Kidins220/ARMS Associates with B-Raf and the TCR, Promoting Sustained Erk Signaling in T Cells

Sumit Deswal, Anja Meyer, Gina J. Fiala, Anja E. Eisenhardt, Lisa C. Schmitt, Mogjiborahman Salek, Tilman Brummer, Oreste Acuto and Wolfgang W. A. Schamel

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Kidins220/ARMS Associates with B-Raf and the TCR, Promoting Sustained Erk Signaling in T Cells


The activation kinetics of MAPK Erk are critical for T cell development and activation. In particular, sustained Erk signaling is required for T cell activation and effector functions, such as IL-2 production. Although Raf-1 triggers transient Erk activation, B-Raf is implicated in sustained Erk signaling after TCR stimulation. In this study, we show that B-Raf is dephosphorylated on its inhibitory serine 365 upon TCR triggering. However, it is unknown how B-Raf activation is coupled to the TCR. Using mass spectrometry, we identified protein kinase D–interacting substrate of 220 kDa (Kidins220)/ankyrin repeat-rich membrane spanning protein, mammalian target of rapamycin, Rictor, Dock2, and GM130 as novel B-Raf interaction partners. We focused on Kidins220, a protein that has been studied in neuronal cells and found that it associated with the pre-TCR, εβTCR, and γδTCR. Upon prolonged TCR stimulation, the Kidins220–TCR interaction was reduced, as demonstrated by immunoprecipitation and proximity ligation assays. We show that Kidins220 is required for TCR-induced sustained, but not transient, Erk activation. Consequently, induction of the immediate early gene products and transcription factors c-Fos and Erg-1 was blocked, and upregulation of the activation markers CD69, IL-2, and IFN-γ was reduced. Further, Kidins220 was required for optimal calcium signaling. In conclusion, we describe Kidins220 as a novel TCR-interacting protein that couples B-Raf to the TCR. Kidins220 is mandatory for sustained Erk signaling; thus, it is crucial for TCR-mediated T cell activation. The Journal of Immunology, 2013, 190: 1927–1935.

Cellular decision processes are tightly regulated, to allow appropriate responses to external stimuli. As in other cell types, the duration of Erk signaling plays a decisive role in multiple T cell outcomes: positive versus negative selection of thymocytes (1, 2), CD4 versus CD8 lineage commitment (3), response to low-affinity versus high-affinity Ags in mature T cells (4), or Th1 versus Th2 differentiation of effector T cells (5). These developmental or differentiation processes are initiated by signal transduction pathways via the TCR (6, 7). However, the precise molecular mechanisms by which transient or sustained Erk activation is achieved are poorly understood and have puzzled scientists for a long time (8, 9).

The TCR is composed of the Ag-binding TCRαβ heterodimer, two CD3 dimers, and the ζζ homodimer (10). Bi- or multivalent Ag binding to TCRαβ induces conformational changes at CD3 and leads to CD3 and ζ phosphorylation (11). Consequently, the kinase ZAP70 is recruited and activated (12), thereby phosphorylating the downstream adaptor molecules LAT and SLP76. This initiates multiple downstream pathways essential for T cell activation (13). It was suggested that Erk is activated by recruitment of the Grb2–SOS complex to the phosphorylated TCR via the adaptor protein Shc (14). SOS is the classical Ras guanine-nucleotide exchange factor activating Ras. Alternatively, the Grb2–SOS complex can be activated by recruitment to phosphorylated LAT (15). In addition, phospholipase Cγ is activated by recruitment to LAT and SLP76 and generates the second messengers Ca2+ and diacylglycerol. The latter can activate the Ras guanine-nucleotide exchange factor RasGRP1 (16). Then, active Ras binds to the serine/threonine kinase Raf-1, which, in turn, triggers Erk phosphorylation and activation of its downstream effectors (17). Thus, multiple pathways seem to exist that couple TCR signaling to Raf-1 and Erk.

In addition to Raf-1, Erk can be activated by its paralog B-Raf (18–20). TCR-induced activation of Raf-1 leads to a transient Erk signal, whereas activation by B-Raf leads to sustained Erk activation in T cells (20, 21). Although sustained Erk signaling is crucial for IL-2 production by T cells (20, 22, 23), it is not understood how TCR triggering leads to B-Raf activation.

The protein kinase D (PKD)-interacting substrate of 220 kDa (Kidins220) (24), also known as ankyrin repeat-rich membrane spanning protein (25), is an integral membrane protein with four transmembrane domains in a topology in which both the N- and C-terminal domains are exposed to the cytoplasm. Its sequence is
conserved from nematodes to humans, suggesting an evolutionarily preserved function (24). Kidins220 possesses several protein–protein interaction motifs, such as 11 ankyrin repeats, a proline-rich region, a sterile α-motif domain, a kinesin L chain–interacting motif, phosphorylatable tyrosines, and a PDZ (PSD-95, Discs-large, ZO-1)—binding motif (Fig. 2A). Initially, Kidins220 expression was identified only in neurons, where it interacts constitutively with the nerve growth factor receptor (NGFR) and is rapidly phosphorylated upon nerve growth factor stimulation (25).

