Binding of Cytoplasmic Proteins to the CD19 Intracellular Domain Is High Affinity, Competitive, and Multimeric

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Binding of Cytoplasmic Proteins to the CD19 Intracellular Domain Is High Affinity, Competitive, and Multimeric


CD19 is required for the development of B1 and marginal zone B cells, for Ab responses, and for B cell memory. CD19 immunoprecipitates contain a complex of cytoplasmic proteins, including Lyn, Vav, phospholipase Cγ2 (PLCγ2), Grb2, and the p85 subunit of phosphatidylinositol 3-kinase. Which of these bind directly to CD19 and the strengths of the interactions are unknown. These issues are important in understanding the signaling functions of CD19, which are crucial for normal B cell physiology. Using purified, recombinant proteins, we now show that each of these signaling proteins contains at least one Src homology 2 (SH2) domain that interacts directly with the phosphorylated CD19 cytoplasmic domain. The affinities of binding of the SH2 domains of Vav, p85, and Grb2 to CD19 are each in the nanomolar range by surface plasmon resonance (Biacore) analysis. Binding of Lyn and PLCγ2 do not fit 1:1 modeling. However, analyses of binding data (Lyn) and competition experiments (PLCγ2) suggest that these bind with comparable affinity. Competition experiments demonstrate that SH2 domains whose binding is dependent on the same CD19 tyrosine(s) compete for binding, but these SH2 domains do not impede binding of different SH2 domains to other CD19 tyrosines. We conclude that binding to the CD19 cytoplasmic domain is multimeric, high affinity, and competitive. The high affinity of the interactions also suggests that tyrosines that were nonessential in vivo are nevertheless functional. A preliminary structural model suggests that CD19 forms a signaling complex containing multiple cytoplasmic proteins in close proximity to each other and to the plasma membrane.


CD19 is a coreceptor expressed on B cells that regulates signaling initiated through the B cell Ag receptor (BCR) (1). CD19 is required for the development/maintenance of B1 and marginal zone B cells, efficient germinal center formation, T-dependent Ab responses, and B cell memory (2–6). CD19 is phosphorylated on one or more of its nine cytoplasmic tyrosines after cross-linking of the BCR. Tyrosine-phosphorylated CD19 can then bind Src homology 2 (SH2)-containing cytoplasmic proteins, which link CD19 to downstream signaling cascades. We and others have shown that CD19 forms a complex that contains Vav, phospholipase Cγ2 (PLCγ2), Grb2/Sos, the p85 subunit of phosphatidylinositol 3-kinase (PI3K), and Lyn in response to stimulation (7–15).

The studies analyzing CD19 associations primarily used coimmunoprecipitation or peptide precipitation from B cell lysates. Such methods give no information about whether an association is direct or mediated by other proteins. Knowing which interactions are direct is essential for planning and interpreting any attempts at specific blockade, whether dominant negative proteins or small molecules. Immunoprecipitations of proteins involved in membrane microdomains may also detect proteins that associate only by lipid interactions. Finally, the precipitations from cell lysates provide little information about the avidity of interactions. A weak band could represent either a low affinity interaction or the availability of only a limited amount of the binding partner(s) in a given cellular compartment.

These issues are particularly relevant for CD19. Multiple interactions have been described as reported above, but the relative strengths of these interactions are unknown. This becomes important in light of our data showing that mutation of certain CD19 tyrosines, Y482 and Y513, cripples CD19 function in vivo, whereas other CD19 tyrosines were nonessential (16). Perhaps the interactions mediated by the nonessential tyrosines, detected in coprecipitation studies, are of low affinity and hence not physiologically relevant. A second issue is that current data suggest that multiple proteins can interact with the same CD19 tyrosines: PLCγ and Vav with CD19 tyrosine (Y) 391, and Lyn and p85 with Y513. To understand these interactions, we need to know the relative affinities of the binding of different SH2-containing proteins to CD19. If binding affinities are similar, then competition between different signaling proteins is likely, and different proteins are likely to bind the same tyrosines on different CD19 molecules. Significantly different affinities would suggest that binding of one protein would be dominant, except where there is compartmentalization.

To determine which, if any, of these CD19 associations is direct, we performed binding assays using purified proteins expressed in bacteria. A His-tagged fusion of the cytoplasmic portion (cyto) of CD19 was coexpressed in bacteria with a promiscuous tyrosine kinase, elk, to yield tyrosine-phosphorylated (pY)-His-CD19-cyto. The SH2 domains of cytoplasmic signaling molecules that associate with CD19 were expressed in bacteria as GST fusions. These purified proteins were then used in two-component binding assays.

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0022-1767/04/$20.00
Using these techniques, we show that CD19 directly binds the SH2 domains of multiple cytoplasmic signaling proteins.

