins not only at the cell surface, but also in the intracellular vesicles and LAMP-1 compartments. This suggests that the BCR may have the ability to continue signaling inside cells and that BCR signaling may influence the endocytic system through the downstream tyrosine kinases. The intracellular signaling of the BCR may regulate the acidification and fusion of endosomes (39), leading to a rapid targeting of the BCR to the peptide-loading compartment. However, the exact nature and roles of BCR intracellular signaling remain to be examined.

The findings presented in this work may help to explain how the BCR distinguishes Ags with different properties, particularly structural differences. A major characteristic of T cell-independent type 2 (TI-2) Ags is their multiple, repeating antigenic determinants. TI-2 Ags include the outer membrane molecules displayed on the surface of microbial pathogens, such as Ags displayed on the surface of Neisseria meningitidis and influenza that cause severe diseases (9). B cell responses to TI-2 Ags are essential for immune protection against microbial pathogens. Self Ags, such as dsDNA, actin filaments, collagens, and cell surface molecules, are often presented in polyvalent forms. Unresponsiveness of B cells to self Ags is critical for immune tolerance. TI-2 Ags need to activate B cells in the absence of T cell help. Without signals provided by T cells, signaling initiated by the interaction between Ags and the BCR probably becomes the major driving force for B cell activation, and B cell responses to TI-2 Ags are likely to be sensitive to the magnitude and duration of the signaling events. The binding of polyvalent Ags stabilizes the surface-signal microdomains and reduces BCR internalization, which provides a way to enhance BCR signaling and ensure B cell activation in the absence of T cell help. Besides BCR-initiated signaling and signals provided by Th cells, additional signals provided by T cell-independent sources, such as Toll-like receptors and complement receptor CD21, are important for the outcome of Ag-BCR interaction (9, 40). These additional signals can be triggered by surface molecules of microbial pathogens, bacterial DNA, and complement factors associated with pathogens. In the absence of additional signals from either T cells or T cell-independent sources, extensive cross-linking of the BCR by polyvalent Ags has been shown to induce apoptosis (17, 18, 41), which maintains the self-tolerance of B cell responses. The relationship between the stability of B cell surface-signaling microdomains and the fate of B cells needs to be further examined. We can predict that both Ag structural properties and costimulatory signals regulate the stability and composition of BCR surface-signaling microdomains, which influence the fate of B cells. Future studies should focus on differences in the composition of BCR...
surface-signaling microdomains induced by Ags with different valencies and cellular machineries involved in the formation of BCR-signaling microdomains, which will further delineate the molecular mechanism for differential B cell responses to different Ags.

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References