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Essential Roles for Dok2 and RasGAP in CD200 Receptor-Mediated Regulation of Human Myeloid Cells

Robin Mihrshahi, A. Neil Barclay, and Marion H. Brown

The CD200 receptor (CD200R) acts as a negative regulator of myeloid cells by interacting with its widely expressed ligand CD200. Using mutants expressed in U937 cells, we show that inhibition is mediated by the PTB domain binding motif (NPLY) in the receptor’s cytoplasmic region. The adaptor protein downstream of tyrosine kinase 2 (Dok2) bound directly to the phosphorylated NPLY motif with a 10-fold higher affinity (K_d of ~1 μM at 37°C) than the closely related Dok1. Both of these proteins have been suggested to play a role in CD200R signaling in murine cells. Dok2 was phosphorylated in response to CD200R engagement and recruited RAS p21 protein activator 1 (RasGAP). Knockdown of Dok2 and RasGAP by RNA interference revealed that these proteins are required for CD200R signaling, while knockdown of Dok1 and the inositol 5-phosphatase SHIP did not affect CD200R-mediated inhibition. We conclude that CD200R inhibits the activation of human myeloid cells through direct recruitment of Dok2 and subsequent activation of RasGAP, which distinguishes this receptor from the majority of inhibitory receptors that utilize ITIMs and recruit phosphatases. The Journal of Immunology, 2009, 183: 4879–4886.

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3 Abbreviations used in this paper: CD200R, CD200 receptor; Dok, downstream of tyrosine kinase; PTB, phosphotyrosine binding; RasGAP, RAS p21 protein activator 1; RU, response unit; SH2, SRC homology 2; SHP, SRC homology 2 domain-containing phosphatase; siRNA, small interfering RNA; SPR, surface plasmon resonance.

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Materials and Methods

Antibodies

Polyclonal goat (sc-8130) and rabbit anti-human Dok2 (sc-13952), monoclonal mouse anti-human RasGAP (sc-63), and monoclonal mouse anti-human SHIP (sc-8425) Abs were from Santa Cruz Biotechnology. A polyclonal rabbit anti-human Dok1 Ab (25) was a gift from D. Davidson and A. Veillette. The monoclonal mouse anti-human CD200R Ab OX108 has been described previously (2). Biotinylated mouse monoclonal anti-phosphotyrosine Ab (B1531) and peroxidase-conjugated polyclonal anti-mouse, anti-rabbit, and anti-goat Abs and ExtrAvidin were from Sigma-Aldrich. PE-conjugated donkey anti-mouse IgG Fab’2 fragment (715-116-151) was from Jackson ImmunoResearch Laboratories.
CD200-COMP

Pentameric human CD200 (CD200-COMP) consisting of the extracellular region of human CD200 (2) linked to domains 3 and 4 of rat CD4 followed by an 11-aa linker sequence (NSGGSGGGGGT) and the rat CD4 (car-tilage oligomeric matrix protein) oligomerization domain was generated as previously described (26). 293T cells were transiently transfected with pEF-BOS vector containing the CD200-COMP construct, and tissue culture supernatant was collected, concentrated, and dialyzed into PBS. Protein activity was tested by surface plasmon resonance (SPR) on a Biacore 3000, which showed strong binding to recombinant human CD200R with binding characteristics similar to those of OX108 mAb (26). Titrations of the concentrate were used in IL-8 assays with CD200R-transduced U937 cells as described below to determine optimal working dilutions.