Given the multiple interactions, we assessed the affinity and kinetics of these interactions by surface plasmon resonance. GST-SH2 domain fusion proteins were used as ligands, and pY-His-CD19-cyto was used as analyte. In some cases, binding of the SH2 domain to CD19 occurred, but no first-order reaction kinetics could be determined, which suggests that these interactions are complex. In those cases where first-order binding reactions could be analyzed, the avidity of binding of SH2 constructs from different cytoplasmic proteins was comparable, suggesting competitive interactions. Such competition was then confirmed by experiments using native cytoplasmic proteins. SH2 domains of cytoplasmic proteins competed with SH2 domains of other proteins that bound the same CD19 tyrosines, but did not interfere with the binding of other SH2 domains that interact with other CD19 tyrosines. Thus, the interactions of cytoplasmic signaling molecules with the intracellular domain of CD19 are high affinity and competitive at specific tyrosines, but permit the formation of multimeric complexes involving different CD19 tyrosines. This provides a model for how transmembrane proteins function to assemble signaling complexes at the membrane.

Materials and Methods

Materials, cDNA constructs, protein expression, and purification

Rabbit polyclonal Ab to the CD19 cytoplasmic domain and biotinylated, tyrosine-phosphorylated, CD19-derived peptides were produced as previously described (10, 16). pGex Lyn SH2 (aa 119–230) and pGex Lyn kinase (aa 231–512) were gifts from Dr. O. Miura (Tokyo Medical and Dental University, Tokyo, Japan) (17). pGex p85 SH2 (aa 312–444) and C-SH2 (aa 612–722) were gifts from Dr. T. Pawson (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada) (18). pGex p85 dual technology containing the N-terminal and C-terminal SH2 domains and the intrash2 domain, was obtained from Dr. G. Mills (M. D. Anderson Cancer Center, Houston, TX). pGex Grb2 SH2 (aa 49–168) has been described previously (19). pGex PLCγ2 N-SH2 (aa 498–636) and pGex PLCγ2 C-SH2 (aa 636–744) were gifts from Dr. S. P. Watson (University of Oxford, Oxford, U.K.) (20). pGex Vav SH2 (aa 670–765) was provided by Dr. A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA) (21). To produce pET-CD19 cyto, a PCR product of human CD19 cDNA encoding residues 326–556 was cloned in frame with a 5′ His tag into pET-28a (Novagen, Madison, WI). To express His-CD19-cyto, pET-CD19 cyto was transfected into competent BL-21 for unphosphorylated or TKB1 (Stratagene, La Jolla, CA) for tyrosine-phosphorylated (pY) His-CD19-cyto. Protein was purified from cleared lysate using ProBond resin (Invitrogen, Carlsbad, CA), and resin was washed with 25 mM imidazole in 20 mM Tris (pH 8) and 500 mM NaCl. Protein was eluted with 80 mM imidazole in 20 mM Tris (pH 8) and 500 mM NaCl. Protein quantity was assayed using Coomassie Protein Assay Reagent (Pierce, Rockford, IL), and >95% purity was determined by SDS-PAGE.

Binding assays

The binding efficiency of pY-His-CD19-cyto was determined by incubating 40 µl of washed glutathione beads, to which various molar ratios of GST-SH2 protein were affinity, with 5 µg of pY-His-CD19-cyto in 500 µl of Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris (pH 7.4), 80 mM KCl, 10 mM EDTA, 5 mM iodoacetamide, 1 mM sodium orthovanadate, 10 mM sodium molybdate, and a mixture of protease inhibitors) for 1 h at 4°C. Molar ratios of GST-SH2:pY-His-CD19-cyto were 0.1, 1, 5, 1/2, 1/3, 1/5, 1/10, and 1/100. Beads were spun down, and 30 µl of supernatant was run out by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit polyclonal anti-CD19 cyto, followed by anti-rabbit IgG-HRP (Southern Biotechnologies, Birmingham, AL). The binding efficiency of GST-SH2 proteins was determined by incubating 40 µl of ProBond resin, to which varying molar ratios of pY-His-CD19-cyto protein were affinity, with a fixed amount (5 µg) of GST-SH2 in 500 µl of Nonidet P-40 lysis buffer for 1 h at 4°C. Molar ratios were the same as above. Beads were spun down, and 30 µl of supernatant was run out by SDS-PAGE and transferred to nitrocellulose, and unbound protein was detected with HRP-conjugated anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA).

For binding assays in which GST-SH2 fusions acted as bait, 40 µl of washed glutathione-Sepharose beads were loaded with 5 µg of GST-SH2 protein for 2 h at 4°C and then washed twice with 50 mM Tris-HCl, pH 8, and once with Nonidet P-40 lysis buffer. Five micrograms of unphosphorylated His-CD19-cyto or tyrosine-phosphorylated pY-His-CD19-cyto was added to the loaded beads in 0.5 ml of Nonidet P-40 lysis buffer and incubated for 2 h at 4°C. Beads were washed five times with Nonidet P-40 lysis buffer, boiled for 5 min in SDS-PAGE sample buffer, run out on 10% SDS-PAGE, transferred to nitrocellulose, and probed with rabbit polyclonal anti-CD19 cyto, followed by HRP-conjugated anti-rabbit IgG to detect bound His-CD19-cyto. Probed blots were developed using SuperSignal West Pico (Pierce), and images were recorded and analyzed using FluorChem 8800 (Alpha Innotech, San Leandro, CA). Blots were subsequently stripped and probed with HRP-conjugated anti-GST to verify loading.