Importantly, Kidins220 was shown to promote sustained Erk activation in neuronal cells, most likely by activating B-Raf (26). In this study, we sought to identify interaction partners of B-Raf in T cells. Using mass spectrometry (MS), we found Kidins220/ankyrin repeat-rich membrane spanning protein to be a novel B-Raf interaction partner in T lymphocytes. In analogy to the NGFR, Kidins220 is constitutively associated with the TCR. Upon TCR triggering, Kidins220 is required for sustained Erk signaling and, subsequently, for expression of IL-2, CD69, and IFN-γ.

Materials and Methods

**Cells**

The murine αβ T cell lines 2B4, T1, and T1.4 (27), the human αβ T cell line Jurkat, the human pre-T cell line SupT1, and the mouse γδ T cell line F30L31 were maintained in complete RPMI 1640 medium supplemented with 5% FBS. C57BL/6 mice were killed, and single-cell suspensions of splenocytes were prepared. After erythrocyte lysis by incubation in ACK solution for 2 min, cells were resuspended in serum-free RPMI 1640 medium. We used the DCEK cell line stably transfected with I-Ek and CD80 expression vectors (kindly provided by R. Germain, National Institutes of Health, Bethesda, MD) as APCs.

**Abs, peptide MHC tetramer, and peptide**

The following Abs were purchased: anti-TCRβ (597-H57; BD Pharmingen), anti-CD3 (145-2C11, J. Bluestone, University of California, San Francisco, San Francisco, CA), anti–B-Raf (F7, Santa Cruz Biotechnology), anti–p-S259–Raf-1 (used in this study for p-S365–B-Raf, rabbit polyclonal; Cell Signaling Technology), anti-Kidins220 (P220 1F8/3; Abcam), anti-Kidins220 (rabbit polyclonal; Abnova), anti–p-tyrosine (4G10; Upstate Biotechnology), anti–B-Raf (F7; Santa Cruz Biotechnology), anti–p-S259–Raf-1 (used in this study for p-S365–B-Raf, rabbit polyclonal; Cell Signaling Technology), anti–B-Raf (F7; Santa Cruz Biotechnology), anti–p-S259–Raf-1 (used in this study for p-S365–B-Raf, rabbit polyclonal; Cell Signaling Technology), anti–p-S259–Raf-1 (used in this study for p-S365–B-Raf, rabbit polyclonal; Cell Signaling Technology), anti–p-S259–Raf-1 (used in this study for p-S365–B-Raf, rabbit polyclonal; Cell Signaling Technology), and anti–B-Raf (F7; Santa Cruz Biotechnology). The rabbit anti-TCR antisemur M49 was obtained as described for 44b4 (28). IRP-conjugated secondary Abs were purchased from Pierce, and the H2Kk–pepBA tetramers were purchased from Imunomab Lässcher (29). The peptide cytochrome C peptide had the sequence KAERADILIAYLKQATK.

**Cell stimulation and Western blotting**

Cells were stimulated in RPMI 1640 medium without serum after starvation for 1 h at 37°C prior to stimulation (stimulation up to 1 h) or with 1% FCS (stimulation >1 h). Stimulation was done with 5 mM pervanadate; a mixture of the anti-CD3e Ab 145-2C11 and the anti-TCRβ Ab 597-H57, both at 5 μg/ml (referred to as anti-TCR in the text); 25 nM H2Kk-pepBA tetramers; or 20 nM pigeon cytochrome C peptide–loaded DCEK cells (APCs) for different time points at 37°C. Where indicated, cells were lysed in 1 ml lysis buffer containing 0.3% Brij96, as described (30). Immunoprecipitation (IP), SDS-PAGE, and Western blotting (WB) were done using standard procedures, as described (31). Western blots were quantified using the ImageQuant LAS 4000mini system (GE Healthcare) or classical autoradiography films and ImageJ (National Institutes of Health).

**Mass spectrometry**

After Colloidal Blue (Carl Roth) staining of the gel, bands a–d (Fig. 1A) were excised; proteins were reduced and alkylated with 10 mM DTT and 55 mM iodoacetamide, as described (32), and digested with 12.5 ng/μl trypsin (proteomeX grade; Sigma). The resulting peptides were acidified to 1% trifluoroacetic acid (TFA); reagent grade; Sigma) and loaded onto Stage Tips (33). Peptides bound to the solid phase were washed with 50 μl 1% TFA and then eluted with 50 μl buffer B (80% acetonitrile, 0.1% TFA) and lyophylized in a SpeedVac Concentrator. Lyophylized peptides were resuspended in 0.1% TFA and analyzed by nano-liquid chromatography–tandem mass spectrometry. An LTQ Orbitrap mass spectrometer (Thermo Electron) was coupled online to a nano-LC Ultimate 3000 (Dionex). To prepare the analytical column, C18 material (ReproSil-Pur C18 AQ 3 μm; Macherey) was packed into a spray emitter (75 μm inner diameter, 8 μm opening, 70 mm length; New Objectives) using a high-pressure packing device (Nanobeaume; Western Fluids Engineering). Mobile phase A consisted of water, 5% acetonitrile, and 0.5% acetic acid, and mobile phase B consisted of acetonitrile and 0.5% acetic acid. Peptides were eluted from the reverse phase by a gradient of solvent B, from 5 to 50% in 22 min. The five most intense peaks in the first MS scan were selected for second MS scans, where fragmentation occurred. Raw data files were processed using DTA-SuperChrom (http://msquant.sourceforge.net/). Peak lists were searched against a target-decoy concatenated International Protein Index human 3.50 database using Mascot 2.2 software with the following parameters: monoisotopic masses, 10 ppm on MS and 0.5 Da on MS/MS; electrospray ionization trap parameters, fully tryptic specificity, cysteine carbamidomethylated as fixed modification, oxidation on methionine, phosphorylation on serine, threonine, tyrosine, proline N-acetylation and deamidation on glutamine and asparagine as variable modifications. Three missed cleavage sites were allowed.

