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Downstream of Tyrosine Kinase 1 and 2 Play Opposing Roles in CD200 Receptor Signaling

Robin Mihrshahi and Marion H. Brown

The CD200 receptor (CD200R) negatively regulates myeloid cells by interacting with its widely expressed ligand CD200. CD200R signals through a unique inhibitory pathway involving a direct interaction with the adaptor protein downstream of tyrosine kinase 2 (Dok2) and the subsequent recruitment and activation of Ras GTPase-activating protein (RasGAP). Ligand engagement of CD200R also results in tyrosine phosphorylation of Dok1, but this protein is not essential for inhibitory CD200R signaling in human myeloid cells. In this paper, we show that CD200R-induced phosphorylation of Dok2 precedes phosphorylation of Dok1, and that Dok2 and Dok1 recruit different downstream proteins. Compared with Dok2, Dok1 recruits substantially less RasGAP. In addition to binding RasGAP, Dok2 recruits the adaptor molecule Nck in response to ligand engagement of CD200R. CD200R-induced phosphorylation of Dok1 results in the recruitment of CT10 sarcoma oncogene cellular homologue-like (CrkL), whereas the closely related CT10 sarcoma oncogene cellular homologue interacts constitutively with Dok1. Knockdown of Dok1 or CrkL expression in U937 cells resulted in increased Dok2 phosphorylation and RasGAP recruitment to Dok2. These data are consistent with a model in which Dok1 negatively regulates Dok2-mediated CD200R signaling through the recruitment of CrkL.


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This paper is dedicated to Dominique Davidson and André Veillette.

CD200R ligation also causes phosphorylation of the closely related Dok1 (22–24), but unlike Dok2, this protein is not essential for inhibitory CD200R signaling in human myeloid cells (22).

We now provide evidence of a regulatory role for Dok1 in CD200R signaling by analyzing the kinetics of phosphorylation of Dok2 and Dok1, and characterizing differences in their interactions downstream of CD200R. Compared with Dok2, CD200R-induced phosphorylation of Dok1 was delayed. RasGAP and the adaptor protein Nck were preferentially associated with Dok2 and the closely related adaptor proteins CT10 sarcoma oncogene cellular homologue (Crk) and Crk-like (CrkL) with Dok1. Knockdown of either CrkL or Dok1 resulted in enhanced phosphorylation of Dok2 and increased recruitment and activation of RasGAP. These data fit a model in which Dok1 is recruited indirectly through Dok2 in CD200R signaling and initiates a CrkL-dependent negative feedback loop to regulate inhibition by CD200R.

Materials and Methods

Abs

Polyclonal rabbit anti-Crk (sc-289) and anti-Grb2 (sc-255), polyclonal goat anti-Dok2 (sc-8130), and monoclonal mouse anti-RasGAP (sc-63) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-CrkL and anti-phosphotyrosine (4G10) were from Millipore (Bedford, MA). Polyclonal rabbit anti-phospholipase Cγ1 (anti-PLCγ1) and peroxidase-conjugated goat anti-rabbit Abs were from Cell Signaling Technology (Beverly, MA). Monoclonal anti-Nck was from BD Biosciences (San Jose, CA). Polyclonal rabbit anti-human Dok1 Ab (25) was a kind gift from Dominique Davidson and André Veillette. Peroxidase-conjugated polyclonal phosphatases Src homology 2 domain-containing phosphatase 1 (SHP1), SHP2, or the inositol phosphatase SHIP (20). The cytoplasmic tail of CD200R contains three conserved tyrosines, of which the most membrane distal one is part of a phosphotyrosine-binding (PTB) domain recognition motif (NPxY) (21). Phosphorylation of this tyrosine motif is essential for inhibitory CD200R signaling (22, 23) and binds directly to the PTB domain-containing adaptor downstream of tyrosine kinase 2 (Dok2) (22). Phosphorylation of CD200R-bound Dok2 results in the recruitment and activation of Ras GTPase-activating protein (RasGAP) and the subsequent inhibition of Ras-Erk signaling (22–24). CD200R ligation also causes phosphorylation of the closely related Dok1 (22–24), but unlike Dok2, this protein is not essential for inhibitory CD200R signaling in human myeloid cells (22).

