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Nore1B regulates TCR signaling via Ras and Carma1

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Abstract

Nore1A was originally identified as a potential Ras effector, and Nore1B is an alternatively spliced isoform. Both share a Ras/Rap association domain (RA domain) but only Nore1A contains sequence motifs that predict SH3 domain binding and diacylglycerol/phorbol ester binding in the amino-terminal region. Here we report that Carma1 binds to Nore1A and Nore1B through the RA domain and that Carma1 interacts with active Ras in the presence of Nore1B. RNA interference against Nore1B attenuates NF- κ B activation induced by T cell receptor (TCR) ligation, but not NF- κ B activation induced by TNF α or lipoteichoic acid. In addition, Nore1B is also required for KiRas GV12-mediated ERK1 activation and Elk1 reporter activity in T cells. We also provide evidence that knockdown of Nore1B also impairs polarized redistribution of Ras at the B cell-T cell immune interface. Together, these findings suggest that endogenous Nore1B recruits active Ras to the APC-T cell interface and mediates the interaction between Ras and Carma1.

Keywords

T-lymphocytes; NF- κ B; Ras proteins; MAGUK proteins; Lymphocyte activation

1. Introduction

T lymphocyte signaling is initiated by a specific interaction with antigen-presenting cells via the T cell receptor (TCR) complex. This process induces the activation of cytosolic non-receptor tyrosine kinases such as Lck/Fyn and ZAP-70, which phosphorylate adaptor molecules including LAT, SLP-76, Gads and Grb2 [1-3]. These phosphorylated adaptor molecules recruit various effector proteins such as the small GTPase Ras, phospholipase C- γ 1, Vav1 and PKC θ , leading to calcium mobilization, activation of protein (serine/threonine) kinase cascades and ultimately to the activation of transcription factors including NFAT, AP-1 and NF- κ B [3-6].

Recent studies have demonstrated that Carma1 (originally called CARD11) is an essential lipid raft-associated regulator of TCR-induced NF- κ B activation [7-11]. Deficiency of Carma1 results in impairment of TCR-induced NF- κ B activation, but has no effect on TNF α -induced NF- κ B activation [12-15]. T cell proliferation, CD69 upregulation and IL-2 production are defective in Carma1-null mice, suggesting that Carma1 is a central molecule in T cell homeostasis. During T cell activation, Carma1 binds to Bcl10 and the ensuing complex is recruited to lipid rafts [9]. Additional kinases associating with Carma1 and regulating NF- κ B activation include PDK1 and TAK1 [16,17].

To identify Carma1 interactors, yeast two-hybrid screening was performed. Using Carma1 as bait, we found that Carma1 interacted with Nore1B, also known as RAPL. Nore1B/RAPL, is encoded by a splice variant of the Nore1/RASSF5 gene that is expressed almost exclusively in lymphoid tissues. Nore1B is a 265 amino acid polypeptide that contains a non-descript, isoform-specific amino terminus followed by Ras-Rap1 binding (RA) domain and a carboxy-terminal coiled-coil region known as a SARAH domain [18,19]. The ubiquitously expressed longer isoform Nore1A was discovered by its ability to bind selectively to the activated forms of Ras and Rap1, and Nore1A endogenous to KB cells was shown to associate transiently with endogenous Ras in response to serum re-addition [18]. Nore1B/RAPL was independently isolated by virtue of its interaction with activated Rap1, and was shown to mediate the Rap1-induced polarized accumulation and increased affinity of LFA-1 in response to TCR activation [20]. Here we characterize the binding of Nore1B/RAPL to Carma1 and examine the functional significance of Nore1B to TCR signaling to the nucleus. RNAi-induced depletion of Nore1B in Jurkat cells reduces by about 50% the robust activation of NF- κ B-directed gene expression induced by anti-CD3 but does not affect the response to TNF α or LTA; this pattern of inhibition is similar to that caused by Ras(Ser17Asn). Over-expression of Ki-Ras(Gly12Val) activates NF- κ B-directed gene expression about three-fold, whereas Rap1b(Gly12Val) is without effect; this response to Ras is abolished by concomitant expression of Nore1B RNAi. Depletion of Nore1B also attenuates anti-CD3 activation of ERK1, abolishes anti-CD3 stimulation of Elk1-directed gene expression and prevents the migration of endogenous Ras into the immune synapse. Our results indicate that in addition to its role in Rap1-induced LFA-1 clustering, Nore1B/RAPL participates in the recruitment of activated Ras into the immune synapse, and is necessary for optimal Ras signaling in response to TCR activation.