For binding assays in which biotinylated peptides acted as bait, 40 µl of washed Neutravidin beads (Pierce) were loaded with 5 µM biotinylated peptides, washed, and then incubated with GST-SH2 fusion protein. Beads were washed five times in Nonidet P-40 lysis buffer, boiled for 5 min in SDS-PAGE sample buffer, run out on 10% SDS-PAGE, transferred to nitrocellulose, and probed with HRP-conjugated anti-GST to reveal bound GST-SH2. Probed blots were developed as described above.

Surface plasmon resonance

A Biacore 2000 (Biacore AB) was used to measure surface plasmon resonance. A capture system was used to attach the ligand (GST-SH2 fusions) indirectly to the surface of a CM5 chip via an anti-GST Ab. The GST Capture Kit (Biacore) was used according to the manufacturer's instructions. GST-SH2 fusion proteins were then captured onto the surface by injection at 5–10 µg/ml and 5 µl/min for 2–5 min to >2000 resonance units. No detectible off rate was observed for the captured GST-SH2 ligands. For analyte, pY-His-CD19-cyto was diluted in HBS-EP (Biacore) and injected at a flow rate of 5–20 µl/min at 20°C in various concentrations, and binding was monitored. The surface of the chip was regenerated by injection with 10 mM glycine, pH 2.2, for 1 min. A GST-only control flow cell was subtracted from all sensorgrams to remove bulk shift and nonspecific binding. Analysis was performed using BioEvaluation version 3.2. The Kd was calculated as koff/kon.

Immunoprecipitation and competition assay

Duadi cells (2 × 10^7/condition) were held in buffer or were treated with pervanadate solution (HBSS with 10 mM pervanadate, 10 µM H_2O_2, 0.1% BSA, 1 mM MgCl2, and 1 mM CaCl2), then solubilized with Nonidet P-40 lysis buffer and cleared by centrifugation as previously described (10). For CD19 coprecipitation assays, Lyn, PI3K, Vav, and PLCγ2 were immunoprecipitated by incubation of the lysate with the appropriate Ab, followed by UltraLink-immobilized protein A. Beads were washed with Nonidet P-40, and bound proteins were eluted by boiling in SDS-PAGE sample buffer. Eluates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were blocked with 3% milk or 2% BSA and probed with polyclonal Ab to CD19, followed by HRP-conjugated anti-rabbit Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized with SuperSignal West Dura (Pierce), then imaged and analyzed on FluorChem 8800. Blots were stripped and reprobed with anti-phosphotyrosine primary Ab, followed by HRP-conjugated anti-mouse Ab (Jackson ImmunoResearch Laboratories) and imaged as described above. Finally, blots were cut into pairs of lanes and probed with a mixture of Abs to Lyn and PI3K or to Vav and PLCγ2, followed by secondary Ab and imaged. For competition assays, CD19 was immunoprecipitated with ADF4.2 anti-hCD19 mAb, followed by UltraLink immobilized protein A beads (Pierce). Beads were washed once with Nonidet P-40 lysis buffer and then incubated with unstimulated Duadi lysates (2 × 10^7/condition) in the presence of 50 µg of GST only or fusions of GST and Grb2 SH2, PLCγ2 C-terminal SH2, or Vav SH2. Beads were washed, and precipitate was eluted by boiling in SDS-PAGE sample buffer. Immunoblots were prepared, probed, and imaged as described above. For determination of the fraction of maximum precipitation, the amount of precipitated protein in the absence of competitor, as measured by average pixel density of the appropriate band in the image, was assigned a value of 0. For all other conditions, the average pixel density of the appropriate band was normalized to this scale.

Structural modeling

Models were derived using Insight II software on a Silicon Graphics server linked to a OCTANE workstation (Silicon Graphics, Mountain View, CA). The Homology module automatically builds and refines a three-dimensional model of the TH domain of the CD19 tyrosine-kinase domain and the extracellular domain.
model based on comparison of the amino acid sequence of the protein by comparison with the known structures of related sequences in public databases, using standard techniques of backbone building and loop modeling. The Visualizer module was used for graphical display and output.

Repli
cations
Experiments were replicated at least three times.

Results
Direct interaction of CD19 with the SH2 domains of Grb2, p85, and Vav and the N-terminal SH2 domain of PLCγ2
After ligation of the BCR, CD19 becomes associated with PI3K, Lyn, Vav, PLCγ2, and Grb2 (7–9, 10, 11, 12, 22). Our previous studies with mutant forms of CD19, which contain phenylalanine substitutions for different tyrosines, demonstrated that specific CD19 tyrosines, once phosphorylated, are necessary and sufficient to form complexes with different cytoplasmic proteins, and that more than one cytoplasmic protein can be found associated with a specific CD19 phosphotyrosine. For example, PI3K and Lyn associate with CD19 Y482 and Y513, whereas Vav and PLCγ2 interact with CD19 Y391. Complexes could form at different tyrosines by either direct interaction of different proteins with a given CD19 phosphotyrosine or an initial binding of one cytoplasmic protein to the CD19 phosphotyrosine, and then a second interaction of another cytoplasmic protein with the first (indirect).