Generation of CD200R mutant cell lines

Mutants of human CD200R were generated by overlap extension PCR mutagenesis. Fragments were amplified from the wild-type gene using terminal and internal primers, with the N-terminal primer introducing a BglII restriction site and the internal primers overlapping and containing a single base change (A to T) to change a tyrosine to a phenylalanine codon of each of Y291, Y294, and Y302, referred to hereafter as Y1, Y2, and Y3. To generate a truncated mutant of the receptor, a stop codon followed by a Sall restriction site was inserted, resulting in the removal of the last 40 residues (aa 286–325) of the cytoplasmic tail of human CD200R. The resulting PCR products were digested with BglII and Sall and cloned into a BamHI and Xhol cut bicistronic, embryonic (Emn) enhanced GFP-expressing pHIR-SIN-BX-IREs-Emn lentiviral expression vector. The constructs were transfected into 293T cells at 60% confluence in 175-cm² culture flasks. Fifteen micrograms of expression vector, 20 ng/ml recombinant human IL-4 (PeproTech), alternately activated by culturing for an additional 2 days in the presence of 10 ng/ml recombinant human IFN-γ (Pharmingen). Monocytes (>90% pure) were isolated from healthy donor buffy coats (Bristol Blood Donor Services) by two-step centrifugation over Ficoll and 46% Percoll gradients (27) and differentiated into macrophages for 7 days on 40% Percoll gradients (27) and differentiated into macrophages for 7 days on 40% Percoll gradients (27). 293T cells were transiently transfected with the indicated Abs as described.

Cytokine assays

For IL-8 assays using CD200R-transduced U937 cells, OCT108 mAb diluted at the indicated concentrations in PBS was immobilized overnight at 4°C to wells of a 96-well tissue culture plate. The plate was then blocked with PBS/5% FCS at room temperature for at least 2 h, followed by addition of 5 × 10⁵ U937 cells per well. For experiments using soluble OCT108 or pentameric human CD200 (CD200-COMP), these reagents were added at the indicated concentrations to wells containing 5 × 10⁵ U937 cells. In experiments using Maja cells, a CD200-positive human B cell line, to engage CD200R on the surface of U937 cells, 4 × 10⁵ Maja cells that had been irradiated at 30 Gy were added to 10⁵ U937 cells in the wells of a 96-well tissue culture plate. After 30 min incubation on ice, LPS, ethanol-killed Neisseria meningitides cells (28), or IFN-γ were added at the indicated concentrations to stimulate IL-8 production. The total volume of media was 200 μl per well. After overnight incubation at 37°C, supernatants were collected and assayed for the presence of IL-8 by ELISA (BD Pharmingen).

RNA interference

Small interfering RNA (siRNA) duplexes containing two thymidine 3’ overhangs were purchased from Sigma-Aldrich (siDok1, siDok2, siSHIP, and siSer) and NBS Biologicals (siRasGAP and siNeg). The sequences were: 5’-GAAUCCUGCAACCCGGCUAACA-3’ for siDok1, 5’-GGUCAG GUUUCUUUUGCGAG-3’ for siDok1, 5’-GCAAGGUGCUUUCGAA

Table I. Peptides used in Biacore experimentsa

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD200R pY0</td>
<td>Biotin-DEQPYASYTEKNNPYPYDTTN</td>
</tr>
<tr>
<td>CD200R pY1</td>
<td>Biotin-DEQPY(pY)ASYTEKNNNYPYDTTN</td>
</tr>
<tr>
<td>CD200R pY1.2</td>
<td>Biotin-DEQPY(pY)ASYTEKNN(LY)PYPYDTTN</td>
</tr>
<tr>
<td>CD200R pY1.3</td>
<td>Biotin-DEQPY(pY)ASYTEKNNNLYPYPYDTTN</td>
</tr>
<tr>
<td>Dok1 pY146</td>
<td>Biotin-EMEENSL(pY)EPTW</td>
</tr>
<tr>
<td>Dok1 pY159</td>
<td>Biotin-DEQPYPYASYTEKNNPYPYDTTN</td>
</tr>
<tr>
<td>SHIP pY197</td>
<td>Biotin-TTEINPNL(pY)NQVGP</td>
</tr>
<tr>
<td>SHIP pY102</td>
<td>Biotin-EMFENFL(pY)OSLIES</td>
</tr>
</tbody>
</table>

a pY indicates phosphotyrosine.