2. Materials and methods

2.1. Reagents

Anti-CD3 antibody (OKT3) was obtained from the Massachusetts General Hospital Pharmacy. TNF α was purchased from R&D systems (MN). LTA was purchased from Sigma-Aldrich (MO). Anti-Nore1 antibody, which detects both Nore1B and Nore1A, was produced by immunizing rabbits with recombinant Nore1A.

2.2. Expression constructs

Human Carma1 cDNA was a generous gift from Dr. Jürg Tschopp (University of Lausanne), and subcloned into the Peak12-Flag vector, which contains an EF1 α promoter and Flag epitope tag, the GST vector, or into the pCMV-Myc vector (Clontech, CA). Flag-Nore1A and B constructs and GFP- or GST-Ki-RasG12V constructs were made as described previously [21-23]. NF- κ B-dependent *Photinus* luciferase reporter was made as described previously [24]. GAL4-Elk1 expression construct and *Photinus* luciferase reporter containing five tandem repeats of GAL4 binding element were obtained from Stratagene (TX). Constructs expressing short interfering RNA (siRNA) were made as previously described [25], using the following sets of oligonucleotides: 1A targeting nucleotides

546-566 of coding sequence of human Nore1A, 5'-
 gatcccgccaccctcaccgtgacctttcaagagaaggtcacggtgagggtgcttttgaaa-3' and 5'-
 agcttttcaaaaaagcaccctcaccgtgacctttcttgaaggtcacggtgagggtgcggg-3'; 1B targeting
 nucleotides 56-76 of coding sequence of human Nore1B, 5'-
 gatcccgactgcttcttactgctattcaagatagcagtgaagaagcagctttttgaaa-3' and 5'-
 agcttttcaaaaaagactgcttcttactgctatcttgaatagcagtgaagaagcagctgcggg-3'.

2.3. Immunoprecipitation and pull-down experiments

HEK293 cells (1×10^6) were transfected with 1 μ g of Myc-CarMA1 construct and 2 μ g of Flag construct expressing Nore1A or its mutants in 6-well dishes using 6 μ l of Lipofectamine2000 (Invitrogen, CA). One day later, the cells were lysed in lysis buffer (20 mM Tris-HCl pH8.0, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4 , 1% Triton X-100 and 10 μ g/ml leupeptin). The lysates were incubated at 4 °C for 3 h with goat anti-mouse IgG antibody-coated sepharose beads (ICN, OH), which were pre-incubated with monoclonal anti-Flag antibody (Sigma-Aldrich) overnight. The bound proteins were eluted in SDS sample buffer, subjected to SDS-PAGE, transferred to PVDF membranes, and probed. HEK 293 cells were transfected with GST CarMA1 and Flag Nore1B or its mutants in 6 cm dishes and GST beads were used to collect immunoprecipitated protein complexes.

HEK293 cells (1×10^6) were transfected in 6-well dishes using 6 μ l of Lipofectamine2000 with 1 μ g of GST construct (either empty vector or expressing CarMA1) and 2 μ g of Flag construct expressing Nore1B or its mutants, or with 1 μ g of GST construct (either empty vector or expressing Ki-RasG12V), 2 μ g of Flag-CarMA1 construct and 2 μ g of Flag-Nore1B construct. One day later, the cells were lysed in the lysis buffer. The lysates were incubated at 4 °C for 3 h with glutathione-sepharose (Amersham, NJ). The bound proteins were eluted in SDS sample buffer, subjected to SDS-PAGE, transferred to PVDF membranes, and probed.

Jurkat cells (1×10^7) were electroporated with 1 μ g of Myc-CarMA1 construct, 5 μ g of GST-Ki-RasG12V construct and 10 μ g of siRNA construct (vector or 1B), and 2 days later, the cells were lysed in the lysis buffer. The lysates were incubated at 4°C overnight with glutathione-sepharose beads.