Fig. 1 confirms the multiple interactions involving CD19 by illustrating a reverse pulldown of CD19 in association with Lyn, PI3K, Vav, and PLCγ2 from cells that are either unstimulated or treated with pervanadate. The amount of associated CD19 with each of the cytosolic proteins increased after pervanadate treatment (Fig. 1A), but the pattern of recovery and the level of phosphorylation (Fig. 1B) vary. For example, the amount of CD19 associated with Lyn shows the greatest increase after stimulation (Fig. 1A), but little of the associated CD19 is phosphorylated (Fig. 1B). This is consistent with a model in which Lyn is recruited to a lightly phosphorylated CD19, in which one or a few tyrosines are phosphorylated, and this recruitment leads to increased phosphorylation of CD19 by Lyn, after which the association is terminated. In contrast, there is less CD19 associated with PLCγ2, but relatively more is phosphorylated. Recovery of Vav is reduced after activation, consistent with fractionation into compartments that are insoluble in detergents used to analyze coprecipitation (Fig. 1C). Further complicating interpretation is the appearance of additional phosphorylated bands in the precipitates in addition to the target cytosolic protein and CD19 (Fig. 1B, see particularly Vav and PLCγ2 lanes). Thus, such coprecipitation studies demonstrate the association of CD19 with multiple intracellular proteins, but cannot determine whether an interaction is direct or is mediated by intermediates, and, if direct, the strength of the interaction.

To determine which interactions are direct, binding assays were performed using bacterially expressed, purified fusion proteins. Only two proteins were present in the binding reactions: a GST fusion protein containing the SH2 domain of the cytoplasmic protein and the His-tagged CD19 cytoplasmic domain. The CD19 construct was produced in two forms: nonphosphorylated, in BL-21 bacteria (His-CD19-cyto), and in TKB1 bacteria, which coexpress elk, a promiscuous tyrosine kinase, resulting in tyrosine phosphorylation of the His-CD19-cyto fusion protein (pY-His-CD19-cyto). In contrast, serine/threonine phosphorylation was equivalent in the CD19 constructs expressed in the BL-21 and TKB1 bacteria (not shown).

Under the conditions used in this study for protein expression and for the binding assays, the purified proteins are competent to bind, and there is little nonspecific binding to beads or control constructs. To test functionality, preliminary assays were performed to determine the percentage of pY-His-CD19-cyto that is competent to bind. PhosphoY-His-CD19-cyto was incubated in
Bound proteins were resolved by SDS-PAGE and probed (CD19-cyto), or phosphorylated pY-His-CD19-cyto (pY-CD19-cyto). Proteins and incubated with buffer only, unphosphorylated His-CD19-cyto. Ne-Sepharose beads were preloaded with the indicated GST-SH2 fusion protein and incubated with buffer only, unphosphorylated His-CD19-cyto. GST-SH2 was incubated either in buffer only or with ProBond beads, to which a range of pY-His-CD19-cyto had been previously bound. The amount of unbound GST-p85 N-SH2 remaining in the supernatant after incubation and centrifugation was determined by immunoblotting. ProBond beads without absorbed pY-His-CD19-cyto depleted little of the GST-p85 dual-SH2 (Fig. 2, lanes 1 and 2), again demonstrating that there is little nonspecific interaction with particular CD19 tyrosines. Increasing amounts of preloaded pY-His-CD19-cyto on the beads resulted in a dose-dependent decrease in the amount of residual GST-p85 N-SH2 in the supernatant. At 10- and 100-fold excesses of pY-His-CD19-cyto, little or no unbound GST-p85 N-SH2 remained, demonstrating that GST-p85 N-SH2 is folded properly, such that it is competent to bind.

To determine which interactions were direct, glutathione-Sepharose beads were preloaded with the GST-SH2 fusion constructs of different cytoplasmic proteins and then incubated with His-CD19-cyto or pY-His-CD19-cyto. Absorbed proteins were eluted and analyzed by immunoblotting. Fig. 3 demonstrates that pY-His-CD19-cyto bound to the isolated SH2 domains of p85, PLCγ2, Grb2, and Vav. Combined with our previous studies demonstrating the specificity of these interactions with particular CD19 tyrosines, these results demonstrate that different signaling proteins can interact with a specific CD19 tyrosine. For example, both Vav and PLCγ2 require CD19 Y391, and both bind CD19 directly. Phospho-Y-His-CD19-cyto bound both SH2 domains of p85 individually, but only the C-terminal SH2 domain of PLCγ2. In addition, GST-p85 dual-SH2 and GST-Vav SH2 bound unphosphorylated His-CD19-cyto at a much lower level than pY-His-CD19-cyto, suggesting a lower affinity, constitutive interaction.