 Unlike most other inhibitory receptors, CD200R does not contain any ITIMs, which mediate cellular inhibition through the phosphorylation-dependent recruitment of the protein tyrosine kinases.
anti-mouse, anti-rabbit, and anti-goat Abs were from Sigma-Aldrich (St. Louis, MO).

Cell culture

U937 cells expressing wild-type (WT) or signaling-deficient (cytoplasmic tail truncated [Tr]) human CD200R have been described previously (22). In brief, these cell lines were established by lentiviral transduction of U937 cells with constructs containing either full-length human CD200R or a Tr version lacking the last 40 aa of its cytoplasmic tail. Cells were grown in RPMI 1640 supplemented with 5% heat-inactivated FCS, 1 mM sodium pyruvate, nonessential amino acids, and 50 U/ml penicillin, 50 μg/ml streptomycin (all from PAA Laboratories, Teddington, U.K.).

Recombinant proteins

Pentameric human CD200 cartilage oligomeric matrix protein (CD200-COMP) consisting of the extracellular region of human CD200 (23) linked to domains 3 and 4 of rat CD4 followed by an 11-amino acid linker (NSGGGSGGTTG) and the rat COMP oligomerization domain was generated as described previously (22, 26).

Immunoprecipitations

U937 cells expressing WT or Tr CD200R were preincubated at 4˚C under gentle agitation for 30 min at ∼1.5 × 10^7 cells/ml in the presence of saturating amounts of CD200-COMP (concentration diluted 1:100 in RPMI 1640). Cells were then warmed to 37˚C for indicated time periods, pelleted, and lysed in NP-40 lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, 1 mM EDTA, 10% [v/v] glycerol, NaN₃ 0.02% [w/v], 1% [v/v] NP-40, 1 mM sodium pervanadate, and 10% [v/v] protease inhibitor mixture (Sigma-Aldrich)). Lysates were collected every 24 h for a period of 72 h. The fractions collected at 48 and 72 h were immediately filtered and added to U937 cells expressing WT or Tr human CD200R. Twenty-four hours after the last addition of lentiviral supernatant, transduced cells were selected in 3 μg/ml puromycin (Sigma-Aldrich). Transduced cells were grown to obtain sufficient numbers, lysed, and lysates resolved in reducing SDS sample buffer and blotted as described earlier to determine the efficiency of protein knockdown. Cell lines with highest knockdown efficiency were selected for experimental use. Some constructs (Ctr1, Ctr2) that did not result in knockdown of their respective target proteins were used as negative controls. Expression of CD200R was determined by flow cytometry on the same day the cells were used in experiments to ensure that the knockdown procedure had not altered cell surface levels of the receptor.

Vectors containing the shRNA sequences listed in Table I were used in knockdown experiments (29).

Surface plasmon resonance analyses using a BIAcore system (BIAcore, Uppsala, Sweden) were conducted essentially as described previously (22, 27). In brief, ∼4000 response units streptavidin was immobilized at 25˚C on CMS chips by amine coupling followed by immobilization of 50–150 response units biotinylated peptides (Peptide Protein Research, Fareham, U.K.). Flow cells with streptavidin only were used as controls. Increasing concentrations of monomeric (fast protein liquid chromatography-purified), 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. The following peptides derived from human proteins were used in BIAcore experiments: peptide sequences were Biotin-EDP(phos)YDEPEGLAP for Dok1 pY362 and Biotin-RPDHI(phos)YDEPEGLAP for Dok2 pY345.

Short hairpin RNA interference

pLKO.1 lentiviral vectors (28) containing short hairpin (shRNA) sets (five constructs per target protein) against human PLCγ1, Crk (CrkII), CrkL, and Nck1 were from Open Biosystems (Thermo Scientific, Epsom, U.K.). Vectors were transfected into 293T cells together with the pSPAX2 packaging vector and the pMD2G envelope plasmid using polyethylenimine (22). Tissue culture supernatant containing lentiviral particles was collected every 24 h for a period of 72 h. The fractions collected at 48 and 72 h were immediately filtered and added to U937 cells expressing WT or Tr human CD200R. Twenty-four hours after the last addition of lentiviral supernatant, transduced cells were selected in 3 μg/ml puromycin (Sigma-Aldrich). Transduced cells were grown to obtain sufficient numbers, lysed, and lysates resolved in reducing SDS sample buffer and blotted as described earlier to determine the efficiency of protein knockdown. Cell lines with highest knockdown efficiency were selected for experimental use. Some constructs (Ctr1, Ctr2) that did not result in knockdown of their respective target proteins were used as negative controls. Expression of CD200R was determined by flow cytometry on the same day the cells were used in experiments to ensure that the knockdown procedure had not altered cell surface levels of the receptor.

