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Dok-4 is a novel negative regulator of T cell activation¹

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⁶ ***Abbreviations used in this paper:*** Dok, Downstream of tyrosine kinase; PH, pleckstrin homology; PTB, phosphotyrosine-binding.

Abstract

Dok-4 is a recently identified member of the Dok family of adaptor proteins, which are characterized by an amino-terminal pleckstrin homology domain (PH), a phosphotyrosine binding domain (PTB) and a carboxy-terminal region containing several tyrosines and poly-proline-rich motifs. Two members of the Dok family, Dok-1 and Dok-2 have already been described as negative regulators in T cells. However, the function of Dok-4, which is also expressed in T cells remains unknown. In this study, we report that Dok-4 is phosphorylated after TCR engagement and shuttled within the cytoplasm of T cells before being recruited to the polarized microtubule organizing centre (MTOC) after the formation of the immunological synapse. Loss-of-function experiments using RNAi constructs show that Dok-4 is a negative regulator of ERK phosphorylation, IL-2 promoter activity and T cell proliferation. Exogenous expression of wild-type Dok-4 induces a significant activation of Rap1, which is involved in the regulation of ERK. The PH domain of Dok-4 is required both for its cytoplasmic shuttling and relocalization as well as for its inhibitory properties on T cell activation. Thus, Dok4 represents a novel negative regulator of T cells.

Introduction

Adaptor molecules are proteins containing modular binding domains that are devoid of intrinsic enzymatic activity, but that serve as essential scaffolds for the formation of multimolecular signaling complexes in a variety of cell lineages, notably in T cells (1). Dok⁶ proteins are a growing family of adaptor molecules, containing an amino-terminal pleckstrin-homology (PH) domain, a central phosphotyrosine binding (PTB) domain and a carboxy-terminal part with several tyrosine phosphorylation sites leading to interactions with SH2 domains. The role of the first two identified Dok family members : Dok-1 and Dok-2, has been assessed in the T cell lineage (2). These proteins are tyrosine phosphorylated upon TCR triggering or the engagement of the costimulatory molecules (3-5). They are potent inhibitors of T cell activation via their binding to RasGAP, which regulates Ras activation, but also probably via their association with Src and Tec protein tyrosine kinases (PTKs) family members (6, 7).

Overexpression studies involving Dok-1 or Dok-2 molecules provided evidence that these adapter proteins inhibit PLC γ -1 and Ras-ERK/MAPK (extracellular signal-regulated kinase / mitogen-activated protein kinase) signals and can prevent IL-2 promoter activation (4, 7). Knockdown of Dok-1 and Dok-2 in human primary T cells reveals their negative role on T cell functions (3). Upon TCR triggering, T cells from mice lacking both Dok-1 and Dok-2 displayed increased IL-2 production, as well as proliferation (8).

Additional five members of this Dok-related family have been identified and named from Dok-3 to Dok-7 (9-16). Among these members, only the gene encoding for Dok-4 is expressed in resting T cells (14). Based on analysis of phylogenetic trees and exon/intron structure of Dok family members, Dok-4 is part of a group that is distinct from that including Dok-1 and Dok-2. In non-immune cells, Dok-4 appears to be a substrate of c-Ret or insulin receptor tyrosine kinase and Src protein tyrosine kinase family members (11, 12). Functional

studies of Dok-4 have yielded conflicting results with two reports claiming that it enhanced Ret or insulin receptor-mediated ERK activation (11, 12) and one other report finding that it inhibited Ret and Src PTKs-mediated activation of the ERK substrate: Elk-1 (13). These apparent discrepancies may relate to differences in cell types, stimuli, and readouts used in the various studies. In Ret signalling, Dok-4 leads to activation of the small G protein Rap1, which induces a sustained activation of ERK in nerve cells (17). Therefore, it would be useful to extend the studies of Dok-4 to other cell types that expressed it, including T cells, where its function remains unknown. Here, we investigated if Dok-4 could be a substrate of the PTKs involved during T cell activation. We report that Dok-4 accumulates in an intracellular vesicular compartment that is recruited to the polarized MTOC (microtubule organising centre) during T cell activation and that it requires its PH domain to adopt this particular localization. The PH domain was also necessary for Dok-4 ability to inhibit ERK activation and IL-2 promoter activity in T cells.

Material and Methods

Culture cells and transfection

Jurkat JA16 T cell subclone and Raji B cell lymphoma were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, and 1 mM sodium-pyruvate. L cells expressing human B7.1 (B7.1 L cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine and 1 mM sodium-pyruvate (18). Hut-78 T cells were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum (3).

PBMC from healthy donors (Etablissement Français du Sang, Marseille, France) were isolated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradients before purification of the CD4⁺ T cell subset using the naïve CD4⁺ T cell isolation kit (Miltenyi Biotec, Paris, France).

Plasmid constructs

The construct β DNA4HADok-4 (Dok-4) corresponding to the wild-type full-length human Dok-4 cDNA tagged with HA epitope in the 5' end, was generated by subcloning of pGEM-T easy vector containing HA tagged Dok-4, described previously (14), into the β DNA4 vector, using the restriction site NotI. The β DNA4 vector corresponds to the pCDNA3 vector (Invitrogen, Cergy Pontoise, France) where its promoter region has been replaced by the promoter region of chicken β -actin (7). Expression plasmid β DNA4HA- Δ PHDok-4 (Δ PHDok-4), containing the human Dok-4 cDNA tagged with HA epitope in the 5' end lacking the PH domain (deletion of aa 1-119), was generated by PCR amplification of β DNA4HADok-4 using the following primers: sense primer 5'-atgtaccatacagcgtcccagactacgctagtctgggagaacctgacctc-3' (containing HA epitope), antisense primer 5'-tcactgggatggggtcttggcctcac-3'.

Green Fluorescence Protein (GFP) fusion protein expression vectors, Dok4-GFP and Δ PHDok4-GFP were obtained by removing HA epitope and stop codon from β DNA4HADok-4 and β DNA4HA- Δ PHDok-4 respectively, and introducing cDNAs into the BamHI/KpnI sites of the pEGFP-N1 vector (Clontech, Palo Alto, CA).

The PH domain tagged GFP (PHDok4-GFP) (aa 1-122) was amplified from β DNA4HADok-4 with the following primers: sense primer 5'-ccgaattccatggcgaccaatttcagtg-3' and antisense primer 5'-cggtggatcccgtcccagactgatgtcgtga-3'. The PCR product was subcloned into BamHI/KpnI sites of pEGFP-N1 expression vector. The expression vector Dok4PTB*-GFP encodes for a Dok4 -GFP fusion protein containing point mutations into the PTB domain of Dok-4, corresponding to the substitution of Arginine 185 and 186 in Alanine (R185/186A). These mutations were previously described for Dok-1 (19). They were obtained using QuickChange® system (Stratagene, Amsterdam, NL) as from the Dok4-GFP expression vector, with the following primers sense 5'-ccctctgctcaactgggccgcctatggccggg-3' and antisense 5'-gcatccggccatagggccgcctagcagcag-3' (corresponding mutations R185/186A are underlined). The expression vector Dok1-GFP encoding for a Dok1-GFP fusion protein was generated by PCR amplification from β DNA4HADok-1 using the following primers: sense 5'-agatctcgagctatggacggggctgtgatggag-3' and anti-sense 5'-ggccaagtctgagggtccaccgtgacgggtaccgcg-3'. The PCR products were sequenced and cloned in pEGFP-N1 XhoI/KpnI.