2.4. Luciferase reporter assay

Jurkat E6 cells (5×10^6) were re-suspended in 250 μ l of culture medium, and electroporated (250 V, 950 μ F) with expression constructs, *Photinus* luciferase reporter and pRL-TK construct expressing *Renilla* luciferase (Promega, WI). Upon electroporation, the cells were diluted in 2.5 ml of culture medium. 24 to 72 h later, the cells were lysed, and luciferase activities were determined with Dual-Luciferase Reporter Assay System (Promega, WI) according to the manufacturer's instructions. Reporter activities were assessed by normalization of *Photinus* luciferase activity to *Renilla* luciferase activity.

2.5. Immune synapse and immunofluorescent staining

APC-T cell conjugate formation was performed as described previously [26,27]. Briefly, Raji cells were loaded with blue fluorescent cell tracker chloromethylbenzoyl derivative of aminocoumarin (Molecular Probe, OR) according to the manufacturer's instructions, and then pulsed with 5 μ g/ml Staphylococcal enterotoxin E (SEE) (Toxin Technology, FL) for 20 min. Viable Jurkat cells were isolated using Ficoll-Paque PLUS (Amersham Biosciences, Sweden) 3 days after electroporation with 10 μ g of siRNA constructs, and then mixed with an equal number of the Raji cells. The cells were centrifuged at low speed, and incubated for 15 min at 37 °C. Thereafter, conjugates were gently resuspended, and plated onto poly-L-lysine-coated slides. After fixation with 3.5% paraformaldehyde and 0.1% Tween 20 in

PBS, the cells were labeled with mouse anti-Ras antibody (Transduction Laboratories, CA) and rabbit anti-Nore1 antibody, followed by secondary antibodies conjugated with Alexa Fluor (Molecular Probes, OR). The samples were viewed under a fluorescence microscope (Olympus, Japan).

2.6. Determination of ERK phosphorylation

Jurkat E6 cells (5×10^6) were re-suspended in 250 μ l of culture medium, and electroporated (250 V, 950 μ F) with 10 μ g of siRNA constructs. Three days later, the cells were incubated with anti-CD3 antibody (2 μ g/ml) for 0, 5 or 15 min, and then lysed in the lysis buffer. The lysates were subjected to SDS-PAGE, transferred to PVDF membrane, and blotted with anti-phospho-p44/42 MAP kinase antibody (Cell Signaling, CA) or anti-p44/42 MAP kinase antibody (Cell Signaling, CA).

3. Results

In order to identify potential partners of Carma1, we carried out yeast two-hybrid screens of a mouse brain cDNA library using Carma1 as bait. We isolated several positive clones, two of which encoded full-length RASSF5/Nore1B (data not shown): murine Nore1B is a 265 amino acid alternative splice form of the Nore1/RASSF5 gene; Nore1A is a 413 amino acid polypeptide originally discovered as a novel interactor of active Ras [18]. The two polypeptides are identical over their carboxy-terminal 225 amino acids, which encompass a Ras/Rap association domain (RA domain) followed by a carboxy-terminal coiled-coil region called a SARAH domain. Nore1B has a unique but nondescript 40 residue amino-terminal segment, whereas the Nore1A amino-terminus contains a proline-rich region followed by a diacylglycerol/phorbol ester binding domain (DAG_PE domain). Both Nore isoforms are known to bind, via their SARAH domains, to the Ste20-like kinases, MST1 and MST2 [19,21].