Vectors containing the shRNA sequences listed in Table I were used in knockdown experiments (29).
Results
Phosphorylation of Dok2 precedes phosphorylation of Dok1 in CD200R signaling

We and others have previously shown that engagement of CD200R on human and mouse myeloid cells results in the phosphorylation of Dok1 and Dok2, and the recruitment of RasGAP (22–24). To determine the temporal dynamics of these events, WT or signaling-deficient (cytoplasmic tail Tr) CD200R was engaged on the surface of U937 cells for various times using pentameric CD200 (CD200-COMP). Both Dok proteins were phosphorylated in response to ligation of WT CD200R (Fig. 1). Dok2 phosphorylation was readily observed after 2.5-min incubation in the presence of CD200-COMP, but Dok1 was not phosphorylated at this time point (Fig. 1). After 5 min, both Dok proteins showed comparable levels of CD200R-induced phosphorylation, but only the Dok2 immunoprecipitates showed a significant increase in RasGAP (Fig. 1). After 10-min incubation in the presence of CD200-COMP, both Dok proteins were strongly phosphorylated; a very slight increase in RasGAP binding was also observed for Dok1 at this point (Fig. 1). These observations support our previous hypothesis that Dok1 is recruited to the CD200R signaling complex indirectly via Dok2 (22), and suggests that RasGAP is primarily recruited by Dok2 in CD200R signaling.

Crk and CrkL interact with Dok1 in CD200R signaling

The C-terminal tails of Dok1 and Dok2 contain multiple tyrosine residues and proline-rich regions, which can recruit Src homology 2 (SH2) and SH3 domain-containing proteins, respectively. To determine which pathways regulated by Dok1 and Dok2 occur in CD200R signaling, we tested for interactions of Dok proteins with reported binding partners (reviewed in Ref. 30).

Two proteins reported to interact with both Dok1 and Dok2 are the adaptor proteins Crk and the closely related CrkL (31, 32). Crk and CrkL share 57% amino acid sequence identity and consist of one SH2 domain and two SH3 domains that are connected by a proline-rich linker region (33, 34).

To determine whether Crk or CrkL interact with Dok proteins in response to CD200R signaling in human myeloid cells, Dok1 and Dok2 were immunoprecipitated from U937 cells expressing WT or Tr CD200R that had been incubated for 10 min in the presence of pentameric CD200. Precipitates were then blotted with specific Abs to test for coprecipitation of Crk and CrkL. CrkL was found to coprecipitate with Dok1 than with Dok2, but this association was not affected by CD200R signaling because it occurred in cells expressing the WT and the Tr receptor (Fig. 2A). These results are indicative of a constitutive interaction between Dok1 and Crk.

Coprecipitation of CrkL with Dok1 and Dok2, in contrast, was affected by CD200R signaling. More CrkL was found to coprecipitate with Dok2 in cells expressing Tr CD200R, which suggests a constitutive interaction that is inhibited by signaling through the WT receptor (Fig. 2B). On the contrary, coprecipitation of CrkL with Dok1 was substantially stronger in the presence of CD200R signaling, suggesting that this interaction is induced by CD200R-dependent phosphorylation of Dok1 (Fig. 2B).

Analysis of Dok1 and Dok2 immunoprecipitates by Western blotting showed no evidence of CD200R-induced recruitment of Src family kinases (Fyn, Hck, Lyn) or a number of other reported Dok-interacting proteins including Grb2, PLCγ1, P13K, SHIP, Tec kinase, or Vav1 (data not shown).

