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Dok1 and Dok2 play opposing roles in CD200R signaling[1]

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Abstract

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 Dok2 and the subsequent recruitment and activation of 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. Here 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 CrkL, while the closely related Crk interacts constitutively with Dok1. Knockdown of Dok1 and 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.

Keywords

Human; Monocyte/Macrophage; Signal Transduction

Introduction

The CD200 receptor (CD200R) is a type 1 transmembrane glycoprotein of the Ig superfamily present on most leukocytes, especially cells of the myeloid lineage (1, 2). CD200R mediates inhibitory signaling in myeloid cells (3-5) and T cells (6) by interacting with its widely expressed ligand CD200 through its N-terminal V-type domain (7).

CD200 knockout mice have increased numbers of myeloid cells, are more susceptible to induction of auto-immune disorders and die of severe lung inflammation following influenza infection (8, 9). Deficiency of CD200R, likewise, results in exaggerated inflammatory responses to various stimuli (10). *In vitro* experiments show that ligation of CD200R causes inhibition of cellular activation in different cells and tissues including human and mouse mast cells (3), macrophages (4, 5), mixed lymphocyte reactions (6, 11) and basophils (12). High levels of CD200 expression are a characteristic of various human cancers, and this is thought to facilitate evasion of immune recognition by inhibiting the activation of CD200R bearing leukocytes (6, 13-16). CD200 homologues are also expressed by a number of

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viruses and have been shown to inhibit host responses against virally infected cells (5, 12, 17-19).

Unlike most other inhibitory receptors, CD200R does not contain any immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which mediate cellular inhibition through the phosphorylation-dependent recruitment of the protein tyrosine phosphatases Src homology 2 domain-containing phosphatase (SHP)1, 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).

We now provide evidence of a regulatory role for Dok1 in CD200R signaling by analysing the kinetics of phosphorylation of Dok2 and Dok1 and characterising 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 Crk and 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

Antibodies

Polyclonal rabbit anti-Crk (sc-289) and anti-Grb2 (sc-255), polyclonal goat anti-Dok2 (sc-8130) and monoclonal mouse anti-RasGAP (sc-63) antibodies were from Santa Cruz Biotechnology. Monoclonal mouse anti-CrkL and anti-phosphotyrosine (4G10®) were from Millipore. Polyclonal rabbit anti-PLCγ1 and peroxidase-conjugated goat anti-rabbit antibodies were from Cell Signaling Technology. Monoclonal anti-Nck was from BD Biosciences. Polyclonal rabbit anti-human Dok1 antibody (25) was a kind gift from Dominique Davidson and André Veillette. Peroxidase-conjugated polyclonal anti-mouse, anti-rabbit and anti-goat antibodies were from Sigma-Aldrich Ltd.

Cell culture

U937 cells expressing wild-type or signaling deficient (cytoplasmic tail truncated) 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 truncated version lacking the last 40 amino acids of its cytoplasmic tail. Cells were grown in RPMI 1640 supplemented with 5% heat inactivated fetal calf serum, 1 mM sodium pyruvate, non essential amino acids and 50 U/ml penicillin, 50 μg/ml streptomycin (all PAA).

Recombinant proteins

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-amino-acid linker

sequence (NSGGGSGGGTG) and the rat COMP (cartilage oligomeric matrix protein) oligomerization domain was generated as described previously (22, 26).

Full-length recombinant His tagged human Nck1 (catalogue #3976) was provided by Louise Bird (Oxford Module Consortium).

Immunoprecipitations

U937 cells expressing wild-type or truncated CD200R were pre-incubated 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 (concentrate diluted 1:100 in RPMI). 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, 0.02% (w/v), 1% (v/v) NP-40, 1 mM sodium pervanadate and 10% protease inhibitor cocktail for mammalian cells (Sigma)) for 20 min at 4 °C. Lysates were cleared by centrifugation at 16,000 g for 10 min at 4 °C and incubated for 45-90 min at 4°C in the presence of 1 μg of antibody per 10^7 cells. Lysates were then cleared for a second time as above. Protein G Sepharose® beads (Amersham) were washed 3 times in PBS and re-suspended as a 50% slurry in lysis buffer of which 10 μl was added per 10^7 cells. After incubating at 4 °C for 45-90 min, beads were washed 2-3 times in lysis buffer. Immunoprecipitates were eluted by boiling the beads at 95 °C for 10 min in non-reducing SDS sample buffer and resolved on NuPAGE® Bis-Tris gradient (4 to 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).