**Proximity ligation assay**

Cells were resuspended in PBS/3%BSA, transferred onto BSA-blocked slides by cytoospin, fixed with 2% PFA, and permeabilized (0.1% saponin and 1% FCS in PBS). All additional steps were performed according to the manufacturer’s instructions (Olink Bioscience, Uppsala, Sweden). In brief, slides were blocked in Duolink blocking reagent and incubated at 4°C in the two primary Abs. Then, slides were washed and incubated in secondary Abs labeled with the proximity ligation assay (PLA) probes; one with a (+)–strand DNA-oligonucleotide and other with the corresponding (–) strand. Ligation solution was added, and slides were incubated for 30 min at 37°C. Then an amplification solution containing DNA-polymerase was added, and the samples were incubated for 100 min at 37°C. Slides were washed. After drying the slides, DAPI–containing mounting medium was added, and the slides were analyzed by fluorescence microscopy.

**Immunoprecipitation-flow cytometry measurement**

CML latent beads (10 μm diameter; Invitrogen) coupled to anti-TCRβ (597-H57) were added to the cellular lysate, and IP was performed as described (34). After washing, beads were resuspended in a solution containing fluoroaphore-labeled Abs or an unlabeled primary Ab, followed by a fluorophore-labeled secondary Ab, and incubated for 1 h at 4°C in the dark. Samples were washed and resuspended in PBS, and fluorescence intensity was measured using the flow cytometer.

**Generation of stable knockdown cells**

Five lentiviral TRC2-pLKO-pan vectors, one nontarget short hairpin RNA (shRNA) control, and four encoding an shRNA specific for the Kidins220 mRNA (Supplemental Fig. 3B) were purchased from Sigma-Aldrich. To generate lentiviral particles, HEK293T cells were transfected with the lentiviral vectors and the components of the Trans-Lentiviral shRNA Packaging Kit (Open Biosystems/Thermo Fisher). The supernatant containing the virus particles was harvested 48 h after transfection and filtered through a 0.2-μm filter. For transfection, 2B4 cells suspended in RPMI 1640 supplemented with 10% FCS and 8 mg/ml Polybrene were mixed with viral supernatant at a 1:1 ratio. Forty-eight hours later, total cells were selected in 1 μg/ml puromycin for 5 d.

**Calcium influx, CD69 upregulation, and cytokine secretion**

To measure the calcium response, cells were loaded with Indo-1 as described (35). Calcium influx into the cytosol was induced by addition of the inducer stimulating 30 s after addition of Indo-1/unbound Indo-1 with an LSR II fluorescence spectrometer (Becton Dickinson). Data were analyzed with FlowJo 6.1 software. To quantify upregulation of the activation marker CD69, cells were stimulated for 24 h with the indicated stimuli. After harvesting, cells were stained with PE-labeled anti-CD69 Ab (Caltag Laboratories) and analyzed by flow cytometry (FACS-Calibur, Becton Dickinson). To measure IL-2 and IFN-γ production, cells were stimulated as described above for 5 h. The relative amount of IL-2 and IFN-γ was measured in the supernatant on the BioPlex200 system (Bio-Rad), following the manufacturer’s instruction.

**Results**

**Identification of novel B-Raf interaction partners by MS**

To identify novel interactions partners of B-Raf in T cells, cells of the murine αβ T cell line 2B4 were lysed in mild detergent conditions (0.3% Brij96). An anti-hemagglutinin IP was performed.
Kidins220 binds to the TCR in unstimulated T cells

From the newly identified B-Raf interaction partners, we focused on Kidins220, because it has not been implicated previously in T cell signaling. Because Kidins220 is an integral membrane protein (Fig. 2A) and binds to the NGFRs (TrkA, TrkB, and TrkC) in neuronal cells (26), we tested whether Kidins220 also binds to TCR. Resting mouse 2B4 cells were lysed, and the TCR and its interaction partners were purified by anti-CD3 IP (Fig. 2B).

Upon SDS-PAGE separation, WB was performed to detect its interaction partners. As a control, Raf-1 was also bound to B-Raf, as previously reported (39–41). Further, the binding of GM130, Kidins220, and Raf-1 to B-Raf increased upon pervanadate stimulation (Fig. 1D). Raf-1 was also bound to Rictor, mTor, GM130, and Kidins220 (Fig. 1C, 1D, lower panels).