Nck is recruited by phosphorylated Dok2 in CD200R signaling

Dok1 and Dok2 have been shown to bind the adaptor molecule Nck (35, 36). Nck (Nck1/Nckα) is one of two members of the Nck family of proteins that are composed of one SH2 domain and three SH3 domains (37). The interaction between Nck and Dok proteins is mediated by its SH2 domain binding phosphotyrosine 362 of human Dok1 and phosphotyrosine 351 of mouse Dok2 (corresponding to Y345 in human) (35, 36). To determine whether Nck interacts with Dok proteins in response to CD200R signaling in human myeloid cells, Dok1 and Dok2 were immunoprecipitated from U937 cells expressing WT or Tr CD200R that had been incubated for 10 min in the presence of pentameric CD200. Analysis of precipitates by Western blot showed that coprecipitation of Nck with Dok2 was induced by ligand engagement of the WT receptor, whereas coprecipitation with Dok1 was minimal and probably nonspecific (Fig. 3).

Nck binds directly to Dok2

To determine whether coprecipitation of Nck and Dok2 is a consequence of a direct interaction, we measured binding of recombinant full-length Nck to peptides containing the tyrosine phosphorylated Y345 and Y362 motifs in Dok2 and Dok1, respectively, by surface plasmon resonance (Fig. 4). At 37°C, Nck bound to the Dok1 peptide with an equilibrium dissociation constant, $K_D = 0.6 \mu M$, whereas binding to Dok2 was comparatively weaker ($K_D = 3.5 \mu M$) (Fig. 4).

Knockdown of CrkL increases phosphorylation of CD200R signaling complexes

The biochemical data suggest that Crk, CrkL, and Nck are recruited by Dok proteins to the CD200R signaling complex. To investigate...
their functional importance in CD200R signaling, we lentivirally transduced U937 cells expressing WT or Tr CD200R with shRNA against Nck1, Crk, CrkL, and, as a control, PLCγ1. Five different shRNA lentiviral constructs targeting differing areas of the mRNA encoding each of these proteins were tested, and the constructs showing the greatest knockdown efficiency (Table I) were selected for subsequent experimental use (Fig. 5A–D). Analysis by flow cytometry showed no differences in the expression of CD200R between the different knockdown lines (data not shown).

To determine effects of protein knockdown on CD200R signaling, we incubated knockdown cell lines expressing WT or Tr CD200R for 10 min at 37˚C in the presence of CD200-COMP, followed by lysis and immunoprecipitation of Dok2 and Dok1. Blotting of immunoprecipitates with anti-phosphotyrosine Abs showed that CrkL knockdown resulted in increased phosphorylation of both Dok proteins (Fig. 5E, F). Concomitant with an increase in phosphorylated Dok2, enhanced recruitment and phosphorylation of RasGAP was observed in Dok2 immunoprecipitates when CrkL was knocked down (Fig. 5E). These effects were specific to CD200R signaling, as Western blotting of lysates with anti-phosphotyrosine mAb did not reveal any proteins with differentially increased phosphorylation in cells expressing the Tr receptor (data not shown). Moreover, immunoprecipitation of the tyrosine phosphatase SHP1, a protein that is unlikely to be involved in CD200R signaling, from CD200R stimulated cells revealed no effect of CrkL knockdown on its phosphorylation state.