CD19-derived phosphopeptides can coprecipitate Lyn (7), but the mechanism of this interaction has not been determined. Lyn consists of an N-terminal unique region, an SH3, an SH2, and a kinase domain. Lyn can phosphorylate CD19-cytoplasmic domain constructs in vitro (7). As kinase domains are capable of binding their targets, we determined whether Lyn’s SH2 or kinase domain mediates its association with CD19. Binding assays were performed using GST-Lyn SH2 and GST-Lyn kinase domain, immobilized onto glutathione-Sepharose beads. Fig. 4A demonstrates that GST-Lyn SH2 can bind pY-His-CD19-cyto and, to a lesser extent, unphosphorylated His-CD19-cyto. However, GST-Lyn kinase is able to bind both unphosphorylated His-CD19-cyto and pY-His-CD19-cyto in approximately equal amounts. Thus, Lyn binds to CD19 by both the SH2 and kinase domains. To determine which CD19 tyrosine(s) may be involved in binding Lyn’s SH2 domain, biotinylated, tyrosine-phosphorylated peptides derived from CD19 corresponding to tyrosines Y482 and Y513, along with flanking sequence, were affixed to Neutravidin beads (Pierce) and then incubated with GST-Lyn SH2. As shown in Fig. 4B, CD19 Y513 phosphopeptide is able to coprecipitate GST-Lyn SH2, but CD19 Y482 phosphopeptide can bind only weakly. Both Y482 and Y513 phosphopeptides can coprecipitate GST-Lyn kinase. Thus, the CD19 cytoplasmic domain can interact with Lyn through two different mechanisms, via the Lyn kinase and SH2 domains, but these interactions differ. The Lyn kinase domain binds unphosphorylated and tyrosine-phosphorylated CD19 equivalently and interacts with CD19 Y482 and Y513 equivalently. The Lyn SH2
domain, like the SH2 domains of Vav and p85, binds the unphosphorylated CD19 cytoplasmic domain, but less strongly than to tyrosine-phosphorylated CD19, and interacts more strongly with CD19 Y513.

Surface plasmon resonance measurement of the interaction between SH2 domains and tyrosine-phosphorylated CD19
The binding assays demonstrate that the CD19 cytoplasmic domain can interact directly with several cytoplasmic proteins, some of which bind specifically to the same CD19 tyrosine(s). To understand these interactions, we need to know whether the different phosphotyrosine-SH2 interactions are of equivalent strength. In addition, our studies in vivo have suggested that CD19 tyrosines other than 482 and 513 are nonessential and/or redundant, raising the question of whether the binding interactions that involve the other CD19 tyrosines are significant. Therefore, we performed Biacore analyses to determine the kinetics of the interaction between GST fusions of the SH2 domains of Grb2, Vav, and p85 with pY-His-CD19-cyto. Most of the previous studies that used Biacore to analyze SH2-tyrosyl phosphoprotein interactions have used short peptides of 10–20 aa as ligand (affixed to solid surface within the flow cell) and a GST-SH2 fusion protein as analyte (fluid phase). An important reassessment of this experimental setup (23) suggested that prior studies had overestimated the affinity as measured by $K_d$, because of the propensity of GST to dimerize and that the reported $K_d$ actually represented a measure of avidity of the dimerized GST fusion. To avoid potential problems with GST dimerization, the GST-SH2 fusion proteins were fixed to the surface of CM5 chips (23). A GST capture system was chosen due to increased reproducibility and the ability to regenerate the chip surface. After capture, no dissociation of GST-SH2 fusion proteins from anti-GST Abs was detected (not shown). Correct interpretation of kinetics and affinity measurements depends on knowing the functional concentration of the analyte, in this case His-CD19-cyto or pY-His-CD19-cyto. Thus, experiments such as that illustrated in Fig. 2 were used to demonstrate that folding and phosphorylation were functional for binding. PhosphoY-His-CD19-cyto was injected across the surface of the chip at a flow rate of either 5 or 20 µl/min at 20°C. The chip surface was regenerated with a 1-min pulse of 10 mM glycine, pH 2.2. A GST-only flow cell was included as a control for bulk shift and nonspecific binding changes in resonance units and was subtracted from the experimental data before analysis (removing nonspecific binding affinity from the binding curves of the SH2 fusion constructs).