The promoter assay plasmids pIL-2-Luc composed of IL-2 promoter, fused to firefly luciferase reporter gene and p β -actin-Rluc composed of β -actin promoter, fused with *Renilla* luciferase gene were previously reported (20).

For siRNA, the pH1shDNA plasmids were derived from the pH1-XhoI plasmid (a descendant of pBlueScript KS+) and contained a XhoI-flanked fragment containing the human H1 promoter amplified by PCR from human blood mononuclear cell genomic DNA, the template

small hairpin DNA (shDNA) sequences encoding siRNAs. The hairpins contained the 19 nt sense sequence of the target transcript which was separated by a 9 nt loop from the 19 nt antisense sequence of the target mRNA and followed by 5 thymidines as a termination signal, as previously described (21). All constructs were verified by sequence analysis. Two sequences for the hairpins RNA were shown: 5' ccccagacagatcgcttcaatgttcaagagacattgaagcgatctgtctgtttttggaaa^{3'} (19 nt corresponding to pb 403- 421) which reduces the expression of Dok-4 (more than 50% in immunoblot analysis, data not shown). It corresponds to pBChH1-RNAiDok4. The second sequence 5' cccattactcgtatccctgcattcaagagatgcagggatacgagtaatgtttttggaaa^{3'} (19 nt corresponding to pb 760- 778) which does not reduce the expression of Dok-4. This plasmid will be used as a control in our RNAi experiments (pBChH1-Control).

Antibodies and products

CD3 mAbs 289, OKT3 and CD28 mAb 248 have been already reported (18). Polyclonal anti-Dok1 antibodies have been described previously (20). Anti-Dok2 mAb was purchased from BD Transduction laboratory (Le-Pont-De-Claix, France). Polyclonal anti-Dok4 antibodies used in western blot experiments were purchased from Abgent (San Diego, CA) or described previously (13). Polyclonal anti-Dok4 antibodies used in immunoprecipitation experiments were described previously (13). Anti-phosphotyrosine (PY) 4G10 mAb was purchased from Millipore (Molsheim, France). Polyclonal anti-GFP antibodies and anti- α tubulin mAb were purchased from Abcam Limited (Cambridge, UK). Anti- γ tubulin mAb and anti-Rap1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-ERK, anti-ERK, anti-phospho-PLC γ 1, anti-PLC γ 1, anti-phospho-JNK, anti-phospho-p38 polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Super-antigen SEE, CellTrackerTM Orange CMTMR (5-(and-6)-(((4-chloromethyl)-benzoyl-amino)tetramethyl-rhodamine) and Ionomycin were respectively

purchased from Toxin Technology Inc. (Sarasota, FL), Molecular Probes (Eugene, OR) and Calbiochem (VWR International SAS, Fontenay-sous-Bois, France). PMA and poly-L-lysine were purchased from Sigma (St Louis, MO).

Immunofluorescence staining

To distinguish Raji B cells from Jurkat T cells, Raji cells were preincubated in RPMI 10% FCS containing 10 μM CellTrackerTM Orange CMTMR for 30 min at 37 °C, washed and resuspended ($5 \cdot 10^6$ cells/ml) in RPMI 50mM Hepes, as indicated. Raji cells were then incubated for 20 min with or without 5 $\mu\text{g}/\text{ml}$ SEE. Transfected Jurkat cells were mixed at a 2:1 ratio with Raji cells pulsed with or without SEE, and then incubated at 37°C for 45 min. After stimulation, the cells were deposited onto poly-L-lysine coated coverslips, let sediment for 3 min, and then centrifugated at 300 rpm for 1 min. The conjugates were fixed for 5 min in methanol. As indicated, immunofluorescence staining was performed. Cells were permeabilised in PBS 0.1% Triton for 10 min and saturated in PBS 5% BSA for 20 min. The staining with the appropriate antibodies (at the dilution 1:500 in PBS 5% BSA) was performed for 20 min, using goat anti-mouse Alexa 594 as secondary antibody (Molecular Probes Inc., Eugene, OR). Slides were mounted with fluorescent mounting medium (Dako Corporation, Carpinteria, CA). Images were taken and processed using a confocal microscope (LEICA TCS NT Confocal Microscope, Heidelberg, Germany).

Stimulation and cell lysis

Jurkat cells ($10 \cdot 10^6$) were stimulated at 37°C in RPMI 50mM Hepes. Stimulations were carried out for the indicated times with Raji cells (ratio 2:1) pulsed or not for 20 min at 37°C with SEE at 5 $\mu\text{g}/\text{ml}$, CD3 mAb 289 at 10 $\mu\text{g}/\text{ml}$, CD28 mAb CD28.2 at 10 $\mu\text{g}/\text{ml}$ or CD28 ligands expressed at the surface of murine L cells (L-B7.1, ratio 2:1). The cells were pelleted in a microcentrifuge and lysed in buffer containing 50 mM Hepes (pH 7.4), 1% Nonidet P-40,

150 mM NaCl, 20 mM NaF, 20 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml protease inhibitors (protease inhibitors cocktail, Sigma, St Louis, MO) and 1 mM Na₃VO₄ for 10 min at 4°C, then centrifugated at 13000 rpm for 10 min at 4°C.

For fractionation experiments, the pellets were lysed using the ProteoExtract™ Subcellular Proteome Extraction Kit™ (Calbiochem, VWR International SAS, Fontenay-sous-Bois, France), according to the manufacturer's instructions. The efficiency of subcellular fractionation from the Jurkat T cell lysates has been investigated by SDS-PAGE and immunoblotting of selected marker proteins. The CD28 molecule was exclusively found in the membrane fraction, the heat shock protein (HSP)-90 was detected in the cytosol and nucleus fractions, the myc protein was detected in the nucleus fraction and cytoskeleton compounds such as actin, vimentin and α/γ -tubulin were present in both cytoskeleton and cytosol fractions (data not shown).

Immunoprecipitation and western blotting

For immunoprecipitation, lysates were clarified and incubated with purified anti-GFP or anti-Dok4 polyclonal antibodies coupled to protein A-Sepharose, for 2 h at 4 °C. Immunoprecipitates were washed two times in 1 ml of lysis buffer and then boiled in reducing SDS gel sample buffer for 5 min. Samples were resolved by standard 10% SDS-polyacrylamide gels.

For immunoblotting, membranes were blocked and probed with specific antibodies. Blots were then incubated with the appropriate secondary antibodies, anti-rabbit IgG or anti-mouse IgG (Amersham Biosciences Ltd, UK), both conjugated with horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences Ltd, UK). Protein bands on Western blots were quantified with the ImageJ software.

Rap assays

Rap activity was determined as described previously using a GST-RalGDS-RBD fusion protein (22). Briefly, Jurkat cells (10×10^6) were stimulated as indicated with 10 $\mu\text{g/ml}$ CD3 mAb (289), lysed in standard NP40 buffer and clarified by centrifugation at maximal speed in an Eppendorf centrifuge for 10 min at 4°C. Two μg of purified GST-RalGDS-RBD coupled to glutathione-sepharose beads were added to the supernatant and incubated at 4°C for 60 min. Beads were washed four times in lysis buffer and denaturated with SDS.