**FIGURE 5.** Knockdown of CrkL increases phosphorylation of CD200R signaling complexes. PLCγ (A), Crk (B), CrkL (C), and Nck (D) expression was knocked down by shRNA in U937 cells expressing WT (Wt) or Tr CD200R. Cells were lysed after incubation for 10 min at 37˚C in the presence of CD200-COMP. Dok2 (E) or Dok1 (F) was immunoprecipitated from the lysates and precipitates blotted with anti-phosphotyrosine mAb to determine the phosphorylation state of Doks and coprecipitated proteins. After extensive washing, membranes were reprobed for RasGAP and Dok proteins. Results are representative of three independent experiments conducted using separately established knockdown cell lines.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Hairpin Sequence (5'-3')</th>
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<tbody>
<tr>
<td>PLCγ</td>
<td>CCAGCCAGTCTGATACCTCTGGAGGTTATACCTGTGATCTGGTTTTTT</td>
</tr>
<tr>
<td>Crk</td>
<td>CCAGCCGTTTTACCAGATTCTTACAACTGAGTTTGGAGTAATCCGCTCTTTTT</td>
</tr>
<tr>
<td>CrkL</td>
<td>CCAGGCTGAAAGTCAAGAGATGAAAAGCTCTGGTTTGGATCTGGTTTTTT</td>
</tr>
<tr>
<td>Nck</td>
<td>CCAGGCCATTGTAAATACCAAGTATCTCGAGATACTTGGTATTTACAATGGCTTTTT</td>
</tr>
<tr>
<td>Dok1</td>
<td>CCAGGCCGTAACAGGATACCCAGCCTACCTGAGTTTGGAGTAATCCGCTCTTTTT</td>
</tr>
<tr>
<td>Ctr</td>
<td>CCAGCCGCTTGGAGTTACTGCTTACAAAGACGAATTCAGGGTTACTGATCTGGTTTTTT</td>
</tr>
<tr>
<td>Ctrl1</td>
<td>CAGGGAGGAGTACAAAAGGAGAAAGTACCTGAGTTTGGAGTAATCCGCTCTCTTTTT</td>
</tr>
<tr>
<td>Ctrl2</td>
<td>CAGGGAGGAGTACAAAAGGAGAAAGTACCTGAGTTTGGAGTAATCCGCTCTCTTTTT</td>
</tr>
</tbody>
</table>

Control (Ctr) is a scrambled sequence derived from PLCγ (29). Ctrl1 and Ctrl2 are constructs targeting Dok1 and CrkL, respectively, which did not cause any detectable knockdown of their respective target proteins.
This suggests a specific effect of CrkL knockdown on the phosphorylation of proteins involved in CD200R signaling.

Knockdown of Nck, Crk, or the control PLCγ did not have any significant effects on CD200R-dependent phosphorylation of Dok proteins (Fig. 5).

Dok1 inhibits Dok2-mediated CD200R signaling

An association between CrkL and Dok1 was induced by CD200R signaling and knockdown of CrkL inhibited CD200R-dependent recruitment of the effector enzyme RasGAP. If CrkL regulates CD200R inhibitory signaling via its association with Dok1, knocking down CrkL and Dok1 should have equivalent effects. We thus examined the effect of Dok1 knockdown on CD200R signaling. Dok1 expression in U937 cells expressing WT or Tr CD200R was knocked down by shRNA (Fig. 6A). Dok1 knockdown cells were then stimulated for 10 min in the presence of CD200-COMP followed by lysis and immunoprecipitation of Dok2. Analysis of precipitates by phosphotyrosine immunoblotting showed a substantial increase in CD200R-induced Dok2 phosphorylation under conditions of Dok1 knockdown (Fig. 6B). Dok2-mediated recruitment and phosphorylation of RasGAP was, likewise, increased in Dok1 knockdown cells (Fig. 6B). These effects were not due to changes in CD200R expression in Dok1 knockdown lines or any nonspecific effects of Dok1 knockdown on overall tyrosine phosphorylation (data not shown). Knocking down CrkL or Dok1 thus had similar effects in enhancing recruitment of the effector enzyme RasGAP to CD200R signaling complexes, and this is in agreement with our observation that CrkL recruitment is dependent on Dok1. Together, these data are consistent with Dok1 inhibiting Dok2-mediated CD200R signaling through recruitment of CrkL (Fig. 7).

Discussion

The CD200 receptor signals through a unique inhibitory pathway, which is dependent on direct binding to the adaptor protein Dok2 and the subsequent Dok2-mediated recruitment of the effector enzyme, RasGAP (22, 24) (this is illustrated schematically in Fig. 7). Ligand engagement of CD200R also results in tyrosine phosphorylation of Dok1, an adaptor protein closely related to Dok2.
These molecular pathways are conserved between primary human macrophages and U937 cells (22), thus making this cell line a suitable model system for the study of human CD200R signaling. We have previously suggested that Dok1 is recruited indirectly via Dok2 in CD200R signaling (22), and the current observation that CD200R-induced phosphorylation of Dok1 is delayed compared with Dok2 supports this hypothesis.