BIAcore™ analysis

Surface plasmon resonance analyses using a BIAcore™ 3000 were carried out essentially as described previously(22, 27). In brief, ~4000 response units (RU) of streptavidin were immobilized at 25 °C to CM5 chips by amine coupling followed by immobilization of 50-150 RU of biotinylated peptides (Peptide Protein Research Ltd.). Flow cells with streptavidin only were used as controls. Increasing concentrations of monomeric (FPLC 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 plotted and fitted to hyperbolas. The following peptides derived from human proteins were used in BIAcore™ experiments:

Peptide sequences were Biotin-EDPI(phos)YDEPEGLAP for Dok1 pY362 and Biotin-RPDHI(phos)YDEPEG for Dok2 pY345.

shRNA interference

pLKO.1 lentiviral vectors (28) containing short/small hairpin (sh)RNA sets (five constructs per target protein) against human PLCγ, Crk (CrkII), CrkL and Nck1 were from Open Biosystems (Thermo Scientific). 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 wild-type or truncated human CD200R. Twenty-four hours after the last addition of lentiviral supernatant, transduced cells were selected in 3 μg/ml puromycin (Sigma). Transduced cells were grown to obtain sufficient numbers, lysed and lysates resolved in reducing SDS sample buffer and blotted as described above 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 following shRNA hairpin sequences were used in knockdown experiments:

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Construct	Hairpin sequence
PLCγ	CCGGCCAGATCAGTAACCCTGAATTCTCGAGAATTCAGGGTTACTGATCTGGTTTTT
Crk	CCGGGCTTTACTGGAATTCTACAAACTCGAGTTTGTAGAATTCCAGTAAAGCTTTTT
CrkL	CCGGCGTGAAAGTCACAAGGATGAACTCGAGTTCATCCTTGTGACTTTCACGTTTTT
Nck	CCGGGCCATTGTAAATACCAAGTATCTCGAGATACTTGGTATTTTACAATGGCTTTTT
Dok1	CCGGGCCTACCGATAACCCACCTAACTCGAGTTAGGTGGGTTATCGGTAGGCTTTTT
Ctr	GCGCGCTTTGTAGGATTCGTTCAAGAGACGAATCCTACAAAGCGCGCTTTTTT
Ctr1	CCGGGAGGGAGTACAACGGAAGAAACTCGAGTTTCTTCCGTTGTACTCCCTCTTTT
Ctr2	CCGGGACCTGTCTTTGCGAAAGCAACTCGAGTTGCTTTCGCAAAGACAGGTCTTTTT

Control, 'Ctr' is a scrambled sequence derived from PLCγ (29). Ctr1 and Ctr2 are constructs targetting Dok1 and CrkL respectively, which did not cause any detectable knockdown of their respective target proteins.

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, wild-type or signaling deficient (cytoplasmic tail truncated) 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 wild-type 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 and 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 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 (30)).

Two proteins reported to interact with both Dok1 and Dok2 are the adaptor proteins 'CT10 sarcoma oncogene cellular homolog' (Crk) and the closely related Crk-like (CrkL) (33, 34). 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 (31, 32).

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 wild-type or truncated CD200R that had been incubated for 10 min in the presence of pentameric CD200 (CD200-COMP). Precipitates were then blotted with specific antibodies to test for co-precipitation of Crk and CrkL (Fig. 2). Substantially more Crk was found to co-precipitate with Dok1 than with Dok2, but this association was not affected by CD200R signaling as it occurred in cells expressing the wild-type as well as the truncated receptor (Fig. 2A). These results are indicative of a constitutive interaction between Dok1 and Crk.