3.1. Nore1A and 1B bind to Carma1

We performed co-immunoprecipitation studies to verify the ability of Nore1 to interact with Carma1. Recombinant Carma1 co-precipitates both Nore1B and Nore1A (Fig. 1A and B). The amino-terminal fragment of Nore1A (1-266) is unable to bind to Carma1 (Fig. 1A, 1-266), whereas deletion of the common Nore1 carboxy-terminus (363-413) does not reduce Nore1 co-precipitation with Carma1 (Fig. 1A, 1-362). Replacing Nore1A RA domain residues 301-304 (LKKF) with alanines substantially reduces association of Nore1A with Carma1 (Fig. 1A, LKKF), whereas replacement of two cysteines (132 and 135) in the Nore1A DAG_PE domain with alanines does not reduce the Carma1-Nore1A association (Fig. 1A, DAG_PE). We next confirmed that Nore1B interaction with Carma1 required the RA domain. Fig. 1B shows that GST Carma1 interacts with Flag tagged Nore1B. Deletion of the RA domain or inactivation of the RA domain by mutating the LKKF motif of Nore1B abolished interaction with Carma1. These results indicate that the RA domain, which is common to the Nore1A and Nore1B isoforms, is required for binding to Carma1. Vavvas and colleagues previously demonstrated that Nore1A interacted with active Ras through the RA domain [18]. As expected, Nore1B co-precipitates the active Ras mutant Ki-RasG12V (data not shown). These results indicate that both Carma1 and Ki-RasG12V interact with Nore1 through the Nore RA domain. We therefore inquired whether Nore1B could bind Carma1 and Ki-RasG12V simultaneously. Whereas GST-Ki-RasG12V associates with co-expressed Nore1B (Fig. 1C, lane 2), no binding of GST-Ki-RasG12V to co-expressed Carma1 was detected (Fig. 1C, lane 4) unless Nore1B was also transfected (Fig. 1C lane 3). Thus Nore1B mediates the binding of Ki-RasG12V to Carma1. Moreover, the co-expression of Carma1 with Ki-RasG12V and Nore1B does not interfere with binding of Nore1B to Ki-RasG12V (Fig. 1C compare lanes 2 and 3).

The ability of Nore1B to bind both Ras-GTP and Carma1 suggests that Nore1B may modulate signaling outputs mediated by Ki-RasG12V and Carma1. Consistent with earlier reports [20,28], we find Nore1B to be the dominant isoform expressed in T cells (Fig. 1D); in as much as Carma1 is critical for TCR-induced NF- κ B activation [29], we next investigated the involvement of Nore1B in NF- κ B activation in Jurkat cells.

3.2. Reduction of Nore1B expression inhibits TCR-induced activation of NF- κ B, ERK1 and Elk1

We prepared two siRNA constructs, 1A and 1B, targeted to unique regions of Nore1A and Nore1B, respectively. The siRNA construct targeting Nore1B specifically inhibited expression of Flag-Nore1B, but not that of Flag-Nore1A in 293 cells (Fig. 2A), whereas the siRNA construct targeting Nore1A did not affect the expression level of either Flag-Nore1A or Flag-Nore1B (Fig. 2A). As shown in Fig. 2B, the siRNA construct 1B substantially decreased endogenous Nore1B protein expression in Jurkat cells examined 3 days after electroporation, whereas the siRNA construct 1A did not; neither construct affected endogenous Ras protein expression (Fig. 2B). In subsequent experiments the siRNA construct 1B was used for knockdown of Nore1B and the siRNA construct 1A was used as a control. Inhibition of Nore1B expression did not alter basal, TNF α - or LTA-stimulated NF- κ B-directed gene expression, but resulted in a 50% reduction of anti-CD3 stimulated NF- κ B-directed gene expression (Fig. 2C-F).

Several studies have demonstrated that oncogenic Ras induces a modest stimulation of NF- κ B activity[30-33]; we observe a two to three fold activation of NF- κ B-directed gene expression by Ki-Ras(Gly12Val), whereas comparable expression of Rap1b(Gly12Val) is without effect (Fig. 3A). Moreover, the ability of the dominant interfering mutant Ras(Ser17Asn) to partially inhibit anti-CD3 stimulated NF- κ B-directed gene expression (Fig. 3B) without affecting the response to TNF α (Fig. 3C) indicates that Ras-GTP contributes to the anti-CD3 activation of NF- κ B.

We next investigated whether Nore1B expression in T cells influenced Carma1 interaction with activated Ki-Ras (Gly12-val). Since commercially available antibodies were not suitable for immunoprecipitation of endogenous proteins we investigated if knockdown of endogenous Nore1B interfered with Carma1 and Ki-Ras (Gly12val) interaction in T cells. As shown in Fig. 4A depletion of endogenous Nore1B expression in Jurkat cells disrupted Ki-Ras (Gly12val)-Carma1 complex formation. Moreover knockdown of endogenous Nore1B attenuated the synergistic NF- κ B activation mediated by Ki-Ras (Gly12val) and Carma1 (Fig. 4B). Collectively, these studies support that Nore1B levels regulate Ras participation in signaling complexes that impinge on NF- κ B activation.