Immunoprecipitations

U937 cells expressing wild-type or truncated CD200R were preincubated on ice for 30 min at ~1.5 × 10⁵ cells/ml in the presence of CD200-COMP concentrate diluted 1/100 in RPMI 1640. Cells were then warmed to 37°C for 5 min, washed in ice-cold PBS containing 1 mM sodium pervanadate, and lysed for 10 min at 4°C. Lysates were cleared by centrifugation at 16,000 × g for 10 min at 4°C and equal amounts of protein (as determined by Bradford assay) were resolved under nonreducing conditions on NuPAGE Bis-Tris gradient (4–12%) gels (Invitrogen). Proteins were transferred to nitrocellulose membranes in a Novex XCell II blot module and Western blotted using the SNAP i.d. protein detection system (Millipore) to determine the effect of RNA interference on protein expression.

Biacore analysis

Recombinant human Dok1 PTB domain (aa 140–268) and Dok2 PTB domain (aa 133–259) were provided by L. Bird (Oxford Modulc Consortium; www.omicsox.ac.uk). SPR analyses using a Biacore 3000 were conducted essentially as described previously (31). In brief, SPR response units (RU) of streptavidin were immobilized at 25°C to CMS chips by amine coupling followed by immobilization of 50–150 RU of biotinylated peptides (Peptide Protein Research and Sigma-Aldrich). Flow cells with non-phosphorylated peptides or with streptavidin only were used as controls. Increasing concentrations of monomeric, recombinant, soluble protein were then passed over the chip at 37°C to determine equilibrium binding. Response units from empty control flow cells were subtracted from those of experimental flow cells and the resulting data points were plotted and fitted to hyperbolas. Sequences of peptides from human proteins used in Biacore experiments are shown in Table I.
Results

Generation of cell lines expressing mutant and wild-type human CD200R

To generate a cellular system in which to dissect the signaling pathway of human CD200R, we used lentiviral transduction to create stable lines of U937 cells expressing wild-type or mutant (cytoplasmic domain truncated or with each of its three tyrosines mutated to phenylalanines (Y1F, Y2F, Y3F)) human CD200R. Expression of wild-type and mutant CD200R is shown after magnetic (MACS) beads separation using OX108 mAb. Cells were stained with OX108 followed by PE-conjugated anti-mouse IgG F(ab\(^\prime\))\(_2\).

CD200R engagement causes inhibition of IL-8 secretion from activated U937 cells

One previous study showed that ligation of CD200R on transfected U937 cells inhibits the secretion of various proinflammatory mediators including the neutrophil-chemoattractant IL-8 (CXCL8) (8). We confirmed this finding using U937 cells expressing wild-type and truncated human CD200R (Fig. 2). Generation of cells expressing a signaling-deficient form of CD200R allows for the use of the same extracellular conditions in both samples and controls. This eliminates potential artifacts caused by different degrees of cell-cell contact or differences in Fc-receptor stimulation by Abs. The inhibitory effect we observed did not depend on the method of receptor ligation; plate-bound or soluble mAb with or without secondary cross-linking Ab, soluble multimeric CD200 or CD200-expressing cells (Fig. 2A), or the type of activation stimulus; LPS, IFN-γ, or ethanol-killed N. meningitides (Fig. 2B). U937 cells express no or minimal levels of CD200 and cells were maintained at densities that minimized cell contact. The inhibition caused by soluble reagents (CD200-COMP and OX108), which can block the CD200-CD200R interaction (data not shown and M. Foster-Cuevas and A. Neil Barclay, unpublished observations) is, therefore, not due to blocking the engagement of CD200R by endogenously expressed CD200, but rather represents an agonistic effect. Cross-linking with OX108 mAb thus mimics the effect of natural ligand engagement in our assays.

The effect of CD200R ligation on other cytokines was also investigated. LPS stimulation of CD200R-transduced U937 cells did not elicit TNF and IL-1β secretion. IL-10 secretion was detectable and, similar to IL-8, was inhibited by soluble or plate-bound OX108 or CD200-COMP (data not shown). However, the effect on IL-10 was more variable and less pronounced than that on IL-8 secretion.

CD200R signaling is dependent on its third intracellular tyrosine residue

To determine the relative contribution of each of the three cytoplasmic tyrosine residues of human CD200R to signaling, we incubated U937 cells expressing wild-type or mutant (truncated, CD200R mutants are expressed on transduced U937 cells. U937 cells were lentivirally transduced to express wild-type (Wt) or signaling-deficient (cytoplasmic domain truncated (Tr) or with each of its three tyrosines mutated to phenylalanines (Y1F, Y2F, Y3F)) human CD200R. Expression of wild-type and mutant CD200R is shown after magnetic (MACS) beads separation using OX108 mAb. Cells were stained with OX108 followed by PE-conjugated anti-mouse IgG F(ab\(^\prime\))\(_2\).