Identification of novel interaction partners of B-Raf in T cells

FIGURE 1. Identification of novel interaction partners of B-Raf in T cells. (A) Resting 2B4 cells were lysed prior to consecutive anti-hemagglutinin (control) and anti-B-Raf IP. After three washing steps, the immunopurified proteins were separated by reducing SDS-PAGE, and those proteins that were enriched in the B-Raf IP (bands a–d, Fig. 1A) were identified by MS (Fig. 1B, Supplemental Table I). We identified the known B-Raf interaction partners IQGAP1 (36) and Hsp90 (37), as well as B-Raf itself, validating our experiment.

We performed co-IP studies to confirm the interaction of the putative novel binding partners to B-Raf. 2B4 cells were left untreated (Fig. 1C, 1D) or stimulated with 5 mM pervanadate (Fig. 1D) and lysed in mild detergent conditions (38). Specific IPs were performed using Abs against the new candidates, and the Western blots were developed with anti–B-Raf Abs (upper panels). Rictor, mTor, GM130, and Kidins220 were verified as novel B-Raf interaction partners. As a control, Raf-1 was also bound to B-Raf, as previously reported (39–41). Further, the binding of GM130, Kidins220, and Raf-1 to B-Raf increased upon pervanadate stimulation (Fig. 1D). Raf-1 was also bound to Rictor, mTor, GM130, and Kidins220 (Fig. 1C, 1D, lower panels).

Kidins220 also associated with these receptors. We also show that Kidins220 bound to the TCR from primary mouse splenocytes (Fig. 2D). Taken together, we conclude that Kidins220 is associated with TCRs at different developmental stages and T cell lineages.

The Kidins220–TCR interaction is disrupted upon stimulation

Next, we tested the effect of TCR stimulation on the Kidins220–TCR interaction. 2B4 cells were left unstimulated or were stimulated with anti-CD3 Abs, and the lysates were subjected to anti-CD3 IP. Although no effect was detected upon 1 min of stimulation, the amount of Kidins220 copurified with the TCR was clearly decreased after 10 min of TCR triggering (Fig. 3A).

To test Kidins220’s association with the TCR following stimulation with peptide MHC ligands, we stimulated T1.4 cells with the H2Kd-pepABA tetramers for different lengths of time; after lysis, the TCR was immunopurified using anti-CD3 Abs (Fig. 3B). As before, TCR stimulation disrupted the TCR–Kidins220 interaction. Anti–p-tyrosine WB served as a control for the stimulation. Next, the DCEK cell line was loaded with the agonistic peptide PCC and used as an APC to stimulate 2B4 cells for 10 min (Fig. 3C, lanes 3 and 6). As controls, 2B4 cells were left untreated (lanes 2 and 5), or the APC was used without 2B4 cells (lanes 1 and 4). After lysis, anti-CD3 or anti-Kidins220 IP, as well as WB, were performed as indicated. Again, the Kidins220–TCR interaction was reduced upon APC stimulation.

Next, we wanted to test whether the dissociation of Kidins220 from the TCR was mediated by phosphorylation events. To induce a strong increase in cellular protein phosphorylation, we used the phosphatase inhibitor pervanadate (42, 43). 2B4 T cells (Fig. 3D) were left unstimulated or were stimulated for 10 min with 5 mM pervanadate. Lysates were subjected to anti-Kidins220 IP. The WB analysis showed that ζ was copurified with Kidins220 in resting T cells (Fig. 3D, lane 2). Furthermore, the amount of TCR copurified with Kidins220 decreased upon stimulation with pervanadate, suggesting that the phosphorylation of the TCR and/or SupT1 and the γδ T cell line F30L31 (Fig. 2C). Copurification of the pre-TCR and the γδ TCR with Kidins220 indicated that Kidins220 also associated with these receptors. We also show that Kidins220 bound to the TCR from primary mouse splenocytes (Fig. 2D). Taken together, we conclude that Kidins220 is associated with TCRs at different developmental stages and T cell lineages.
Kidins220 might disrupt the Kidins220–TCR association. This was also observed with the human T cell line Jurkat (Supplemental Fig. 1B). To substantiate this result, we used a more quantitative approach: immunoprecipitation measured by flow cytometry (Supplemental Fig. 2A) (34, 44). 2B4 cells were stimulated with 5 mM pervanadate or were left untreated. Proximity of the proteins of interest resulted in a red fluorescent product upon an in situ PLA reaction. The nuclei were counterstained with DAPI, and the slides were analyzed by fluorescence microscopy. The interaction of Kidins220 with the TCR in resting cells is visualized by the presence of specific red dots (Fig. 4A); the number of dots is a relative measurement of the extent of colocalization (51). Upon pervanadate stimulation the interaction was reduced, as seen by a reduction in the number of red dots (Fig. 4B, Supplemental Fig. 2C). As a positive control, we used the well-known interaction between TCR and ZAP70 in stimulated cells (Fig. 4C). To control the specificity of the reaction, either of the two primary Abs was omitted, and red dots were no longer detected (Fig. 4D, 4E). These controls also show that there is no cross-reactivity between the secondary Abs; thus, the red dots corresponded to the specific association of Kidins220 with the TCR in situ.