Luciferase assays

Jurkat cells (10×10^6) were electroporated at 960 μF and 250 V using a Bio-Rad Gene Pulser with 12 μg of pIL-2-Luc or NF-AT-Luc plasmid, 1 μg of p β -Actin-Rluc, and 15 μg of the other expression plasmids. Stimulations with APC were carried out for 8 h with Raji cells (ratio 1:1) pulsed for 20 min at 37°C with SEE at 10 ng/ml. Stimulation with antibodies were carried out for 16 h, using CD28 mAb (248; ascites fluid at a 1:400 dilution) and CD3 mAb (289; ascites fluid at a 1:4000 dilution). Stimulations with drugs were carried out for 16 h, using PMA (50 ng/ml) and Ionomycin (2 $\mu\text{g/ml}$). Following cell lysis, proteins were quantified by Bradford reagent (Bio-Rad), and 30 μl of cell lysates were then subjected to dual luciferase reporter assay according to the manufacturer's instructions (Promega France SARL, Charbonnières, France). Results were corrected by the activity of firefly luciferase standardized by that of Renilla luciferase and quantification of proteins in lysates.

CD69 expression and proliferation assay

CD4⁺ PBL-T cells were transfected with the Amaxa Nucleofector technology (Köln, Germany) according to the manufacturer's instructions and were used 18 h after transfection

(<http://www.amaxa.com/>). CD4⁺ T cells (10⁵ cells/well) were plated in Cellstar U-bottom 96-well culture plates (Greiner Bio-One SARL, Poitiers, France) and left unstimulated or stimulated with CD3 mAb (OKT3 at 0.5 µg/ml) plus CD28 mAb (CD28.2 at 0.5 µg/ml) or PMA at 1 ng/ml plus Ionomycin at 1µg/ml. After 24 h of culture, T cells were incubated with PE-conjugated CD69 mAb from BD Biosciences (Pont-de-Claix, France). The detection of CD69 expression was done by flow cytometry. After 72 h of culture, wells were pulsed with 1 µCi [³H]-thymidine (Amersham France, Les Ulis, France) for the remaining 24 h and then harvested onto glass fiber filters. Thymidine incorporation was measured in a direct beta counter (Matrix 9600, Packard Instruments, France).

Results

Expression and intracellular localization of Dok-4 proteins in T cells

We have previously reported DOK4 gene expression in resting T cells (14). An anti-Dok4 polyclonal antiserum (see “Materials and Methods”) was produced to determine the levels of Dok-4 protein in cells including T cells. COS cells transiently transfected or not with an expression vector containing the Dok-4 cDNA demonstrated the specificity of this antibody. As shown in Fig. 1A, strong expression of a 37 kDa protein, consistent with the expected MW of Dok-4, was detected in lysates from Dok4-transfected compared to control COS cells. The weak signal detected in non-transfected COS-7 cells was thought to correspond to endogenous Dok-4 expression, which had been previously reported in several epithelial cell lines (13). Dok-4 protein was also detected in peripheral blood T cells (PBL-T) and in the Jurkat T cell line confirming previous mRNA expression data (14). To determine the subcellular localization of Dok-4 in T cells, three different fractions of Jurkat T cell lysates corresponding to cytosol, membranes or cytoskeleton, were analyzed by anti-Dok4 immunoblotting (Fig. 1B, top panel). Endogenous Dok-4 was detected only in the cytosol and cytoskeleton fractions and was absent from membranes. This distribution pattern paralleled that of actin, vimentin and α/γ -tubulin that are present in both cytoskeleton and cytosol fractions (data not shown). Unfortunately, using different polyclonal anti-Dok4 antibodies, we were not able to detect the endogenous Dok-4 protein in T cells by confocal microscopy. To determine the intracellular localization of Dok-4, we produced a Dok4-GFP fusion protein, which we expressed in Jurkat T cells. Anti-GFP immunoblots confirmed that the transiently expressed Dok4-GFP was largely contained in the cytosol and in the cytoskeleton fractions like the endogenous protein (Fig. 1B, top versus bottom panel). Under confocal microscopy, Dok4-GFP was primarily localized in a condensed area of Jurkat T cell cytoplasm (Fig. 1C). Since Dok-4 contains a PH domain that might be recruited by phosphoinositides, localization

experiments were duplicated in a T cell line with a normal phosphatidylinositol 3-kinase (PI3K) activation, Hut-78. Consistent with the previously reported insensitivity of the Dok-4 PH domain to the activation of PI3K (23), a similar distribution of Dok4-GFP was observed in both Jurkat and Hut-78 T cells (Fig. 1C). Notably, the level of Dok4-GFP overexpression in these experiments was moderate, as documented by comparison of the 62 kDa Dok4-GFP fusion protein versus the 37 kDa endogenous Dok-4 signal, on the same immunoblot (Fig. 1D). These observations confirm that Dok-4 is expressed in specific compartments in T cells, prompting further investigation into the effect of T cell activation upon Dok-4.

Dok-4 is tyrosine phosphorylated upon TCR stimulation

Other members of Dok family such as Dok-1 are substrates of protein tyrosine kinases (PTKs) in T cells (4, 7, 24). In Jurkat T cells, Dok-1 is prominently tyrosine phosphorylated upon engagement of CD28 by its B7-1 (CD80) ligand, but poorly phosphorylated upon TCR stimulation with CD3 mAbs (25). Therefore, we examined the tyrosine phosphorylation state of Dok-4 under TCR or CD28 stimulation. Jurkat cells were stimulated with CD3 mAbs or with L cells expressing B7-1 ligand. Lysates obtained from these cells were subjected to immunoprecipitation with anti-Dok4 (Fig. 2A, top panels) or anti-Dok1 (Fig. 2A, bottom panels) antibodies followed by anti-phosphotyrosine immunoblotting. As previously described, Dok-1 was more prominently phosphorylated upon CD28 stimulation than TCR stimulation (Fig. 2A, bottom panels). In contrast, Dok-4 was tyrosine phosphorylated only when the TCR/CD3 complex was stimulated, though detection of this phosphorylation required long exposure of the immunoblots. TCR-mediated phosphorylation was also detectable in Hut-78 T cells (supplementary Fig. 1). To determine the phosphorylation state of Dok-4 during TCR-mediated cell-cell contacts, Jurkat T cells expressing Dok4-GFP or Dok1-GFP were activated by superantigen (SEE)-pulsed Raji B cells as APCs. Cell lysates were

subjected to immunoprecipitation with anti-GFP antibodies followed by anti-phosphotyrosine immunoblotting. Basal tyrosine phosphorylation of both overexpressed Dok-1 and Dok-4 proteins was detectable in unstimulated Jurkat cells (Fig. 2B). As previously described (25), tyrosine phosphorylation of Dok-1 increased more prominently in Jurkat T cells in the absence than in the presence of a TCR stimulus (Fig. 2B, bottom panels). In contrast to Dok-1, tyrosine phosphorylation of Dok-4 was most strongly increased upon presentation of SEE by Raji B cells (Fig. 2B, top panels). Dok-4 phosphorylation was still detectable after 20 min of contact with SEE-pulsed Raji B cells (supplementary Fig. 2). Thus, these data suggest that the Dok-4 protein is phosphorylated upon TCR engagement.