**Table 1.** Affinity constants of associations between pY-His-CD19-cyto and the SH2 domains of Grb2, p85, and Vav

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Analyte</th>
<th>$k_a$ ($10^5$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ ($10^{-3}$ s$^{-1}$)</th>
<th>$K_d$ ($10^{-9}$ M)</th>
<th>Fit Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-Grb2 SH2</td>
<td>pY-His-CD19-cyto</td>
<td>3.13 ± 0.77</td>
<td>1.42 ± 0.57</td>
<td>4.51 ± 1.62</td>
<td>Separate $k_a$ and $k_d$, 1:1</td>
</tr>
<tr>
<td>GST-p85 dual-SH2</td>
<td>pY-His-CD19-cyto</td>
<td>3.8 ± 1.71</td>
<td>1.18 ± 0.18</td>
<td>3.4 ± 1.0</td>
<td>Separate $k_a$ and $k_d$, 1:1</td>
</tr>
<tr>
<td>GST-Vav SH2</td>
<td>pY-His-CD19-cyto</td>
<td>3.1 ± 1.5</td>
<td>2.5 ± 0.86</td>
<td>8.8 ± 3.0</td>
<td>Simultaneous $k_a$ and $k_d$, 1:1</td>
</tr>
</tbody>
</table>

**FIGURE 5.** Representative Biacore sensorgrams of interactions between SH2 domains of Grb2, Vav, and p85 with pY-His-CD19-cyto. The indicated GST-SH2 fusions were captured onto the surface of CM5 chips via covalently attached anti-GST Abs. Various concentrations of pY-His-CD19-cyto were injected across the surface, and binding was monitored by surface plasmon resonance. Concentrations of pY-His-CD19-cyto injected were 720, 360, 180, 36, and 7.2 nM (A and B); 360, 90, 36, and 3.6 nM (C); and 360, 90, 36, 18, and 9 nM (D and E).
The interaction of the dual SH2 domain construct, GST-p85 dual-SH2, with pY-His-CD19-cyto had the highest affinity (Fig. 5C). Interestingly, the model used to fit the sensorgram data was a 1:1 Langmuir fit, suggesting that the interaction between p85’s dual SH2 domains and CD19 is concerted, with one binding and one dissociation event, indicating a single binding reaction ordered in one orientation. GST SH2 fusions of the individual N-terminal and C-terminal SH2 domains of p85 were able to bind pY-His-CD19-cyto (Fig. 5, D and E). However, these curves were inimical to analysis using a 1:1 Langmuir fit. The predicted binding motif for both SH2 domains of p85 is the same, YxxM (24), and CD19 tyrosines 482 and 513 both exhibit this motif. Most likely, each SH2 domain of p85 can bind either tyrosine 482 or 513 of CD19, but the two different interactions lead to non-first-order kinetics.

The Biacore sensorgrams of the Lyn SH2 and kinase domains showed clear evidence of binding by pY-His-CD19-cyto (Fig. 6A), but were resistant to modeling as a 1:1 fit. Previous reports and our current data (Fig. 4) demonstrate binding of Lyn to multiple CD19 phosphotyrosines. Although not definitive, we suspect that such multiple binding interferes with kinetic analysis. Biacore analysis calculates affinities by measuring the rates of binding and dissociation. The $K_a$ can also be measured by Scatchard analysis, and the necessary data (relative binding in arbitrary resonance units at equilibrium and concentration) are available from the Biacore analysis, with the caveat that full equilibrium is not achieved. Therefore, we compared Scatchard curves derived for the binding of the SH2 domains of p85 and Lyn, respectively, and found that the slopes were within an order of magnitude (Fig. 6B). Thus, although we cannot calculate a single affinity of binding of Lyn SH2 to pY-His-CD19-cyto, the average affinity appears comparable to that of p85. Finally, the analyses of binding of the C-terminal SH2 of PLCγ2 to pY-His-CD19-cyto and of Vav SH2 to unphosphorylated His-CD19-cyto (to model the constitutive association, discussed above) resulted in sensorgrams where binding was uninterpretable. This could represent either low affinity or technical difficulties (e.g., unstable baselines).

**Vav and PLCγ2 compete in binding CD19, but Grb2 does not**

Our previous studies have shown that both Vav and PLCγ2 bind CD19 Y391 (10, 12). The binding studies (Fig. 5) demonstrate that both the Vav SH2 and the PLCγ2 C-terminal SH2 independently bind CD19 directly. If so, then Vav and PLCγ2 would compete for binding of Y391. Additional binding studies were performed with native proteins to address two related questions: whether a protein that binds at particular phosphotyrosines competes with other proteins, and whether the affinity of PLCγ2 for binding CD19 is comparable to that of Vav. Native CD19 was immunoprecipitated from the lysates of Daudi cells that were stimulated with the phosphatase inhibitor, pervanadate, which induces strong phosphorylation of CD19. The phosphorylated CD19 was incubated with lysates of unstimulated Daudi cells, which serve as a source of the native, cytosolic binding partners. We have previously used this technique to amplify the detection of interactions (10). In the current experiments we also added GST only or GST fusions of Grb2 SH2, PLCγ2 C-SH2, or Vav SH2 to the mixture of phosphorylated CD19 and Daudi lysates. We measured the ability of these SH2 domains to compete with the native cytoplasmic proteins present in the unstimulated lysates for binding to CD19. The presence of either GST-PLCγ2 C-terminal SH2 or GST-Vav SH2 resulted in reduced coprecipitation of both PLCγ2 and Vav with CD19 (Fig. 7A). Thus, the two SH2 domain-containing proteins compete for binding to the same phosphotyrosine(s), defined previously as primarily CD19 Y391 (10). The recovery of Grb2 was not reduced in the presence of GST-Vav SH2 or PLCγ2 SH2 (Fig. 7A). However, the amount of coprecipitated native Grb2 was reduced in the presence of added GST-Grb2 SH2, demonstrating the expected competition (Fig. 7B). In contrast, the binding of GST-Grb2 to CD19, which is mediated by CD19 Y330, did not hinder the binding of Vav and PLCγ2, which interact with Y391. As an additional control, the SH2 of Syk, which is not known to bind CD19, did not interfere with the binding of Grb2, Vav, or PLCγ2 (not shown). Thus, different cytosolic proteins that bind the same CD19 tyrosine compete for binding with each other, but different CD19 tyrosines...
can bind other SH2 domains independently and without interference from occupancy of other tyrosines.