Co-precipitation of CrkL with Dok1 and Dok2, on the other hand, was affected by CD200R signaling. More CrkL was found to co-precipitate with Dok2 in cells expressing truncated CD200R, which suggests a constitutive interaction that is inhibited by signaling through the wild-type receptor (Fig. 2B). On the contrary, co-precipitation 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 blot revealed 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, PI3K, 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 wild-type or truncated CD200R that had been incubated for 10 min in the presence of pentameric CD200. Analysis of precipitates by western blot showed that co-precipitation of Nck with Dok2 was induced by ligand engagement of the wild-type receptor, while co-precipitation with Dok1 was minimal and probably non-specific (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 μM, while binding to Dok2 was comparatively weaker (K_D = 3.5 μM) (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, U937 cells expressing wild-type or truncated CD200R were lentivirally transduced with short/small hairpin RNA (shRNA) against Nck1, Crk and 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 highest knockdown efficiency 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, knockdown cell lines expressing wild type or truncated CD200R were incubated 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 antibodies revealed 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 truncated 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 (data not shown). 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 examined the effect of Dok1 knockdown on CD200R signaling. Dok1 expression in U937 cells expressing wild-type or truncated 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 revealed 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 non-specific effects of Dok1 knockdown on overall tyrosine phosphorylation (data not shown). Knocking down CrkL or Dok1 thus had similar effects in preventing 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.

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) and 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 (30)). Nevertheless, the current study shows that RasGAP and Nck are primarily recruited to Dok2 tyrosines in response to CD200R engagement while CrkL inducibly binds to Dok1. Although, the affinity of Nck for the phosphorylated tyrosine motif Y362 in Dok1 was higher 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 (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).

Similarly to Nck, many of the Crk and CrkL-mediated signaling pathways involve a rearrangement of the actin cytoskeleton (reviewed in (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, while the phosphorylation-dependent interaction between CrkL and Dok1 presumably occurs through the SH2 domain of CrkL. Since SH2-mediated interactions are usually of a higher affinity than SH3-dependent interactions, the dissociation of CrkL from Dok2 that was observed upon 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 down-modulates 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 the finding that Dok1 is not a major recruiter of RasGAP in CD200R signaling in U937 cells (Fig. 1, Fig. 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). Since Ras is capable of binding and activating phosphatidylinositol 3-kinase (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 PH 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, since 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, the current report provides evidence for opposing roles of Dok1 and Dok2 in CD200R signaling. While 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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Abbreviations used in this paper

Cbl	Casitas B-lineage lymphoma
CD200R	CD200 receptor
Crk	CT10 sarcoma oncogene cellular homologue
CrkL	Crk-like
Dok	downstream of tyrosine kinase
Grb2	growth factor receptor-bound protein 2
PH	pleckstrin homology
PLCγ	phospholipase C-γ
PTB	phosphotyrosine binding
RasGAP	
SH2	SRC homology 2
SHP	Src homology 2 domain-containing phosphatase

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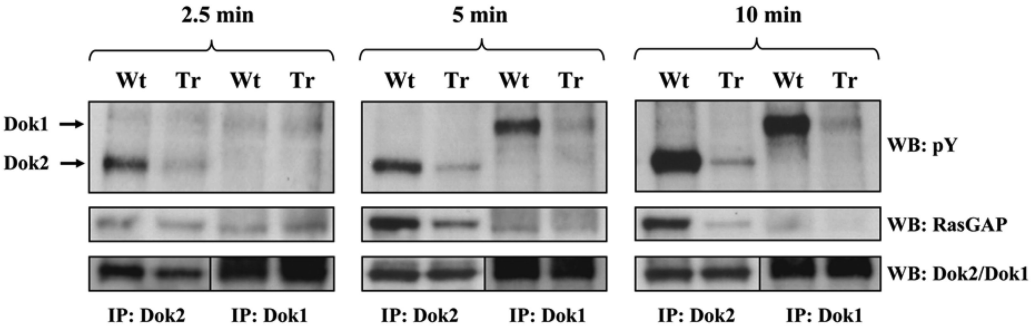

Fig. 1.