FIGURE 1. CD200R mutants are expressed on transduced U937 cells. U937 cells were lentivirally transduced to express wild-type (Wt) or signaling-deficient (cytoplasmic domain truncated (Tr) or with each of its three tyrosines mutated to phenylalanines (Y1F, Y2F, Y3F)) human CD200R. Expression of wild-type and mutant CD200R is shown after magnetic (MACS) beads separation using OX108 mAb. Cells were stained with OX108 followed by PE-conjugated anti-mouse IgG F(ab\(^\prime\))\(_2\).

FIGURE 2. CD200R engagement inhibits activation of U937 cells. A, Wild-type (black bars) and truncated (gray bars) CD200R were engaged on U937 cells using different reagents as indicated. Plate-bound OX108 or isotype control were immobilized at 40 μg/ml, soluble OX108 was used at 10 μg/ml, and cross-linking anti-mouse IgG was used at 20 μg/ml. Cells were then stimulated overnight with 20 ng/ml LPS and culture supernatants assayed for IL-8 by ELISA. B, Wild-type or truncated CD200R was engaged on U937 using 100 μg/ml plate-bound OX108 mAb and cells were stimulated overnight using different reagents as indicated. Neisseria indicates ethanol-killed N. meningitides diluted in culture medium. *, p < 0.05 according to two-tailed Student’s t test; **, p < 0.005, N.S., nonsignificant. Results are expressed as means of duplicate or triplicate wells ± SD and are representative of two or more independent experiments.
Y1F, Y2F, and Y3F) CD200R in the presence of plate-bound OX108 mAb and 20 ng/ml LPS. IL-8 secretion was strongly inhibited in the presence of wild-type CD200R, while the Y3F mutation completely abrogated this effect. The Y1F mutation relieved inhibition only slightly, while the Y2F mutation had no functional effect (Fig. 3). Taken together, these results show that CD200R mediates active inhibitory signaling, which is dependent on its third intracellular tyrosine residue. The first tyrosine plays a minor role and the second one is dispensable in this assay.

The third intracellular tyrosine residue of CD200R binds Dok2 with higher affinity than Dok1

Previous studies suggest that the cytoplasmic domain of murine CD200R, when phosphorylated, can recruit the adaptor molecules Dok1 and Dok2 (23, 24). We used SPR to determine whether these interactions occur in human cells and whether they are direct or indirect. We measured direct binding of both Dok proteins to phosphorylated peptides corresponding to parts of the human CD200R cytoplasmic domain (Fig. 4). Dok2 and Dok1 both bound to two phosphopeptides (pY3 and pY1,3), but Dok2 bound with a 10-fold higher affinity ($K_D$ of $\sim$1 μM at 37°C; Fig. 4B) than Dok1 (Fig. 4C). The difference in binding affinity between Dok1 and Dok2 was not due to differences in protein activity, as Dok1 bound more

![Figure 3](http://www.jimmunol.org/) The inhibitory effect of CD200R is mainly mediated by its third intracellular tyrosine. CD200R (wild-type and mutants) was engaged on U937 cells using 100 μg/ml plate-bound OX108 mAb and stimulated and assayed as in Fig. 2. *, $p < 0.05$; ***, $p < 0.0005$; N.S., nonsignificant. Results are expressed as means of triplicate wells ± SD and are representative of six experiments. No significant effects of tyrosine mutations were observed in the absence of OX108 mAb or in the presence of an isotype control mAb (data not shown).