The Biacore experiments suggested that binding of PLCγ2 to the phosphorylated CD19 cytoplasmic domain was comparable to that of Vav, but the binding kinetics did not fit a first-order equation. Therefore, we compared the ability of the SH2 domains to compete for binding with the native cytosolic proteins. In these experiments we analyzed recovery of Vav in a two-step precipitation, described above, in the presence of a range of concentrations of GST fusions of Vav SH2, PLCγ2 C-terminal SH2, or Vav SH2. A, Precipitates were resolved by SDS-PAGE and sequentially probed for PLCγ2, Vav, and CD19. B, A parallel blot was probed for Grb2 and reprobed for CD19. C, CD19 precipitates were prepared as described in A with a range of concentrations of GST fusions of Vav SH2 ( ), PLCγ2 C-terminal SH2 ( ), or Grb2 SH2 ( ) and probed for recovery of Vav. The amount of Vav was measured by luminescence imaging and calculation of mean pixel density of the appropriate band. Vav recovery was normalized to a scale in which 1 represents the amount of Vav recovered in the absence of GST fusion constructs, and 0 represents the amount of Vav recovered in the presence of 2.5 μM GST-Vav-SH2. The vertical dotted lines indicate the intercept of the x-axis at 50% inhibition of recovery of Vav for GST-Vav-SH2 and GST-PLCγ2 C-terminal-SH2.

FIGURE 8. Structure of the human CD19 cytoplasmic domain predicted by short sequence homologies. Side chains of tyrosine residues with homologous flanking regions are indicated in the same color.

Discussion

In this study we provide confirmation of the interaction between CD19 and the downstream effectors Lyn, the p85 subunit of PI3K, Vav, and Grb2 and show that these interactions are all direct. In analyzing these interactions, we have found that several different types of interactions occur. The first type is that mediated by the Lyn kinase domain, which differed from that of SH2-mediated interactions in that it bound equally to the unphosphorylated and phosphorylated CD19 cytoplasmic domains. Secondly, SH2 domains can mediate an interaction with nonphosphorylated CD19. We have previously shown that Vav binds to CD19 constitutively, from unactivated cells, in immunoprecipitation studies. We now show that this interaction is direct and reconstituted by the SH2 domain alone, but, consistent with our previous results, appears to be of low affinity. Interactions of SH2 domains with unphosphorylated CD19 were also detected with Lyn and p85. Thus, there may be preassembly of components of the complex at the membrane in unstimulated cells, when phosphorytrosines are limited. After cells are activated, the SH2-mediated binding shifts to the third type of interaction, the high affinity interaction of SH2 domains with phosphorylated tyrosines. We previously demonstrated that the association of Vav with a form of CD19 lacking all cytoplasmic tyrosines was reduced when such cells were activated, suggesting a relocation of Vav to the phosphorytrosines induced on other molecules. Vav, Grb2, and Lyn each have a single SH2 domain that mediates the interaction with phosphorytrosines.

Both p85 and PLCγ2 have dual SH2 domains. Numerous inducible protein-protein interactions are mediated by tandem pY-SH2 interactions (25, 26). However, only one SH2 domain of PLCγ2 can bind CD19. This is consistent with the consensus target sequence of the SH2 domain binding partners, as determined by combinatorial library, which predict that the N-terminal and C-terminal SH2 domains of PLCγ2 have different preferences (24, 27). In contrast, the individual N-terminal and C-terminal SH2 domains of p85 are both able to bind pY-His-CD19-cyto, as shown by binding assays and Biacore (data not shown). This is consistent with the similar predicted consensus binding motif (YxxM) for each of the p85 SH2 domains (23). The Biacore binding and dissociation curves for the individual p85 SH2 domains failed analysis by a 1:1 Langmuir model, which would result if both were undergoing two binding reactions to the two different tyrosines.
More surprisingly, the Biacore sensorgrams that were generated by interaction of the GST fusion of the dual SH2 domains of p85 and pY-His-CD19 fit a 1:1 model of interaction despite the fact that the ligand has two SH2 domains and the analyte has two tyrosines that can bind either. The fact that this interaction was successfully analyzed using a 1:1 Langmuir model suggests that the binding and dissociation of the two p85 SH2 domains from/to CD19 occur as single events, implying that binding occurs in only one orientation. This would serve to orient the kinase subunit, p110, in relation to both the membrane and other associated proteins.