To better quantify the interaction between TCR and Kidins220, a slightly different protocol was followed, and the intensity of red fluorescent PCR products/cell was measured by flow cytometry (Fig. 4F). Again, Kidins220 was in close proximity to the TCR in untreated cells, and this association was reduced upon pervanadate stimulation.

Next, we treated 2B4 cells with anti-TCR Abs on ice, so that only cell surface-localized TCRs, and not intracellular TCRs, bound to the Abs. Nonbound Abs were removed by centrifugation and washing steps. After lysis of the cells, Ab-bound TCRs were purified with protein G–coupled beads. Proteins were detected by WB. (B) Lysates of 2B4 (lanes 2 and 3) were used for either an anti-CD3 (145-2C11) IP (lane 2) or a control IP with protein G–coupled beads alone (lane 3). As an additional control, the anti-CD3 IP was performed with lysis buffer (lane 1). Purified proteins were separated by reducing SDS-PAGE, and Kidins220 and the ζ chain were detected by WB analysis using specific Abs. (C) The pre-T cell line SupT1 or the γδ T cell line F30L31 were lysed followed by anti-Kidins220 IP or a control IP with protein G–coupled beads. Proteins were detected by WB. (D) Lysates of C57BL/6 splenocytes were used for IP as in (C). The H chain of the immunoprecipitating Ab and an anti-actin WB of the lysate served as controls.

Kidins220 regulates ERK signaling in T cells

To test whether Kidins220 influences Erk signaling in T cells, we generated 2B4 cell lines in which Kidins220 was stably knocked down by the expression of Kidins220-specific shRNAs using lentiviral vectors. Four stable lines were generated using different Kidins220-specific shRNA sequences. The stable line named shRNA-2 showed the strongest reduction in Kidins220 expression activity of B-Raf often does not reflect the biological activity in the cell (19, 45–47). Thus, we monitored its phosphorylation status at serine 365, because B-Raf and Raf-1 require dephosphorylation at this site for activation (47, 48), and dephosphorylation of this site in Raf-1 correlates with an increase in its activity (49). In 2B4 cells, B-Raf is phosphorylated at this inhibitory residue in nonstimulated cells but becomes rapidly dephosphorylated within the first 3 min of stimulation, which also coincides with the rapid onset of Erk activation (Fig. 3G). The activation of the B-Raf/Mek/Erk module is also reflected by its decreased electrophoretic mobility shift (already visible at 3 min), which is caused by its increased phosphorylation status (50).

Pervanadate stimulation reduces the binding of Kidins220 to the TCR in situ

Next, we analyzed the Kidins220–TCR interaction using a detergent-free approach. To this end, we performed an in situ PLA. With this assay, the proximity (with a distance < 40 nm) of two proteins can be detected by fluorescence microscopy and flow cytometry (Supplemental Fig. 2B) (51).

For a qualitative approximation, 2B4 cells were stimulated with 5 mM pervanadate for 5 min or were left untreated. Cells were transfected onto microscope slides, fixed, and permeabilized. Proximity of the TCR with either Kidins220 or with ZAP70 was detected by anti-CD3e plus anti-Kidins220 or anti-ZAP70 Abs, respectively. Secondary Abs coupled to DNA were then used. Proximity of the proteins of interest resulted in a red fluorescent product upon an in situ PLA reaction. The nuclei were counterstained with DAPI, and the slides were analyzed by fluorescence microscopy. The interaction of Kidins220 with the TCR in resting cells is visualized by the presence of specific red dots (Fig. 4A); the number of dots is a relative measurement of the extent of colocalization (51). Upon pervanadate stimulation the interaction was reduced, as seen by a reduction in the number of red dots (Fig. 4B, Supplemental Fig. 2C). As a positive control, we used the well-known interaction between TCR and ZAP70 in stimulated cells (Fig. 4C). To control the specificity of the reaction, either of the two primary Abs was omitted, and red dots were no longer detected (Fig. 4D, 4E). These controls also show that there is no cross-reactivity between the secondary Abs; thus, the red dots corresponded to the specific association of Kidins220 with the TCR in situ.

To better quantify the interaction between TCR and Kidins220, a slightly different protocol was followed, and the intensity of red fluorescent PCR products/cell was measured by flow cytometry (Fig. 4F). Again, Kidins220 was in close proximity to the TCR in untreated cells, and this association was reduced upon pervanadate stimulation.

Kidins220 is required for sustained Erk signaling and CD69 upregulation

In neuronal cells, Kidins220 is involved in sustained Erk signaling. To test whether Kidins220 influences Erk signaling in T cells, we generated 2B4 cell lines in which Kidins220 was stably knocked down by the expression of Kidins220-specific shRNAs using lentiviral vectors. Four stable lines were generated using different Kidins220-specific shRNA sequences. The stable line named shRNA-2 showed the strongest reduction in Kidins220 expression...
Kidins220 positively regulates Ca2+ influx

To test whether other TCR-induced signaling pathways are affected by silencing of Kidins220, we measured Ca2+ influx upon TCR stimulation. Kidins220 knockdown (shRNA-2) and control cells were loaded with the Ca2+-binding dye Indo-1. Stimulation was induced by the addition of an anti-CD3 Ab. Ca2+ influx was measured by the ratio of Ca2+-bound Indo-1/unbound Indo-1. Upon Ab stimulation, shRNA-2 cells showed less Ca2+ influx than did the control cells (Fig. 6A), whereas ionomycin-stimulated cells showed equal Ca2+ influx, indicating equal staining with Indo-1 (Fig. 6A).