Dok-4 relocates to the T cell MTOC during APC contact.

Several intracytoplasmic PTK substrates are recruited to the T cell plasma membrane in the context of a T cell – APC contact (26, 27). The cell contact zone has been termed the "Immunological Synapse" (IS) (28). To determine whether Dok-4 is recruited to the IS, we analyzed by confocal microscopy, cell contacts between Jurkat T cells expressing Dok4-GFP and SEE-pulsed Raji B cells labelled in red with a celltracker dye (Fig. 3A). Twenty minutes after contact, Dok-4 protein was located in an intracytoplasmic compartment opposite to the IS. More prolonged contacts (45 min) resulted in the relocalization of Dok-4 in close proximity to the IS. This change in Dok-4 localization did not occur in the absence of antigen, suggesting that it was induced by TCR stimulation. This late distribution pattern evoked similarities with a polarized MTOC, which has been described before in this position (29). To determine whether Dok-4 was located within the MTOC, the centrosomal protein γ -tubulin, a key factor in microtubule nucleation, was used as a marker (30). In support of Dok-4 localization in the MTOC, we found that, in cell fractionation experiments, Dok-4 and γ -tubulin presented the same distribution pattern (Fig. 3B). To confirm this, confocal analysis

was performed in cell conjugates formed between Dok4-GFP-transfected Jurkat T cells and SEE-pulsed Raji B cells. In unstimulated T cells, γ -tubulin was condensed in a cytoplasmic dot-like structure typical of a MTOC, whereas Dok-4 was in a distinct compartment in close proximity to the MTOC (Fig. 3C, top panels). Upon contact with SEE-pulsed Raji B cells (time of contact: 20 - 45 min), a polarized MTOC was detected in the vicinity of the IS on both T cell and APC side (Fig. 3C, middle and bottom panels). During the early phase of cell contact formation, Dok-4 was preferentially found opposite to the IS and MTOC in a membrane or sub-membrane compartment (Fig. 3C, middle panels). Later on, Dok-4 re-localized within the MTOC itself (Fig. 3C, bottom panels). Upon synapse formation, tyrosine phosphorylation of Dok-4 was consistently detectable until Dok-4 joined the polarized MTOC, at which point Dok-4 was no longer phosphorylated (supplementary Fig. 2 & Fig. 3D). These results indicate that Dok-4 is specifically shuttled during T cell activation, being first excluded from the IS, but then moving back to its vicinity into the polarized MTOC after a phase of transient recruitment at the plasma membrane (Fig. 3C & Fig. 3D). Nocodazole treatment (a microtubule-disrupting drug) confirmed that Dok-4 migration to the vicinity of the IS was dependent upon microtubule network formation (supplementary Fig. 3).

The PH domain regulates Dok-4 localization in T cells

Dok-4 contains two protein:protein interaction domains, the PH and PTB domains (11, 12, 14). Both domains are important for Dok-4 plasma membrane localization in epithelial cells (13). To test the role of these domains on Dok-4 localization in T cells, we generated different mutants of Dok-4 fused to GFP, lacking either the PH domain (Δ PHDok4-GFP) or the PTB and the carboxy terminal part (PHDok4-GFP). We also developed a PTB mutant of Dok-4 containing a substitution of critical residues of the PTB domain (Dok4PTB* -GFP), these arginine to alanine mutations are usually able to abolish the binding functions of the PTB

domains as described for the PTB domain of Dok-1 (19). The localization of the different Dok-4 mutants in unstimulated Jurkat T cells or in contact with SEE-pulsed Raji B cells, was determined by confocal microscopy. Deletion of the PH domain (Δ PHDok4-GFP) resulted in a diffuse cytosolic distribution in stimulated or unstimulated Jurkat cells (Fig. 3D, top panels). The isolated PH domain of Dok-4 (PHDok4-GFP) was localized in a cytosolic and membrane fraction, and was not recruited to the MTOC during IS formation (Fig. 3D, middle panels). Point mutations in the PTB domain (Dok4PTB* -GFP) did not alter the distribution of Dok4 (Fig. 3D, bottom panels). These results indicate that the PH domain of Dok4 is necessary for the basal localization of Dok4, and for its recruitment in the MTOC compartment during T cell / APC junction.

Dok-4 regulates T cell signaling

To determine the role of the Dok-4 molecule in signaling events downstream of TCR stimulation, we analyzed ERK1/2 activation in Jurkat cells upon contact with SEE-pulsed Raji B cells. Different research groups reported that Dok-4 was involved in ERK activation and downstream transcription factors such as Elk-1 (11-13, 17). However studies found Dok-4 to be an activator of ERK pathway in CHO cells or nerve cells (11, 12, 17) and other studies found that Dok-4 inhibited the activation of Elk-1 in Caco-2 epithelial cells (13, 31). To resolve this issue in T cells, we developed an alternative approach to knock down Dok4 expression using RNA interference (RNAi). As shown in Fig. 4A, transfection with a plasmid containing a Dok-4 RNAi sequence resulted in almost complete inhibition of the endogenous expression of Dok-4 in Jurkat T cells after one day whereas transfection with an empty vector or a construct containing an inefficient Dok-4 RNAi sequence did not affect Dok-4 expression. Expression of Dok-1 and Dok-2 was not affected in Dok-4 RNAi-treated cells (Fig. 4A, middle and bottom panel), confirming the specificity of Dok-4 RNAi. In Jurkat cells

stimulated by Raji B cells, ERK phosphorylation was detected only upon presentation of SEE (Fig. 4B, top panels). In Dok-4 RNAi-treated Jurkat cells, TCR engagement resulted in enhanced phosphorylation of ERK as compared to control RNAi-treated cells. In contrast, tyrosine phosphorylation of PLC- γ , was not altered by Dok-4 RNAi (Fig. 4B and 4C, bottom panels). The other MAPK pathways involved in T cell activation were also examined and relative protein phosphorylation level was calculated (supplementary Fig. 4). This showed that JNK phosphorylation was mildly altered by Dok-4 RNAi (JNK phosphorylation is decreased at 30 min), whereas surprisingly p38 MAPK phosphorylation was decreased in the presence of downregulated Dok-4 (Fig. 4C, center panels).

In complement to the Dok-4 RNAi approach, we examined the effect of Dok-4 overexpression on MAPK pathways in Jurkat T cells. As shown in Fig. 5A, overexpression of Dok-4 markedly decreased ERK phosphorylation induced by CD3 plus CD28 engagement (relative phosphorylation intensity is shown in supplementary Fig. 5). Consistent with Dok-4 RNAi experiments (Fig. 4C), Dok-4 overexpression appeared to slightly increase JNK and p38 MAPK phosphorylation under CD3 plus CD28 co-stimulation (Fig. 5A and supplementary Fig. 5). In contrast to wild-type Dok-4, overexpression of the PH-deleted mutant of Dok-4 induced an increase of ERK activation and it had little effect in general on activation of the other MAPK pathways (Fig. 5A, right side and supplementary Fig. 5). Taken together these results suggest that, in T cells, Dok-4 negatively regulates ERK activation, but positively regulates p38 MAPK activation. Because Dok-4 has been reported to activate the Ras-related GTPase Rap1 in nerve cells (17) and because Rap1 negatively regulates ERK activation in T cells (32), we investigated the impact of Dok-4 overexpression on Rap1 activation in T cells. Rap1 activity assays were performed on cell lysates of Jurkat T cells transfected with wild-type Dok-4 or the PH-deleted mutant of Dok-4 (Fig. 5B). Rap1 was activated upon CD3 stimulation and this activation was enhanced in the presence of Dok-4

overexpression. In contrast, overexpression of the PH-deleted mutant of Dok-4 blocked Rap1 activation in TCR-activated T cells. Thus, activation of Rap1 by Dok-4 could plausibly account for our observation of Dok-4-mediated inhibition of ERK in T cells.