Phosphorylation of Dok2 precedes phosphorylation of Dok1 in CD200R signaling. U937 cells expressing wild-type (Wt) or truncated (Tr) CD200R were incubated for 2.5, 5 or 10 min at 37 °C in the presence of CD200-COMP. Cells were then lysed and Dok2 or Dok1 was immunoprecipitated from lysates. Immunoprecipitates were blotted with anti-phosphotyrosine mAb. Membranes were then washed extensively and re-probed with specific antibodies against the indicated proteins. Results are representative of three independent experiments.

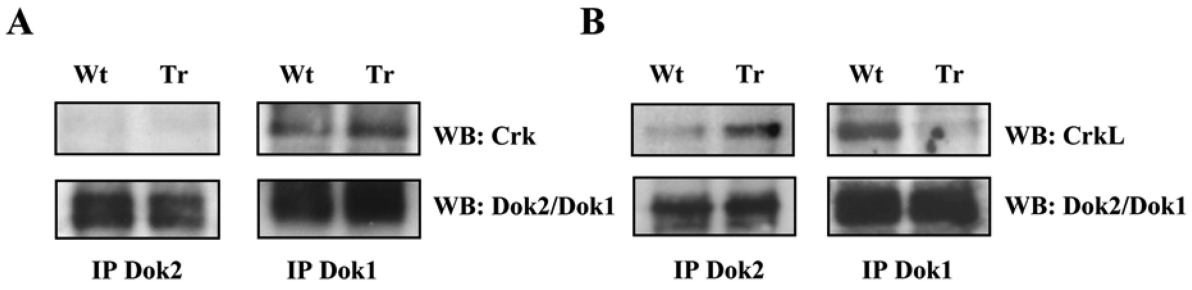

Fig. 2.

Crk and CrkL interact with Dok1 in CD200R signaling. U937 cells expressing wild-type (Wt) or truncated (Tr) CD200R were incubated for 10 min at 37 °C in the presence of CD200-COMP. Cells were then lysed and Dok2 (A) or Dok1 (B) was immunoprecipitated from lysates. Immunoprecipitates were blotted with specific antibodies against Crk or CrkL. After extensive washing, membranes were re-probed with antibodies against Dok2 or Dok1 to control for loading. Results are representative of three or more independent experiments.

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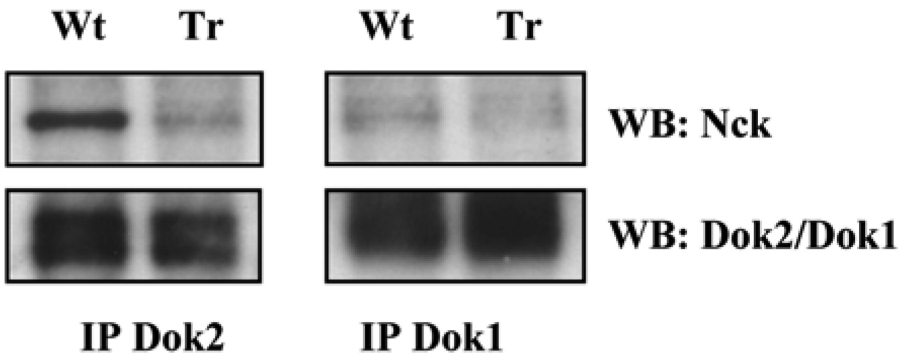

Fig. 3.

Nck is recruited to phosphorylated Dok2 in CD200R signaling. U937 cells expressing wild-type or truncated CD200R were incubated for 10 min at 37 °C in the presence of CD200-COMP. Cells were then lysed and Dok2 and Dok1 were immunoprecipitated from lysates. Immunoprecipitates were blotted with mAb against Nck. After extensive washing, membranes were re-probed with antibodies against Dok2 or Dok1 to control for loading. Results are representative of three independent experiments.