The PTB domains of the closely related Dok1 and Dok2 proteins differ in their specificities for binding to various phosphotyrosine motifs, including those found in the cytoplasmic tail of CD200R (22). We now show that the C-terminal regions of Dok1 and Dok2 can, likewise, differ in their specificities for downstream effectors. RasGAP, Nck, and CrkL are all capable of interacting with both Dok proteins via their SH2 domains (reviewed in Ref. 30). Nevertheless, this study shows that RasGAP and Nck are primarily recruited to Dok2 tyrosines in response to CD200R engagement, whereas CrkL inductively binds to Dok1. Although the affinity of Nck for the phosphorylated tyrosine motif Y362 in Dok1 was greater than for Y345 in Dok2, only Dok2 was found to recruit this adaptor molecule in CD200R signaling. This suggests that not all Dok tyrosines are phosphorylated in CD200R signaling, but rather that phosphorylation patterns are specific and different for the two Dok proteins.

Many of the proteins that interact with the SH3 domains of Nck participate in various cellular processes involving cytoskeletal rearrangement (reviewed in Ref. 37). Both Dok1 and Dok2 have been shown to play a role in promoting cellular spreading and migration in various cells and tissues through their ability to recruit Nck (38–40). Recruitment of Nck via Dok2 may thus enable CD200R to affect cellular processes involving cytoskeletal rearrangement. This hypothesis is supported by the observations that the ability of retinal microglia to migrate in response to injury is dependent on CD200R signaling (41) and by the fact that CD200R has been shown to play a role in macrophage fusion (42). Lack of an effect of Nck knockdown on the tyrosine phosphorylation state of proteins involved in CD200R signaling may be explained by tyrosine-independent interactions of the Nck SH3 domains (37) or functional compensation by the closely related Nck2 (43).

Similar to Nck, many of the Crk- and CrkL-mediated signaling pathways involve a rearrangement of the actin cytoskeleton (reviewed in Ref. 44). Given the structural and functional similarities between Crk, CrkL, and Nck, it is thus conceivable that these proteins collaborate in connecting CD200R signaling to various cellular processes involving cytoskeletal rearrangement. The activation-independent interactions between Crk and Dok1 and between CrkL and Dok2 are likely to be SH3 domain-mediated, whereas the phosphorylation-dependent interaction between CrkL and Dok1 presumably occurs through the SH2 domain of CrkL. Because SH2-mediated interactions are usually of a higher affinity than SH3-dependent interactions, the dissociation of CrkL from Dok2 that was observed on ligand engagement of CD200R (Fig. 2) may be a result of CD200R-induced phosphorylation of Dok1.

CrkL knockdown resulted in a profound increase in the phosphorylation of Dok proteins and enhanced Dok2-binding and phosphorylation of RasGAP. Similar results were observed under conditions of Dok1 knockdown. This suggests that the Dok1–CrkL complex downmodulates Dok2-mediated inhibitory CD200R signaling by inhibiting the recruitment and activation of the effector enzyme RasGAP. This hypothesis is supported by our previous observation that Dok1 knockdown does not prevent CD200R-mediated inhibition of LPS-stimulated IL-8 secretion in U937 cells (22), and by the finding that Dok1 is not a major recruiter of RasGAP in CD200R signaling in U937 cells (Figs. 1 and 5).

CrkL can activate Ras through recruitment of the guanine nucleotide exchange factor Sos (45) and may thus be capable of counteracting the RasGAP-mediated inhibition of Ras activity that has been shown to occur in CD200R signaling (24). Because Ras is capable of binding and activating PI3K (46), it is also possible that PI3K-mediated changes in the phosphorylation state of membrane lipids may affect the recruitment of Dok proteins. The pleckstrin homology domains of Dok1 and Dok2 have been shown to bind more strongly to phosphoinositides phosphorylated at the five-than at the three-hydroxyl group (47). Alternatively, because CrkL has been shown to interact constitutively with the E3 ubiquitin ligase, Cbl in U937 cells (48), recruitment of CrkL may result in Cbl-mediated degradation of Src family kinases required for the phosphorylation of the CD200R signaling complex.

In summary, this report provides evidence for opposing roles of Dok1 and Dok2 in CD200R signaling. Although Dok2-mediated recruitment of RasGAP is required for the inhibitory function of CD200R (22), a complex between Dok1 and CrkL appears to initiate a negative feedback loop in this receptor’s signaling pathway (Fig. 7).

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Disclosures

The authors have no financial conflicts of interest.

References