Kidins220 knockdown reduces TCR-induced cytokine secretion

To further test the effect of Kidins220 downregulation on T cell activation, we measured the release of the cytokines IL-2 and IFN-γ upon T cell activation. The shRNA-2 or control cells were stimulated with anti-TCR Abs for 5 h, and the presence of the cytokines in the supernatant was measured using a bead-based cytokine assay (52). shRNA-2 cells showed slightly decreased IL-2 and IFN-γ production (52). As a control, anti-Kidins220 IP was performed with lysis buffer alone (lane 1). Signals from the L chain of the immunoprecipitating Ab used for the IP served as a loading control (left panels). Quantification of the band intensities of five experiments is displayed (right panel), error bars show mean ± SEM. (A) 2B4 cells were stimulated with 5 mM pervanadate for 5 min (gray curve) or were left untreated (black curve). Mean fluorescence intensity (MFI) is shown in the bar graph (right panel). Quantification of the MFI of triplicates is displayed; error bars show mean ± SEM. (B) 2B4 cells were stimulated with 5 mM pervanadate for the time points indicated or left untreated. Phospho-Kidins220 was detected by anti–p-tyrosine WB after an anti-Kidins220 IP. The H chain of the immunoprecipitating Ab served as a loading control. (C) 2B4 cells were stimulated with anti-TCR Abs for different time points and an anti–B-Raf IP was performed. The immunoprecipitates and the lysates were separated by reducing SDS-PAGE and WB done with anti–p-S259 Raf-1 Ab, which detects phosphorylation of its equivalent S365 in B-Raf, anti–B-Raf, anti–p-Mek, and p-Mek signals present in control cells were absent in shRNA-2 cells (Fig. 5A). However, at long stimulation times (30 and 40 min), the p-Mek and p-Erk signals present in control cells were absent in shRNA-2 cells. Kidins220 knockdown was shown by the amount of Kidins220 in control and shRNA-2 cells (Fig. 5B). These results indicate that Kidins220 is involved in sustained Erk signaling in T cells. Consequently, induced expression of the Erk target genes c-Fos and Egr-1 was absent in shRNA-2 cells (Fig. 5C). Likewise, TCR-triggered CD69 upregulation, an early T cell activation marker, was significantly reduced upon Kidins220 knockdown (Fig. 5D). These results demonstrate the importance of Kidins220-mediated sustained Erk signaling in T cell activation. A transient knockdown of Kidins220 using small interfering RNAs (siRNAs) also showed that the presence of Kidins220 is required for TCR-induced Erk phosphorylation and CD69 upregulation (Supplemental Fig. 3C–E).

Kidins220 positively regulates Ca2+ influx

To test whether other TCR-induced signaling pathways are affected by silencing of Kidins220, we measured Ca2+ influx upon TCR stimulation. Kidins220 knockdown (shRNA-2) and control cells were loaded with the Ca2+-binding dye Indo-1. Stimulation was induced by the addition of an anti-CD3 Ab. Ca2+ influx was measured by the ratio of Ca2+-bound Indo-1/unbound Indo-1. Upon Ab stimulation, shRNA-2 cells showed less Ca2+ influx than did the control cells (Fig. 6A), whereas ionomycin-stimulated cells showed equal Ca2+ influx, indicating equal staining with Indo-1 (Fig. 6A).