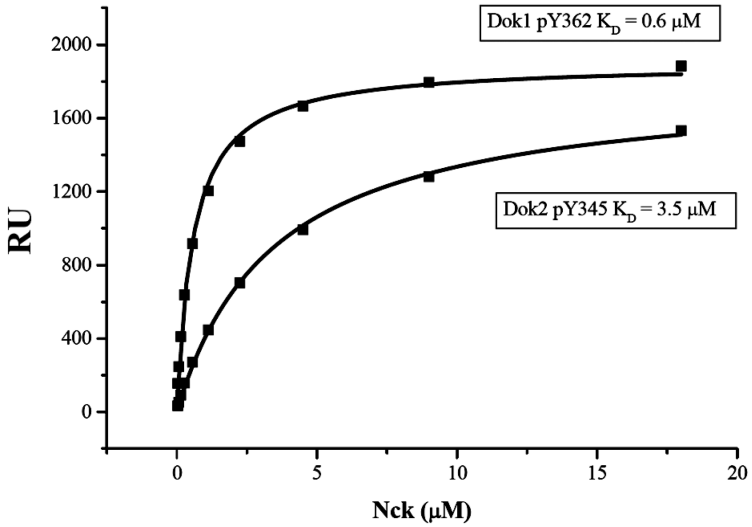

Fig. 4.

Nck binds directly to Dok2. Phosphorylated peptides corresponding to sequences around Tyr362 of Dok1 or Tyr345 of Dok2 were immobilized on a BIAcore™ chip and recombinant human Nck SH2 domain was passed over flow cells at different concentrations at 37 °C. Equilibrium binding at each concentration of Nck binding to the phosphopeptides is shown. The hyperbolas represent best fits used for affinity calculation of equilibrium dissociation constants. Results are representative of two or more independent experiments.

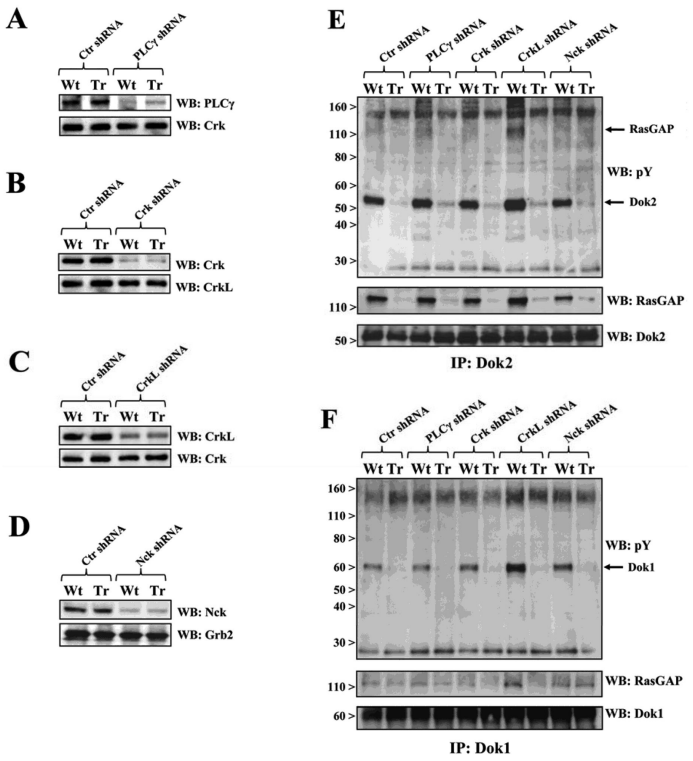

Fig. 5.

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 wild-type (Wt) or truncated (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 co-precipitated proteins. After extensive washing, membranes were re-probed for RasGAP and Dok proteins. Results are representative of three independent experiments conducted using separately established knockdown cell lines.