![Figure 4](http://www.jimmunol.org/) Dok2 binds the cytoplasmic tail of CD200R with higher affinity than Dok1. Singly (pY3) or doubly (pY1,3) phosphorylated peptides corresponding to parts of the cytoplasmic tail of human CD200R were immobilized on a BIACore chip. A, Binding of various concentrations (μM) of recombinant PTB domain of human Dok2 passed over flow cells containing immobilized unphosphorylated (pY0) control or pY1,3 phosphopeptide. B and C, Equilibrium binding values at each concentration for Dok2 (B) and Dok1 (C) binding to pY3 and pY1,3 phosphopeptides. The hyperbolas represent best fits used for affinity calculations. Results are representative of two independent experiments.
strongly than Dok2 to several other peptides (Table II and data not shown). The interaction between CD200R and Dok2 was dependent on phosphorylation of the functionally most important third tyrosine residue. Neither Dok1 nor Dok2 bound strongly to the first or second tyrosine when these were phosphorylated alone or in combination (Table II), although cophosphorylation of the first tyrosine caused a slight increase in affinity compared with phosphorylation of the third tyrosine alone (Fig. 2).

**Hierarchy of interactions suggests indirect recruitment of Dok1 and SHIP**

Dok1 and Dok2 have been reported to form phosphorylation-dependent homo- and heterodimers resulting from an interaction between their PTB domains and Tyr \(^{146}\) of Dok1 or Tyr \(^{139}\) of Dok2 (32, 33). To investigate the possibility of indirect recruitment of Dok1 to the CD200R signaling complex, we measured interactions between both Dok proteins and peptides corresponding to sequences surrounding these tyrosines. Dok2 did not bind to either of the two peptides. The PTB domain of Dok1, on the other hand, was found to interact with both peptides, but with a slight preference for the Dok2-derived sequence (Table II). This suggests that Dok1 may be recruited to CD200R indirectly via Dok2.

Dok1 has been shown to interact directly with the inositol phosphatase SHIP, and this interaction was dependent on both the PTB domain of Dok1 and the SRC homology 2 (SH2) domain of SHIP (34). The C-terminal part of SHIP contains two NPxY motifs that are likely to be involved in the formation of this complex. We measured interactions between Dok proteins and phosphopeptides corresponding to sequences surrounding these NPxY motifs. Dok2 did not bind to the sequence surrounding Tyr \(^{1022}\) and only very weakly (\(K_d\) of \(>30\) \(\mu\)M) to the sequence surrounding Tyr \(^{917}\) of SHIP. The PTB domain of Dok1 bound both of the SHIP NPxY motifs, although this interaction was not particularly strong (Table II). Sequence analysis of Dok1 and Dok2 in the context of a published SHIP SH2 domain consensus sequence (35) suggests that SHIP is more likely to bind to Dok1 (especially Tyr \(^{377}\) and Tyr \(^{398}\)) than to Dok2.

**Engagement of CD200R on U937 cells causes phosphorylation of Dok2 and recruitment of RasGAP**

As recruitment of downstream effector molecules via Dok2 depends on phosphorylation of its C-terminal tail (36), we tested whether CD200R engagement results in phosphorylation of Dok2 in human myeloid cells. Wild-type or truncated CD200R was engaged on the surface of U937 cells using soluble CD200-COMP, followed by lysis and immunoprecipitation of Dok2. Analysis of precipitates by Western blot showed that engagement of wild-type but not truncated CD200R caused phosphorylation of Dok2 (Fig. 5A).

Concomitant with phosphorylation of Dok2, engagement of CD200R resulted in recruitment of RasGAP (Fig. 5A). Phosphorylation of Dok2 and recruitment of RasGAP were dependent on signaling by the intact cytoplasmic region of CD200R. The levels of SHIP coprecipitated with Dok2 from lysates of cells expressing wild-type or truncated CD200R were the same (Fig. 5A), suggesting that SHIP coprecipitation was nonspecific. Lack of a specific effect of CD200R on SHIP activity was confirmed by immunoprecipitation of SHIP (Fig. 5B), which showed no difference in SHIP phosphorylation or its interaction with other phosphoproteins in response to CD200R engagement (Fig. 5B).