The ability of Nore1B to bind selectively to Ras-GTP led us to inquire as to whether Nore1B participates in the regulation of classical Ras outputs in Jurkat cells, either in a positive or negative manner. Perhaps the most characteristic downstream response to active Ras is recruitment of the MAPK pathway leading to phosphorylation of ERK, which if sufficiently sustained, translocates to the nucleus and phosphorylates and activates the transcription factor Elk1 [34-36]. We observe that depletion of Nore1B is accompanied by attenuation and shortening of anti-CD3-induced ERK1 activation (Fig. 5A). As shown in Fig. 5B Nore1B deficiency did not alter anti CD3 mediated tyrosine phosphorylation indicating that proximal TCR signaling was not affected in these cells. Moreover, consistent with the very transient activation of ERK1 engendered by anti-CD3 in Nore1B depleted cells, knockdown of Nore1B inhibits completely Elk1 activation induced by TCR stimulation (Fig. 5C). Thus, Nore1B serves a positive role in Ras signaling, both to the MAPK pathway and in the activation of NF- κ B.

3.3. Ras polarization in Jurkat-Raji cell conjugates requires Nore1B

To explore the molecular mechanisms by which Nore1B may regulate Ras signaling, we examined the distribution of endogenous Ras following antigen specific T cell responses. Jurkat cells expressing endogenous Nore1B were incubated with antigen presenting cells (Raji cells) pulsed with Staphylococcal enterotoxin E (SEE). As shown in Fig. 5D, superantigen stimulation of Jurkat cells induces both Nore1B [20] and Ras to redistribute to the B cell-T cell interface (Fig. 5D, compare top and middle panels). However, in Jurkat cells expressing siRNA against Nore1B, Ras recruitment to the B cell-T cell conjugate interface was substantially reduced (Fig. 5D). This failure of Ras recruitment, which is likely responsible for the brevity of ERK1 activation, suggests that Nore1B is responsible, to a significant degree, for the recruitment of Ras-GTP to the immune synapse during T cell activation.

4. Discussion

In this work we show that Nore1 isoforms interact with Carma1. The interaction requires the RA domain shared by Nore1A and 1B. RNAi-mediated knockdown of the lymphocyte specific isoform Nore1B in Jurkat cells attenuated TCR mediated NF- κ B activation and abolished NF- κ B activation by KiRasV12. Nore1B depletion also attenuated TCR activation of ERK1 and abolished activation of the ERK target, Elk1. Nore1B appears to be important for the recruitment of Ras-GTP to the immune synapse of T cells, so as to enable full and sustained Ras signaling.

Recent studies have demonstrated that Nore1B/RAPL is essential for activation of the integrin LFA-1 following TCR engagement [20]. RAPL knockout mice display defects in lymphocyte homing to lymphoid tissue and chemokine triggered adhesion and transmigration [37]. This action of Nore1B/RAPL is dependent on its binding to activated Rap1. The impairment of NF- κ B, ERK1 and Elk1 activation observed in the present studies in consequence of Nore1B depletion are not attributable to the interaction of Nore1B with Rap1 or LFA-1; Rap1 has no role in TCR activation of NF- κ B or MAPK in Jurkat cells. The activation of Ras and Rap are both central to effective T cell signaling [38]. These two GTPases have an identical switch 1 effector loop sequence, and are able to bind many of the same polypeptides in a GTP-dependent manner. They are, however, localized to different membrane compartments, have distinct regulators of their GTPase cycle and contribute to different cellular programs. Our results indicate that in T cells, Nore1B, in addition to its known role as an effector of Rap1-GTP, also functions both upstream and downstream of Ras-GTP, although future studies will be required to elucidate the spatiotemporal pattern of Nore1B interaction with Rap1 and Ras following T cell receptor engagement. In its role upstream of Ras-GTP, Nore1B participates, and may be largely responsible for the recruitment of Ras-GTP into the immune synapse. The mechanism underlying Nore1B localization in the synapse is less clear. It is unlikely that Carma1 is the primary vehicle for Nore1B recruitment into the synapse; deletion of the gene encoding Carma1 essentially abolishes TCR activation of NF- κ B and impairs activation of JNK, but does not alter TCR activation of ERK [14]. Thus the role of Nore1B in Ras-GTP recruitment into the synapse and in the TCR activation of the MAPK pathway is likely to be independent of the Nore1B/Carma1 interaction.