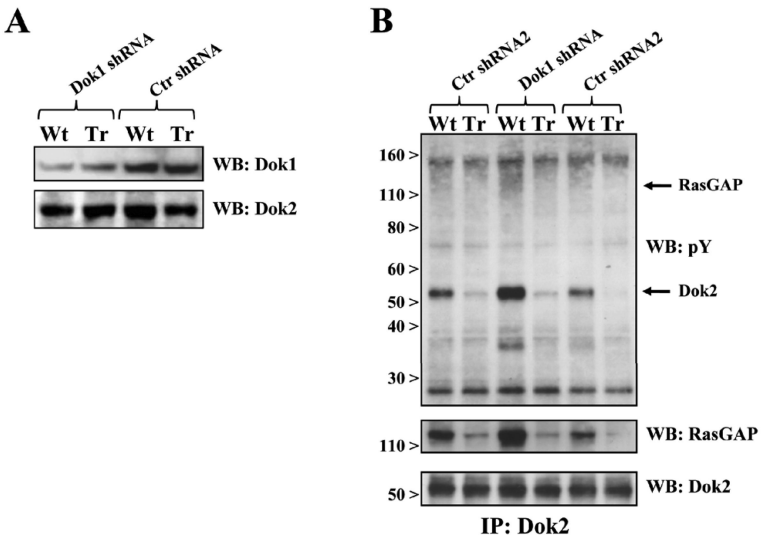

Fig. 6.

Dok1 inhibits Dok2-mediated CD200R signaling. (A) Dok1 expression was knocked down by shRNA in U937 cells expressing wild-type (Wt) or truncated (Tr) CD200R. (B) Cells were lysed after incubation for 10 min at 37 °C in the presence of CD200-COMP. Dok2 was immunoprecipitated from the lysates and precipitates blotted with anti-phosphotyrosine mAb to determine the phosphorylation state of Dok2 and co-precipitated proteins. After extensive washing, membranes were re-probed for RasGAP and Dok2. Results are representative of three independent experiments. Results are representative of three independent experiments conducted using separately established knockdown cell lines.

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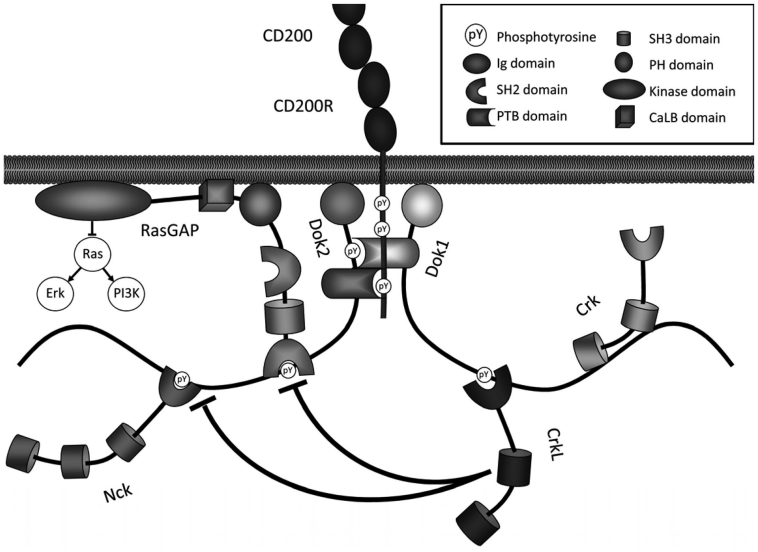

Fig. 7.

Model for CD200R signaling. Dok2 binds to the third phosphotyrosine in the cytoplasmic tail of CD200R via its PTB domain. Subsequent phosphorylation of Dok2 results in the recruitment of RasGAP, Nck and Dok1. RasGAP hydrolyses RasGTP into the inactive form RasGDP, thereby inhibiting PI3K and Erk. Dok1 interacts constitutively with Crk and recruits CrkL upon tyrosine phosphorylation. CrkL subsequently inhibits phoshorylation of Dok2 by an unknown mechanism, resulting in inhibition of RasGAP activation.

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