Kidins220 knockdown reduces TCR-induced cytokine secretion

To further test the effect of Kidins220 downregulation on T cell activation, we measured the release of the cytokines IL-2 and IFN-γ upon T cell activation. The shRNA-2 or control cells were stimulated with anti-TCR Abs for 5 h, and the presence of the cytokines in the supernatant was measured using a bead-based method (Bio-Plex). Upon stimulation, both cytokines were produced in lesser amounts in shRNA-2 cells compared with the control cells (Fig. 6B). Decreased TCR expression on the surface of shRNA-2 cells could be the cause for the lower Erk and Ca2+ signaling and reduced CD69, IL-2, and IFN-γ production (52). Thus, we tested the surface TCR levels in these cells by flow cytometry using anti-CD3 Abs. shRNA-2 cells showed slightly decreased surface TCR expression (Fig. 6A), whereas ionomycin-stimulated cells showed equal TCR expression, indicating equal staining with Indo-1 (Fig. 6A).
FIGURE 4. Pervanadate stimulation reduces Kidins220 binding to the TCR in situ. (A–E) 2B4 cells were stimulated with 5 mM pervanadate for 5 min or were left unstimulated. Cells were transferred onto microscope slides using cytopsin. After fixation and permeabilization, the PLA was performed to quantify the association between the TCR and Kidins220. Primary Abs used were goat anti-CD3ε (M20), rabbit anti-Kidins220, or mouse anti-ZAP70. Two negative controls, in which one of the primary Abs was omitted, were included (D, E). Nuclear DAPI staining is shown in blue and the TCR–Kidins220 interaction (A, B) or the TCR-ZAP70 interaction (C) is shown in red. Scale bars, 10 μm. (F) 2B4 cells were stimulated with 5 mM pervanadate for 5 min (red curve) or were left untreated (blue curve). Fixation, permeabilization, and the PLA reaction were performed in suspension. Graphs show the fluorescence intensity of the red channel, quantifying the Kidins220–TCR interaction. One sample, in which the anti-Kidins220 Ab was omitted, serves as a negative control (green curve). A rabbit anti-Kidins220 Ab (left panel) or a mouse anti-Kidins220 Ab (right panel) was used in combination with the goat anti-CD3ε Ab M20ε. (G) 2B4 cells were incubated with 5 μg anti-CD3 (145-2C11) and 5 μg anti-TCRβ (597-H57) Abs (lane 3) or without Abs (lane 2) on ice for 90 min. After extensive washing, Ab-bound surface TCRs were purified with protein G–coupled Sepharose beads and separated by reducing SDS-PAGE. A control IP was performed only in lysis buffer (lane 1). Kidins220 and ζ were detected by WB in the purified TCR samples and in the lysates.

Discussion
Sustained Erk signaling induced by Ag binding to the TCR is required for full activation of T cells and their effector functions, such as IL-2 production (20, 22, 23). It was reported that B-Raf mediates sustained Erk activation (18–20), but the molecular mechanism of how stimulation of the TCR is coupled to B-Raf is unknown. In this study, we identified Kidins220 as a novel protein involved in TCR signaling and showed that it binds to both the TCR and B-Raf and, thus, promotes sustained Erk activation.

Kidins220 was originally described as a protein specifically expressed in neurons (24, 25). Only very recently, Kidins220 was shown to be expressed in T cells (53), in line with our findings. We demonstrate in this study that silencing of Kidins220 expression by shRNA reduced the sustained TCR-induced Erk, which is particularly obvious at later time points (30 and 40 min). Likewise, Kidins220 is required in neurons for sustained Erk activation (26). Because Kidins220 associates with B-Raf in T cells and because B-Raf is required for sustained Erk activation in T cells (20), we suggest that Kidins220 binds to B-Raf, thereby sustaining Erk signaling. Indeed, for neurons it was suggested that Kidins220 might activate B-Raf via a CrkL–C3G–Rap1 complex (26). B-Raf stimulation by Rap1 is controversial (54). We propose an alternative pathway, in which Kidins220 is constitutively associated with the TCR and is tyrosine phosphorylated upon stimulation. Then, Kidins220 recruits Grb2 or Shc by specific interaction with their SH2 domains. Indeed, upon pervanadate stimulation of the TCR, Kidins220 was tyrosine phosphorylated in 2B4 T cells, as well as in neurons (25). Further, we performed a ScanSite in silico analysis and found that tyrosine 261 of Kidins220 within the fifth cytoplasmic ankyrin repeat (Fig. 2A) is part of a binding motif for Grb2 (ScanSite score 0.28) that is highly conserved in vertebrates. Thus, in T cells (and in neurons), Kidins220 might be phosphorylated upon TCR (or NGFR) triggering and then activate the canonical (Shc)-Grb2-SOS-Ras-B-Raf pathway by binding to both Grb2 and B-Raf.

In line with this, it was shown that B-Raf is activated by TCR signaling (4, 20, 21, 55), that B-Raf deficiency in T cells results in developmental defects in the mouse (21), and that B-Raf is critical for TCR-mediated ERK activation in T cells from rheumatoid arthritis patients (56). In this study, we show that, upon TCR stimulation, the inhibitory N-terminal 14-3-3 binding site of B-Raf (S365) was dephosphorylated, and B-Raf was hyperphosphorylated, again showing that B-Raf is a downstream-signaling molecule of the TCR.

In Kidins220 knockdown cells, the TCR-induced expression of the transcription factors c-Fos and Egr-1 was absent, most likely as a result of abrogated sustained Erk signaling. Indeed, prolonged expression of these transcription factors depends on sustained Erk activation, by first inducing their transcription and then by preventing degradation of the proteins (57–59).

In Kidins220 knockdown cells, the transient Erk activation after TCR triggering (3–5 min) was unaltered, indicating that Kidins220 is not necessary for Raf-1 activation. Indeed, it was shown that the TCR binds to Raf-1 (60), and this might contribute to the Kidins220-independent transient Erk activation (Supplemental Fig. 4). However, Kidins220 bound to Raf-1, and it is not clear whether Raf-1 interaction with Kidins220 is direct or via B-Raf, because B-Raf and Raf-1 are known to form heterodimers (39, 61). Because we show that Kidins220 also plays a role in the activation of calcium signaling, Kidins220 is a major element in the...
TCR-signaling network controlling several pathways that lead to T cell activation (Supplemental Fig. 4). In fact, reduced CD69, IL-2, and IFN-γ upregulation in the absence of Kidins220 was also seen. Kidins220 interacts with the TCR in the resting state and at short stimulation time points, and it might bind to B-Raf in the vicinity of the TCR. Surprisingly, after longer anti-TCR, MHC-peptide tetramer, or APC stimulation times (10 min), Kidins220 dissociates from the TCR. To our knowledge, all other described binding partners of the TCR show the opposite behavior: they only bind to the TCR upon stimulation. The dissociation that we observed by