Nevertheless, the Nore1B/Carma1 interaction may reflect a role for Nore1B acting downstream of Ras-GTP. Carma1 is an essential molecule for TCR-induced NF- κ B activation [29]. Carma1 activates IKK through a signaling pathway that includes Bcl10, MALT1, TRAF6 and TAK1. The modest ability of oncogenic Ras alone to activate NF- κ B is entirely dependent on Nore1B, and it is plausible to propose that the ability of Ras to recruit Carma1 through Nore1B may be responsible for the subsequent activation of NF- κ B.

As regards the significance of the Carma1/Nore1B interaction for the TCR activation of NF- κ B, the present data indicates that interfering with Ras activation with Ras(Ser17Asn) diminished TCR activation of NF- κ B by perhaps one-third, and depletion of Nore1B reduced TCR activation of NF- κ B by about half. Depletion of Nore1B did not interfere significantly with the ability of overexpressed Carma1 to activate NF- κ B, nevertheless the present results support the idea that the supplementary effect of Ras on TCR activation of NF- κ B reflects the participation of Nore1B in the recruitment of Carma1 into the immune synapse mediating NF- κ B signaling.

5. Conclusions

Based on these data, we propose a model in which Nore1B modulates Ras signaling outputs triggered by TCR stimulation via recruiting active Ras to the plasma membrane and by contributing to the localization of Carma1. Further examination of this proposal and of the mechanism by which Nore1B itself is recruited is in progress.

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Fig. 1.

Nore1A and 1B interact with Carma1. (A) HEK293 cells were transfected with Myc-Carma1 construct and Flag constructs expressing full length Nore1A, truncated mutants (1-266, 1-362), LKKF (residues 301-304 to alanines in the RA domain), or DAG_PE (cysteines132 and 135 to alanines in the DAG_PE binding domain). Monoclonal anti-Flag antibody was used for immunoprecipitations. Monoclonal anti-Myc or anti-Flag antibody was used for blotting. (B) HEK293 cells were transfected with GSTconstructs (empty vector or expressing Carma1) and Flag constructs expressing Nore1B or its mutants lacking the entire RA domain, mutant in which the LKKF residues have been mutated to alanines, or Nore1B constructs lacking the C-terminal residues 134-265 and 211-265. Glutathione-sepharose beads were used to pull down GST-Carma1. Monoclonal anti-Flag or anti-GST antibodies were used for blotting. (C) HEK293 cells were transfected with either empty GST vector or with GST Ki-RasG12V together with Flag tagged versions of Nore1B and Carma1. Glutathione-sepharose beads were used to pull down GST-Carma1. GST pulldowns were probed with anti-Flag antibody. Total lysates were blotted with GST and anti-Flag antibodies (panels 2-4). (D) Jurkat cells, mouse primary thymocytes and Hela cells were lysed in the lysis buffer, and equal amounts of protein (40 µg per lane) was loaded into the wells of SDS-PAGE gels for Western blotting with anti-Nore1 antibody.

Fig. 2.

SiRNA construct 1B specifically suppresses Nore1B protein expression, and impairs NF-κB activation induced by anti-CD3 antibody. (A) 293 cells (3×10^5) were transfected with 0.1 μ g of Flag-construct expressing human Nore1A, 0.3 μ g of Flag-construct expressing human Nore1B and 2 μ g of siRNA construct (vector, 1A or 1B) in 12-well dishes using 3 μ l of Lipofectamine2000. Three days later, the cells were lysed, and equal amount of protein (15 μ g per lane) was loaded into the wells of SDS-PAGE gels for Western blotting. (B) Jurkat cells (5×10^6) were electroporated with 10 μ g of siRNA construct (vector, 1A or 1B). Three days later, the cells were lysed, and used for Western blotting. (C, D E and F) Jurkat cells were electroporated with 6 μ g of NF-κB-dependent luciferase reporter, 30 ng of pRL-TK, 10 μ g of siRNA construct (vector, 1A or 1B). Three days later, the cells were incubated in the absence (C) or presence of anti-CD3 antibody (2 μ g/ml) (D), TNFα (1 ng/ml) (E) or LTA (10 μ g/ml) (F) for a further 8 h, and then luciferase activities were determined as described in Materials and methods. Three independent experiments were performed with duplicate samples. Bars represent standard errors.