To determine whether Dok2 and RasGAP are also involved in CD200R signaling in primary human cells, Dok2 was immunoprecipitated from IL-4–activated macrophages that had been treated with CD200R mAb (OX108) or isotype control (OX21). Treatment of macrophages with OX108 resulted in phosphorylation of Dok2 and recruitment and phosphorylation of RasGAP. RasGAP also coprecipitated with Dok2 in lysates of OX21-treated cells, but phosphorylation of Dok2 and RasGAP was substantially stronger with OX108 treatment (Fig. 5C). No phosphoproteins corresponding to the molecular mass of Dok1 or SHIP were detectable (data not shown).

### Table II. Dissociation constants of interactions between Dok PTB domains and biotinylated peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Dok1 PTB ((\mu)M)</th>
<th>Dok2 PTB ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD200R pY0</td>
<td>N.B.</td>
<td>N.B.</td>
</tr>
<tr>
<td>CD200R pY1</td>
<td>14</td>
<td>N.B.</td>
</tr>
<tr>
<td>CD200R pY1,2</td>
<td>8.4</td>
<td>N.B.</td>
</tr>
<tr>
<td>CD200R pY3</td>
<td>7.3</td>
<td>1.4</td>
</tr>
<tr>
<td>CD200R pY1,3</td>
<td>6.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Dok1 pY146</td>
<td>6.9</td>
<td>N.B.</td>
</tr>
<tr>
<td>Dok2 pY139</td>
<td>3.3</td>
<td>N.B.</td>
</tr>
<tr>
<td>SHIP pY197</td>
<td>13</td>
<td>(&gt;30)^b</td>
</tr>
<tr>
<td>SHIP pY1022</td>
<td>13</td>
<td>N.B.</td>
</tr>
</tbody>
</table>

\(^{a}\) N.B. indicates no binding (\(K_d\) of \(>50\) \(\mu\)M or no specific interaction detectable). Results are representative of at least two experiments.

\(^{b}\) Interaction was dependent on high peptide density and was not observed under standard conditions.
Dok2 and RasGAP are essential for CD200R signaling

Our functional analysis of mutant receptors and SPR binding data indicate that Dok2 binding to phosphorylated Y3 and recruitment of RasGAP are essential for signaling by CD200R, while Dok1 and SHIP are less important. To test this, we used RNA interference to knock down expression of Dok1, Dok2, SHIP, and RasGAP in CD200R-transduced U937 cells and assess the effects on CD200R signaling. We compared the effects of the RNA interference on CD200R mAb (OX108)–induced CD200R signaling in cells expressing wild-type and truncated receptor (Fig. 6). Knockdown of Dok2 completely abrogated CD200R-mediated inhibition of IL-8 secretion from LPS-stimulated U937 cells (Fig. 6A). In contrast, knockdown of Dok1 did not affect CD200R signaling in this assay (Fig. 6B).

Knockdown of RasGAP also abrogated CD200R signaling consistent with this being an effector enzyme recruited by CD200R via Dok2. Knockdown of SHIP, on the other hand, had no specific effect on CD200R-mediated inhibition (Fig. 6C), although it increased overall IL-8 production by ~30–50% (data not shown). Thus, our results suggest essential roles for Dok2 and RasGAP, but not Dok1 and SHIP, in human CD200R signaling.
**Discussion**

The CD200R mediates inhibitory signals but is distinguished from the large number of other leukocyte inhibitory receptors that function by recruiting phosphatases through ITIMs (20). We show that the membrane distal tyrosine in CD200R is essential for CD200R-mediated inhibition of IL-8 secretion in U937 cells and that a phosphotyrosine peptide from this region binds the adaptor Dok2 through its PTB domain. The affinity of Dok2 for this peptide ($K_D$ of $\sim 1 \mu M$ at 37°C) is within the range of other functional interactions between phosphotyrosine peptides derived from cell surface receptors and cytoplasmic adaptor or signaling proteins that have been measured at physiological temperature (31, 37). It is about 10-fold weaker than that reported for the PTB domain of Src homology 2 domain-containing transforming protein (Shc), but these measurements were made at lower temperatures (38), and Shc does not bind strongly to CD200R (Ref. 21 and our unpublished observations). The direct interaction between CD200R and Dok2 is thus likely to be crucial in initiating signaling, and its involvement is confirmed by our RNA interference experiments (Fig. 6).