FIGURE 5. Kidins220 is required for sustained Erk signaling and CD69 upregulation upon TCR triggering. (A) 2B4 shRNA-2 (stable Kidins220 knockdown) or control (containing a nontargeting shRNA control vector) cells were stimulated with anti-TCR Abs for the indicated time points or were left untreated. Cells were lysed, and proteins were separated by reducing SDS-PAGE. p-Mek, p-Erk, and actin were detected by WB (left panel). A quantification of the p-Erk/actin ratio after normalization is shown for three independent experiments (right panel). p, 0.05, Student t test. (B) shRNA-2 or control cells were lysed, and an anti-Kidins220 IP was performed. Kidins220 and the H chain of the anti-Kidins220 Ab were detected by WB. Anti-actin WB of an aliquot of the lysate served as a control. (C) shRNA-2 or control cells were stimulated with anti-TCR Abs for the indicated time points or were left untreated. Cells were lysed, and proteins were separated by reducing SDS-PAGE. c-Fos, Egr-1, and actin were detected by WB. (D) shRNA-2 or control cells were stimulated for 24 h with anti-TCR Abs or were left untreated. Cells were stained with a fluorophore-labeled anti-CD69 Ab, and mean fluorescence intensity (MFI) was measured by flow cytometry (left panel). Quantification of the MFI of triplicates is displayed (right panel), error bars show mean ± SEM. ***p < 0.0001, unpaired t test. ns, Nonsignificant.

FIGURE 6. Kidins220 knockdown reduces calcium signaling and TCR-induced cytokine production. (A) shRNA-2 (black) and control (nontargeting shRNA, gray) cells were stained with the dye Indo-1. The ratio of bound Indo-1/unbound Indo-1 was measured by flow cytometry. Stimulation was induced after 30 s of the measurements with the anti-CD3 Ab 145-2C11 (arrow). Samples treated with ionomycin served as a positive control. (B) shRNA-2 or control cells were stimulated with anti-TCR Abs for 5 h, and IL-2 and IFN-γ were measured in the cell supernatant using the Bio-Plex method. Error bars show mean ± SEM of triplicate samples. p < 0.05, unpaired t test. (C) shRNA-2 or control cells were stained with the fluorophore-labeled anti-CD3 Ab 145-2C11 and analyzed by flow cytometry (left panel). A quantification of the MFI of triplicates is shown (right panel), error bars show mean ± SEM.
co-IP experiments and PLA might be regulated by phosphorylation-related events, because pervanadate treatment led to massive detachment of Kidins220 from the TCR. One function of this dissociation at longer times might be to avoid negative-feedback pathways from downstream signaling to the vicinity of the TCR (62). Thus, Erk activation by Kidins220 that is not in the vicinity of the TCR might escape these feedbacks, offering a mechanism for the generation of sustained signaling.

Our finding that Kidins220 plays a role in TCR signaling might also shed light on the function of PKD in T cells, because Kidins220 is the only PKD substrate known (24), other than histone deacetylase 7 (63). PKD is recruited to the plasma membrane upon TCR activation (64), where it could phosphorylate Kidins220 and modulate signaling. Because Kidins220 binds to the αβTCR, pre-TCR, and γδTCR, it might regulate signaling in all T cell subsets. Using MS, we identified additional interaction partners of B-Raf and validated these interactions biochemically. For example, mTor is a kinase involved in controlling cell proliferation and motility in many cell types, including T cells (65, 66). Although, a combined B-Raf and mTor inhibitor therapy was suggested for tumors (67), no direct link between B-Raf and mTor signaling has been reported. We also found that B-Raf interacted with Rictor, a known binding partner of mTor (68). Further, we identified Dock2, a guanine nucleotide exchange factor that activates the small G protein Rac (69), and GM130, a Golgi matrix protein, as binding partners for B-Raf and Raf-1. Erk signaling from different locations in a T cell can have different outcomes (70, 71), and we contemplate that GM130 might be involved in targeting Raf-1 and B-Raf to the Golgi.

By showing that Kidins220 participates in TCR signaling, we enforce the view that signaling pathways in the nervous system and T cells are striking, suggesting that neurons and T cells use similar mechanisms to interpret extracellular cues to mount appropriate responses.

In conclusion, we identify Kidins220 as a novel element in regulating sustained Erk activation. T cell–decision processes rely on sustained versus transient Erk activation. Thus, Kidins220 might be involved in regulating T cell–activation outcomes. Further studies are required to explore the molecular details of the TCR–Kidins220–B-Raf interaction, and they might offer insight into mechanisms that regulate the differential activation of the map cascade by epidermal growth factor and nerve growth factor in PC12 cells. J. Biol. Chem. 276: 18169–18177.