Dok-4 inhibits T cell function

To further determine the role of Dok-4 on T cell activation, we examined IL-2 promoter activation. We had previously shown that SEE-pulsed Raji B cells induced Jurkat cells to transcribe a reporter luciferase gene under the control of an IL-2 promoter sequence (33). In RNAi-treated Jurkat cells, a decrease of approximately 90% in Dok-4 protein expression was achieved (Fig. 6A, right panels) and was accompanied by increased activity of the IL-2 promoter upon contact with SEE-pulsed Raji B cells (Fig. 6A, left panel). These results were consistent with the observed effect of Dok-4 RNAi on ERK activation (Fig. 4B and 4C). Conversely, overexpressing Dok-4 in Jurkat cells (Fig. 6B) reduced IL-2 promoter activity induced by CD3+CD28 co-stimulation. However, Dok-4 overexpression had no impact on IL-2 promoter activity induced by a stimulus (PMA + Ionomycin treatment) that bypasses the early steps in TCR signaling. In contrast to wild-type Dok-4, overexpression of the PH-deleted mutant of Dok4 did not inhibit IL-2 promoter activation (Fig. 6B, left panel). Anti-HA epitope tag immunoblotting confirmed that similar levels of wild-type Dok-4 (HADok4) and PH-deleted mutant (HA Δ PHDok4) were obtained (data not shown). Therefore, deletion of the PH domain abolished the repressive action of Dok-4 on IL-2 promoter activity.

Primary CD4⁺ PBL-T cells isolated from human peripheral blood were also examined. Dok-4 siRNA sequences were introduced in these cells by nucleofection technology, resulting in an approximately 60% reduction in Dok-4 expression after 24 hours (Fig. 6C, right panel). Dok-4 inhibition resulted in an increase of CD69 cell surface expression (Fig. 6C, left panel) and of T cell proliferation (Fig. 6D) in response to CD3+CD28 co-stimulation. Altogether, results

show that Dok-4 is a negative regulator of ERK phosphorylation, IL-2 promoter activation and proliferation in T cells.

Discussion

In this report, we demonstrate that a novel Dok family member Dok-4, is expressed in specific cytoplasmic compartments in T cells, is phosphorylated in response to TCR engagement and negatively regulates T cell activation.

Having previously shown that Dok-4 mRNA is expressed in resting T cells (14), we have now confirmed that Dok-4 protein is expressed in these cells and that it undergoes tyrosine phosphorylation upon TCR stimulation (Fig. 2 and supplementary Fig. 1). Of note, as compared to phosphorylation of Dok-1 and 2, which is quite robust following T cell stimulation (3), tyrosine phosphorylation of Dok-4 was more subtle and was consistently more difficult to detect. In Dok-1 and Dok-2, most sites of tyrosine phosphorylation appear to be contained within the C-terminal region. In contrast, most tyrosine residues within the C-terminal region of Dok-4 sequence are predicted to be poor substrates for PTKs (data not shown). Indeed, the deletion of this C-terminal region did not alter the tyrosine phosphorylation of Dok-4, suggesting that some tyrosine residues in the PH or PTB domain are accessible to the PTKs (13). Among the PTKs so far shown to phosphorylate Dok-4 (11-13), only members of the Src family, such as Fyn, are involved in TCR signaling events.

Subcellular localization might be a limiting factor for tyrosine phosphorylation of Dok-4 in T cells. Dok-4 was primarily located at the vicinity of the MTOC, as identified by γ -tubulin staining (Fig. 3C). Both γ -tubulin and endogenous Dok-4 accumulated in cytoskeletal fractions (Fig. 3B). Following SAg presentation, Dok-4 was initially located at the distal pole of the immunological synapse. Later on, Dok-4 accumulated at the polarized MTOC in the vicinity of the immunological synapse (Fig. 3). Dok-4 was also transiently detected at the plasma membrane (Fig. 3C), which could permit the phosphorylation of Dok-4 by the Src kinase members present in the inner leaflet of the plasma membrane (supplementary Fig. 2).

However, the exact identity of the subcellular compartments containing the Dok-4 adaptor molecule remains under investigation.

Following a prolonged cell contact, Dok-4 was located at the polarized MTOC (Fig. 3C). Several signaling molecules involved in T cell activation, such as protein kinases (like PKC β , FAK, or Pyk-2) colocalize with the MTOC (34, 35). To address the mechanisms responsible for targeting Dok-4 to these intracellular compartments, we examined the role of its PH domain. PH domains are generally considered as phosphoinositide-binding modules and have often been implicated in recruitment of proteins to the plasma membrane (36). Some molecules like the four-phosphate-adaptor protein 1 and 2 (FAPP1 and FAPP2) accumulate via their PH domains in intracellular compartments such as the trans-Golgi network (37). The PH domain of Dok-4 is important to localize Dok-4 at the plasma membrane of the epithelial cells (13). Here, we show that, in T cells, Dok-4 localizes to a unique intracellular compartment through the action of its PH domain (Fig. 3D). However, an isolated Dok-4 PH domain alone could not accumulate in the same intracellular compartments, suggesting that cooperation with another region of Dok-4 is required. This is not surprising given evidence that other PH domains require the cooperative action of another domain for subcellular targeting (38-40). Indeed, Dok-4 itself requires both its PH and PTB domains in order to localize at the membrane in epithelial cells (13). However, using a point mutant of the PTB domain, we show that the localization of Dok-4 to its unique intracellular compartment in T cells appears to be independent of PTB domain function (Fig. 3E). Since specific phosphoinositides control the correct location and timing of many trafficking events (41) and since PH domains are generally recognizing a variety of phosphoinositides (42), defining the phosphoinositide-binding specificity of the Dok-4 PH domain will be important in order to identify its role mediating subcellular targeting of Dok-4.

Our studies show that, in addition to its role in subcellular targeting, the PH domain is also essential to mediate the inhibitory effect of Dok-4 on T cell activation. It has previously been suggested that Dok-4 regulates the ERK signaling pathway (11-13), but conflicting results exist as to its stimulatory versus inhibitory impact. Using a Dok-4 RNAi strategy as well as an overexpression approach, we showed that Dok-4 negatively regulates the activation of ERK without altering other key signalling events such as the tyrosine phosphorylation of PLC- γ 1 or the activation of JNK. How Dok-4 targets the ERK pathway is unclear because, unlike Dok-1/2, Dok-4 does not bind p120RasGAP, an inhibitor of the Ras/ERK pathway (11). However, as had previously been shown in nerve cells stimulated through Ret (17), we found that Dok-4 activates Rap1 downstream of TCR stimulation in T cells (Fig. 5B). In T cells, Rap1 has been generally implicated in the regulation of cell adhesion (43), but it has also been reported to antagonize ERK activation in anergic T cells, thus contributing to repression of IL-2 transcription in these cells (32, 44). To further dissect the inhibitory mechanisms involved in Dok-4 action, it will be essential to identify the partner molecules for Dok-4 in T cells, which are currently unknown.