In Fig. 4, we show that GST fusions of Lyn SH2 as well as the Lyn kinase domain are independently able to bind the cytoplasmic region of CD19. Although the Lyn SH2 domain binds preferentially to phosphorylated tyrosine 513 of CD19, the Lyn kinase domain binds its potential substrates independently of the phosphorylation state. Lyn’s SH2 domain can also bind the C-terminal negative regulatory tyrosine of Lyn, an intramolecular pY-SH2 interaction that results in an inactive conformation typically relieved by dephosphorylation by CD45 (28). Using purified proteins, Src inactivity can be relieved by incubation with a synthetic phosphotyrosine-containing peptide with sequence derived from the inhibitory C-terminal tyrosine of Src, suggesting that inhibition is relieved by competition (29). By analogy, it is possible that CD19 can relieve Lyn repression by binding of CD19 tyrosine 513 with Lyn’s SH2 domain. These data provide a molecular basis for the observation by Fujimoto and colleagues (7) that interaction of Lyn with CD19 enhances Lyn kinase activity.

The competition studies suggest a model in which a single CD19 can bind multiple cytosolic signaling proteins. Binding of a signaling protein at one site (e.g., Grb2 to CD19 Y330) does not interfere with binding of a different protein at another site (e.g., Vav or PLCγ2 at Y391; p85 or Lyn at Y482/Y513). However, different proteins that bind the same tyrosine do compete for binding, and the data do not support the concept of higher order complexes at a given tyrosine, at least for the proteins known to bind CD19. Thus, CD19 can form multicomponent complexes, but the composition of those complexes will be determined by the local concentration of the available, SH2-containing signaling proteins, which will be altered by such factors as the sequestration in or out of lipid microdomains or the presence of phosphotyrosines on other nearby proteins. Understanding the roles of these interactions in the complex regulation of the enzymatic activity of Vav and PLCγ2 will be an important area for future research. However, binding interactions may have other important effects, including functions as adapter proteins through other binding domains and regulation of phosphorylation. Indeed, we have shown that coligation of CD19 and the BCR significantly augments phosphorylation of the total cellular pool of binding proteins, such as Vav and PI3K. In contrast, ligation of CD19 concurrently with, but independently of, the BCR, inhibits B cell activation. Under this condition, binding of cytosolic signaling proteins to CD19 may function as a negative sink for proteins that would otherwise contribute to positive signaling.

We have examined the structure of the CD19 cytoplasmic domain. Although the material produced is of high functional purity (Fig. 2), the yield of purified protein by these systems has been insufficient for crystallization or nuclear magnetic resonance analysis. Taken as a whole, the CD19 cytoplasmic domain has no homologous proteins to permit structural comparisons. However, comparison modeling with shorter fragments of homology from other proteins of known structure permits derivation of a preliminary suggestion. Using the Insight II system, a model of the cytoplasmic portion of CD19 (beginning at methionine, residue 308, through the stop codon) was derived on an automated basis. The results were visualized as the linear backbone plus the side chains of tyrosines (Fig. 8). Tyrosines with homologous flanking residues are shown in the same color. The model predicts a globular structure, which would be close to the membrane, with all but one tyrosine (Y490) predicted to be exposed on the surface of the molecule. One can picture different facets of this structure that would serve to bind the different SH2-containing cytoplasmic proteins. Combined with the competition data, this suggests that different proteins can bind to Y482/Y513 (p85 or Lyn) on the upper right facet (as pictured in Fig. 8), Y391/Y421 (Vav or PLCγ2) on the bottom, Y330/Y360 (Grb2, others?) on the right and rear, and Y403/Y443 (Fyn, Lyn, others?) to the left. All these would be in close proximity to the membrane.

In summary, these observations demonstrate that the interactions of different signaling proteins with different CD19 tyrosines are direct, high affinity, and competitive. These data imply that the interactions with tyrosines whose function was not apparent in mutation analysis studies in vivo are nevertheless likely to mediate signaling. This is consistent with the high level of conservation of the apparently nonessential tyrosines, such as CD19 Y391 (30), and our previous demonstration of important roles for CD19 tyrosines Y330, 391, and 421 in studies of signal transduction in cell lines (12, 13). This suggests that either the crucial function has not been tested or that there is redundancy, either within CD19 or with other proteins. Finally, combined with our preliminary model (Fig. 8), our work suggests a tight, multimeric cluster of signaling proteins in close proximity to each other and the membrane.

Acknowledgments
We thank Dr. Christie Broulliette for providing access to the Biacore 2000, and Drs. Amnon Altman, Osamo Miura, Tony Pawson, Gordon Mills, and Stephen P. Watson for providing pGex SH2 constructs.

References