Studies on murine CD200R suggested that Dok1 and Dok2 are involved in CD200R signaling, as both were precipitated from mouse mast cells using phosphopeptides corresponding to sequences in the cytoplasmic domain of murine CD200R and were phosphorylated and coprecipitated CD200R in response to receptor engagement (23). We also observed phosphorylation of Dok2 (Fig. 5A) and Dok1 (unpublished data) in response to CD200R engagement in human cells. However, we found that Dok1 bound much more weakly to CD200R peptides than Dok2, and RNA interference of Dok1 expression had no effect on CD200R signaling in our assays (Fig. 6). Dok1 and Dok2 form phosphorylation-dependent homo- and heterodimers (32, 33), and our BLAcore experiments show that Dok1 has a 2-fold higher affinity for phosphorylated Dok2 than for CD200R. Dok1 is thus more likely to be recruited to CD200R via Dok2 than by direct association with CD200R.

The first two intracellular tyrosine residues of CD200R are also conserved between human and mouse. An inhibitory role for the phosphorylated membrane proximal tyrosine demonstrated in mouse CD200R signaling (24) was substantiated in our human model. The contribution of Y1 to CD200R signaling was greater in mouse cells (24) than we observed in our human system. This may either be due to species differences or to variations in assay sensitivity. Neither Dok1 nor Dok2 bound strongly to CD200R phosphotyrosine residues at 37°C; Table II), but binding of the SHIP SH2 domain to phosphorylated NPXY motifs in SHIP is rather weak ($K_D$ of $\sim 13 \mu M$ at 37°C; Table II), and our BLAcore experiments show that Dok1 and Dok2 can recruit SH2 domain-containing proteins, most notably RasGAP (42, 43). RasGAP recruitment is essential for the ability of Dok1/2 to inhibit Ras-ERK signaling (32, 36). This interaction involves five tyrosines in Dok1 (44) and at least two tyrosines in Dok2 (36), indicating that RasGAP recruitment is the primary function of these adaptor proteins. RasGAP is one of several Ras GTPase-activating proteins and is an important negative regulator of the Ras-ERK and PI3K signaling pathways (summarized in Ref. 45). The functional significance of these pathways can be inferred from the observation that mutations or mis-expressions of Ras are found in $\sim 30\%$ of all human cancers (46). Our immunoprecipitation and RNA interference experiments suggest that recruitment of RasGAP is the primary mechanism by which Dok2 affects cellular activation in response to CD200R engagement in human myeloid cells.

The inositol phosphatase SHIP has been shown to be phosphorylated and bind Dok1 in response to CD200R signaling in mouse mast cells (23). We did not observe any effect of CD200R engagement on SHIP phosphorylation or its interaction with other phosphoproteins in our human system. Moreover, knockdown of SHIP had no effect on CD200R signaling in our RNA interference experiments. In agreement with studies in mouse cells (23), BLAcore experiments (Table II) and the SHIP SH2 domain consensus sequence (35) suggest that SHIP is more likely to interact with Dok1 than with Dok2. Neither Dok1 nor SHIP, however, was found to play an important role in CD200R-mediated inhibition of IL-8 secretion in our assays. The interaction between Dok1 and SHIP has been shown to be dependent on both the PTB domain of Dok1 and the SH2 domain of SHIP (34). Quantitative binding analyses show that the affinity of the PTB domain of Dok1 for two phosphorylated NPXY motifs in SHIP is rather weak ($K_D$ of $\sim 13 \mu M$ at 37°C; Table II), but binding of the SHIP SH2 domain to phosphorylated tyrosine residues in Dok1 may stabilize this complex. SHIP is thus unlikely to bind to Dok1 if the latter’s PTB domain is already occupied, but it may form complexes with phosphorylated free Dok1.

Our results show that CD200R signals through a pathway involving recruitment and activation of RasGAP by the adaptor molecule Dok2. This pathway is novel in so far as it causes cellular inhibition independent of phosphatases and because it uses Dok2 as a primary signal transducer rather than a modulator of other pathways.

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**Disclosures**

The authors have no financial conflicts of interest.

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