Our findings, taken together with previous studies of Dok-1 and Dok-2 (3, 4, 7, 8), demonstrate that the three Dok-related adaptors expressed in resting T cells (Dok-1/ -2 / -4) are negative regulators of T cell activation, although they likely perform this function through different mechanisms.

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Figure legends

Figure 1. Subcellular localization of Dok-4.

(A) Western blot anti-Dok4 was performed on whole cell lysate of Cos7 cells, Cos7 expressing HADok4, Jurkat cells and peripheral blood T cells (PBL-T).

(B) Fractionation experiment was performed on Jurkat cells or Jurkat cells transfected with Dok4-GFP. 50 µg of lysates were separated by SDS-PAGE and identified by immunoblotting with anti-Dok4 antibodies for untransfected Jurkat cells (Dok4 WB, top panel) or anti-GFP antibodies for transfected cells (GFP WB, bottom panel). Data are representative of three independent experiments.

(C) Jurkat or Hut-78 cells were transfected with Dok4-GFP. 24 h after transfection, cells were plated on poly-L-lysine coated coverslip. 50 GFP⁺ cells are examined and a representative distribution pattern is shown.

(D) Western blot anti-Dok4 was performed on whole cell lysate of Jurkat or Hut-78 expressing Dok4-GFP, arrowheads are indicating the position of the endogenous Dok-4 and the GFP fusion protein.

Figure 2. Dok-4 is tyrosine phosphorylated upon TCR stimulation.

(A) Jurkat cells (10^7) were left unstimulated (NS) or stimulated with CD3 mAb (CD3 condition) or using a coculture with L cells expressing the human B7.1 (CD28 condition) for 10 min. Whole cell lysates were immunoprecipitated with anti-Dok4 or anti-Dok1 antibodies, separated by SDS-PAGE and probed by immunoblotting with anti-phosphotyrosine mAb (PY WB). The membrane was stripped and reprobed with an anti-Dok4 antibody (Dok4 WB). This experiment is representative of three independent experiments.

(B) Jurkat cells were transfected with Dok4GFP or Dok1GFP. 24 h after transfection, cells were stimulated for the indicated time with Raji cells pulsed or not with SEE. Whole cell

lysates were immunoprecipitated with anti-GFP antibodies, separated by SDS-PAGE and probed by immunoblotting with anti-phosphotyrosine mAb (PY WB). The membrane was stripped and western blot anti-GFP was performed (GFP WB). This experiment is representative of three independent experiments.

Figure 3. Dok-4 recruitment during T – APC contact.

(A) Jurkat cells were transfected with pDok4GFP. 24 h after transfection, cells were incubated for the indicated time with Raji or pulsed SEE-Raji cells. Raji cells were preincubated for 30 min with CMT-MR. Contacts were plated on poly-L-lysine coverslips. (B) Jurkat cells were transfected with pDok4GFP. 24 h after transfection, cells were stimulated for 30 min with Raji or pulsed SEE-Raji cells. After fractionation experiments, 50 µg of lysates were separated by SDS-PAGE and probed by immunoblotting with anti-GFP, or anti-Dok4 antibodies. This experiment is representative of at least three independent experiments. (C) Jurkat cells were transfected with pDok4GFP. 24 h after transfection, Jurkat cells were incubated or not for 45 min with pulsed SEE-Raji cells. Contacts were plated on poly-L-lysine coverslips. Immunofluorescence against γ -tubulin was performed. 40-50 GFP⁺ cells were examined for each condition and a representative distribution pattern is shown. (D) **Kinetic of Dok4GFP localization during conjugate formation. Raji cells were labeled with CMT-MR and pulsed with SEE, as described in Materials and Methods. Dok4GFP expressing-Jurkat cells and Raji cells were admixed, plated on poly-L-lysine coverslips and spin-down for 2 min at 1000 rpm to allow synchronization of conjugates formation. At the indicated time, cells were fixed in 4% PFA and stained for α -tubulin. Localization of Dok4GFP has been divided and scored as follow: (1) Dok4GFP was localized at the back of the cell, not found at the IS (black); (2) Dok4GFP was localized along the microtubule, and/or localized both at the back and at the IS (bipolar localization) (dark grey); (3) Dok4GFP was exclusively localized at the**

IS (light grey). Percentages correspond to at least 20 cells examined for each condition. This experiment is representative of two independent experiments. (E) Jurkat cells were transfected with Δ PHDok4GFP, PHDok4GFP or Dok4PTB*-GFP. 24 h after transfection, Jurkat cells were incubated for 45 min with Raji or SEE- pulsed Raji cells. Raji cells are preincubated for 30 min with CMT-MR. Contacts were plated on poly-L-lysine coverslips.

Figure 4. Down-expressing Dok-4 induces hyper-activation of ERK1/2. (A) Jurkat cells were transfected with pBChH1 (empty vector), pBChH1-RNAiDok4 (containing shDNA encoding for a Dok-4 siRNA that downregulates the Dok-4 expression) or pBChH1-Control (containing shDNA encoding for a Dok-4 siRNA that does not alter the Dok-4 expression). 24 h after transfection, cells were left lysed. Whole cell lysates were separated by SDS-PAGE and identified by immunoblotting with anti-Dok1, Dok2 and Dok4 antibodies. This experiment is representative of at least three independent experiments. (B) Jurkat cells were transfected with pBChH1, pBChH1-RNAiDok4 or pBChH1-Control. 24 h after transfection, cells were left unstimulated or stimulated for 15 min with Raji cells or pulsed SEE-Raji cells. Whole cell lysates were separated by SDS-PAGE and identified by immunoblotting with anti-phospho-Erk1/2 or anti-phospho-PLC γ antibodies. The membrane was stripped and anti-Erk, anti-PLC γ immunoblots were performed. This experiment is representative of at least three independent experiments. (C) Jurkat cells were transfected with pBChH1-Control or pBChH1-RNAiDok4. 24 h after transfection, cells were left unstimulated (in contact with unpulsed Raji for 15 min) or stimulated for 5, 15 or 30 min with pulsed SEE-Raji cells. Whole cell lysates were separated by SDS-PAGE and identified by immunoblotting with anti-phospho-Erk1/2, anti-Erk, anti-phospho-JNK, anti-phospho-p38MAPK or anti-phosphoPLC γ antibodies (Relative protein phosphorylation was calculated and shown in

supplementary Fig. 4). The membrane was stripped and anti-Erk, anti-PLC γ immunoblots were performed. This experiment is representative of two independent experiments.