Fig. 3.

Ki-RasG12V activates NF- κ B, whereas Rap1bG12V does not. (A) Jurkat cells were electroporated with 3 μ g of NF- κ B-dependent luciferase reporter, 30 ng of pRL-TK and 4 μ g of Flag construct either empty or expressing Ki-RasG12V or Rap1bG12V. One day later, the cells were lysed, and then luciferase activities were determined as described in Materials and methods. Four independent experiments were performed. Bars represent standard errors. (B and C) Jurkat cells were electroporated with 3 μ g of NF- κ B-dependent luciferase reporter, 30 ng of pRL-TK and 6 μ g of Flag construct either empty or expressing Ki-RasT17N. Twenty hours later, the cells were incubated with anti-CD3 antibody (2 μ g/ml) (B) or TNF α (1 ng/ml) (C) for further 4 h, and then luciferase activities were determined as described in Materials and methods. Three independent experiments were performed with duplicate samples. Bars represent standard errors.

Fig. 4.

Knockdown of Nore1B impairs synergic NF- κ B activation with Ki-RasV12 and Carma1. (A) Jurkat cells were electroporated with 1 μ g of Myc-Carma1 construct, 5 μ g of GST-Ki-RasG12V construct and 10 μ g of siRNA construct (vector or 1B), and 2 days later, the cells were lysed. Glutathione-sepharose beads were used to pull down GST-KiRasG12V. Anti-Nore1 antibody, anti-Myc antibody or anti-GST antibody was used for blotting. (B) Jurkat cells were electroporated with 4 μ g of NF- κ B-dependent luciferase reporter, 30 ng of pRL-TK, 10 μ g of siRNA construct (vector or 1B), and 1 μ g of Flag-Carma1 construct and/or 4 μ g of Flag-Ki-RasG12V construct. The total amount of DNA was equalized with empty vector. Three days later, the cells were lysed, and then luciferase activities were determined as described in Materials and methods. Three independent experiments were performed with duplicate samples. Bars represent standard errors.

Fig. 5.

Knockdown of Nore1B reduces Ras redistribution to the immune synapse and ERK activation. (A) Jurkat cells were electroporated with 10 μ g of siRNA construct (Vector or 1B). Three days later, the cells were incubated with anti-CD3 antibody (2 μ g/ml) for 0, 5 or 15 min, and then lysed. Equal amount of protein (15 μ g per lane) was loaded into the wells of SDS-PAGE gels for Western blotting. Antibodies recognizing ERK and phospho ERK were used for immunoblotting lysates. Three independent experiments showed similar results. (B) Effect of knockdown of Nore1B on anti CD3 mediated tyrosine phosphorylation in Jurkat cells. Proteins from cell lysates were immunoblotted with anti-phosphotyrosine antibodies (4G10) as indicated. These data are representative of three independent experiments. (C) Jurkat cells were electroporated with 6 μ g of GAL4 binding element-dependent luciferase reporter, 3 μ g of construct expressing GAL4-Elk1, 30 ng of pRL-TK, and 10 μ g of siRNA construct (vector, 1A or 1B). Three days later, the cells were incubated in the absence or presence of anti-CD3 antibody (2 μ g/ml) for a further 8 h, and luciferase activities were determined as described in Materials and methods. Three independent experiments were performed with duplicate samples. Bars represent standard errors. (D) Jurkat cells were electroporated with 10 μ g of siRNA construct (vector or 1B). Three days later, the live cells were isolated on a Ficoll gradient, and conjugated with the Raji cells (blue), pre-incubated with or without SEE as described in Materials and methods. Cells were stained with anti-Nore1 (green) and anti-Ras (red) antibodies. Representative images are presented from three independent experiments. Original magnification, $\times 1000$.