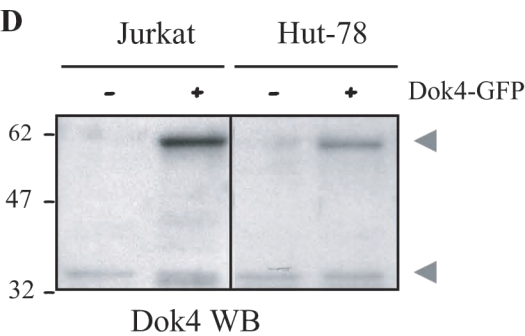
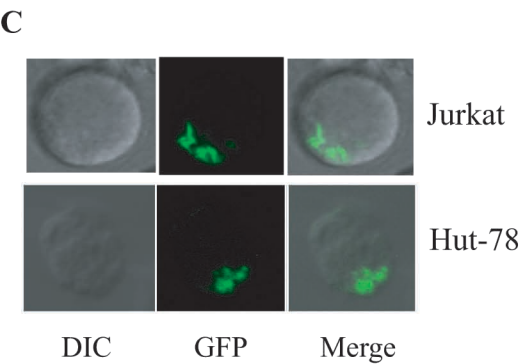
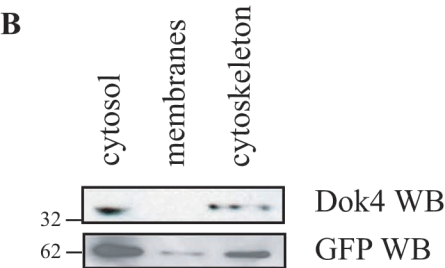
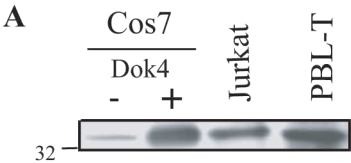
Figure 5. Dok-4 overexpression induces a decrease of ERK1/2 activation and a Rap1 hyper-activation.

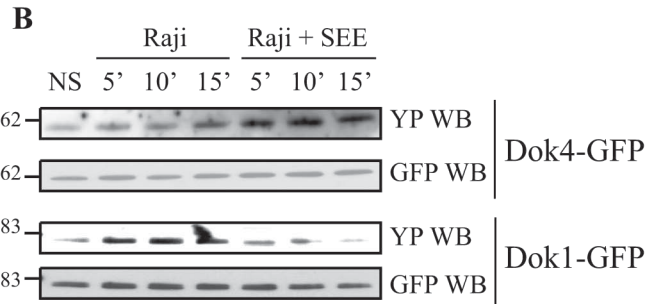
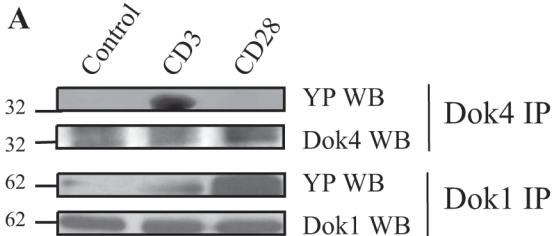
Jurkat cells were transfected with 20 μ g empty vector, Dok4-GFP or Δ PHDok4-GFP. After 24 h of recovery, cells were stimulated with CD3 plus CD28 mAbs (A) for various times (3, 10, 30 min) and lysed, then the levels of phosphorylated MAPKs were determined by western blot analysis with specific antibodies (Relative protein phosphorylation was calculated and shown in supplementary Fig. 5). Three independent experiments have been performed. (B) Transfected Jurkat cells were stimulated with CD3 mAb for 5 or 10 min, then lysates were incubated with GST-RalGDS RBD fusion proteins, beads were washed and finally proteins were subjected to western blot analysis for Rap1 (Rap assay, upper panel), total level of Rap1 was determined from whole cell lysates (Rap total, center panel) or the presence of wild-type Dok-4-GFP or PH-deleted Dok-4-GFP were detected after GFP immunoprecipitation followed by anti-GFP western blot analysis. The position of both Dok-4 forms was indicated by arrowheads (GFP WB, bottom panel). Two independent experiments have been performed.

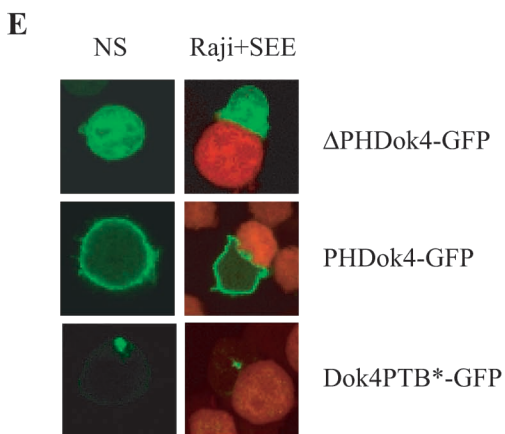
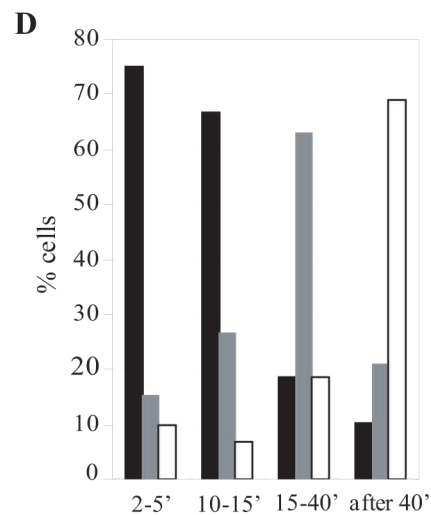
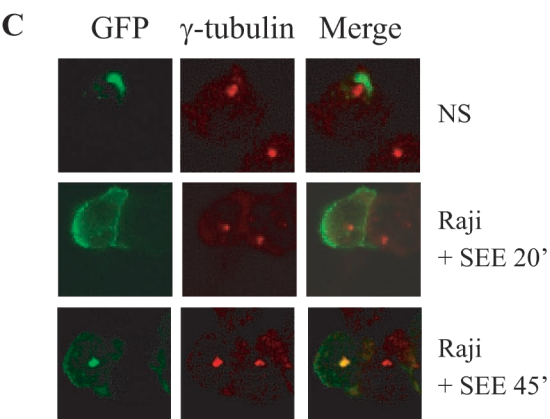
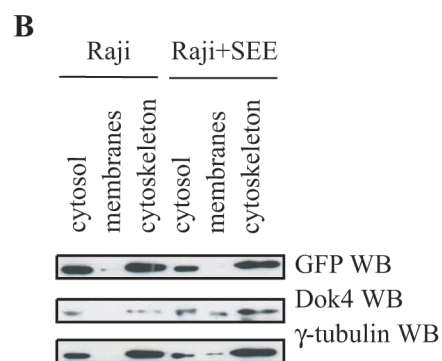
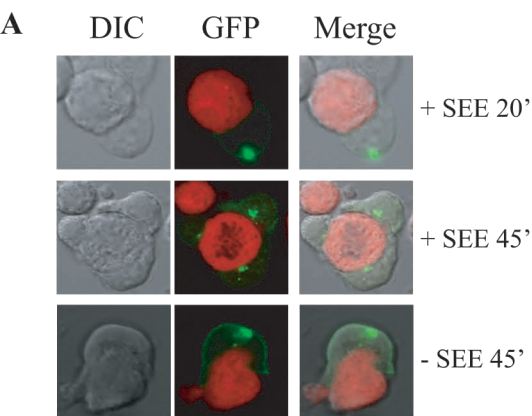
Figure 6. Down-expressing Dok-4 promotes IL-2 promoter activation and T cell proliferation.

(A) Jurkat cells were transfected with 10 μ g pBChH1 or pBChH1-RNAiDok4 and 10 μ g pIL-2-Luc, and 1 μ g β actinR-luc. After 16 h of recovery, cells were left unstimulated or stimulated for 8 h with an equivalent number of Raji cells or Raji cells pulsed with SEE. Three independent experiments have been performed. In control, whole cell lysates were separated by SDS-PAGE and identified by immunoblotting with anti-Dok4 and anti- α tubulin. (B) Jurkat cells were transfected with 10 μ g empty vector, Dok4-GFP or Δ PHDok4-

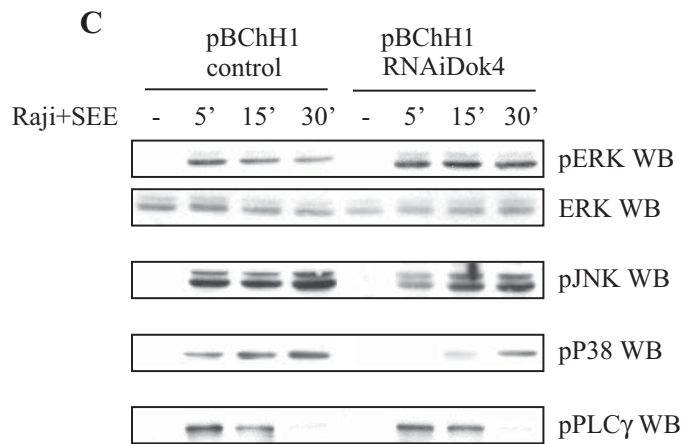
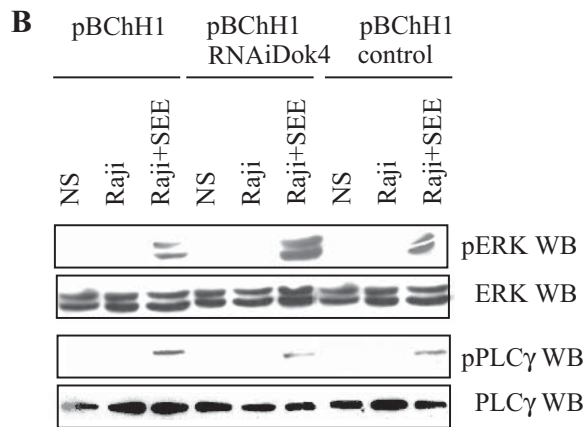
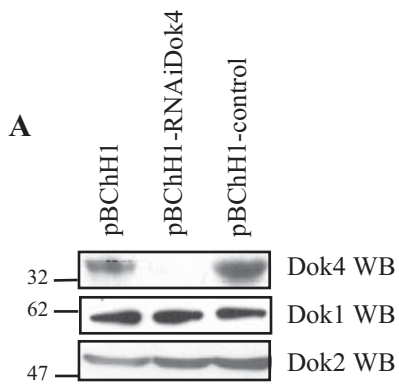
GFP, and 10 μ g pIL-2-Luc, and 1 μ g β actinR-luc. After 2 h of recovery, cells were stimulated with CD3 and CD28 mAbs, or PMA and Ionomycin. After 18 h of stimulation, luciferase assay was performed. Three independent experiments have been performed. **(C-D)** PBL-Ts were transfected with pBChH1 or pBChH1-RNAiDok4. After 24 h of recovery, cells were left unstimulated, or stimulated with CD3 and CD28 mAbs, or PMA and Ionomycin. **(C)** After 1 day of stimulation, CD69 cell surface expression was examined by flow cytometry and expressed as the mean of fluorescence intensity (MFI). Two independent experiments in duplicate were performed. To confirm Dok-4 overexpression, cells were lysed and subjected to immunoblotting with anti-Dok4 and anti- α tubulin antibodies. **(D)** After 4 days of stimulation, [3 H]-thymidine incorporation measurement was performed. Three independent experiments in triplicate were performed.

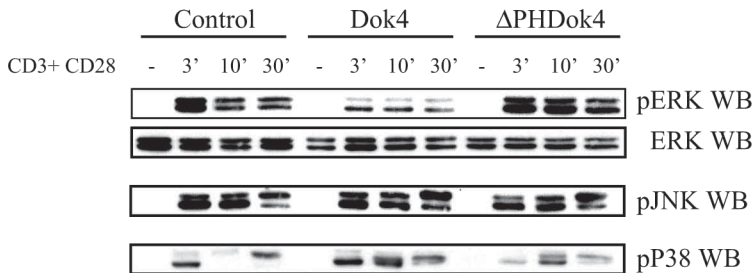
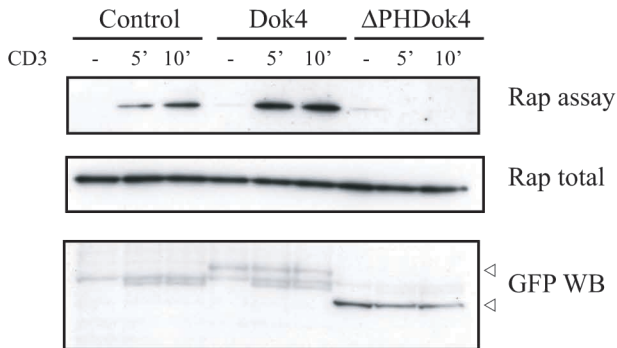


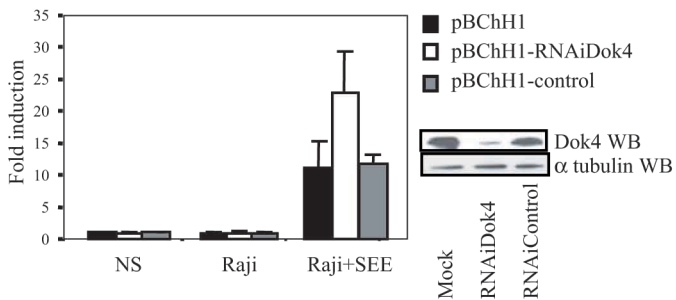
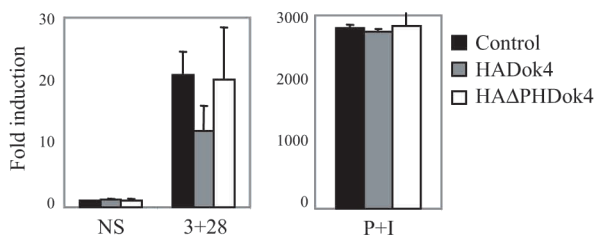
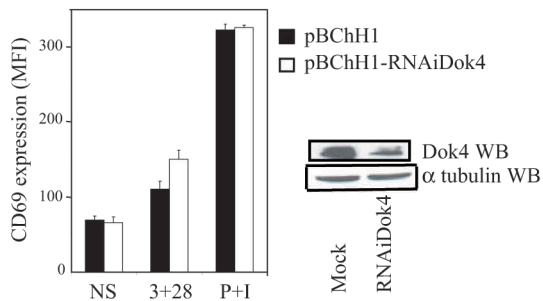




- back/not localized at the IS
- recruitment along the microtubule/bipolar localization
- localization at the IS



A**B**

A**B****C****D**