p62<sub>dok</sub> Negatively Regulates CD2 Signaling in Jurkat Cells

Jean-Guy Némorin, Pierre Laporte, Geneviève Bérubé and Pascale Duplay

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p62<sup>dok</sup> Negatively Regulates CD2 Signaling in Jurkat Cells<sup>1</sup>

Jean-Guy Némorin, Pierre Laporte, Geneviève Bérubé, and Pascale Duplay<sup>2</sup>

p62<sup>dok</sup> belongs to a newly identified family of adaptor proteins. In T cells, the two members that are predominantly expressed, p56<sup>dok</sup> and p62<sup>dok</sup>, are tyrosine phosphorylated upon CD2 or CD28 stimulation, but not upon CD3 ligation. Little is known about the biological role of Dok proteins in T cells. In this study, to evaluate the importance of p62<sup>dok</sup> in T cell function, we generated Jurkat clones overexpressing p62<sup>dok</sup>. Our results demonstrate that overexpression of p62<sup>dok</sup> in Jurkat cells has a dramatic negative effect on CD2-mediated signaling. The p62<sup>dok</sup>-mediated inhibition affects several biochemical events initiated by CD2 ligation, such as the increase of intracellular Ca<sup>2+</sup>, phospholipase Cγ1 activation, and extracellular signal-regulated kinase 1/2 activation. Importantly, these cellular events are not affected in the signaling cascade induced by engagement of the CD3/TCR complex. However, both CD3- and CD2-induced NF-AT activation and IL-2 secretion are impaired in p62<sup>dok</sup>-overexpressing cells. In addition, we show that CD2 but not CD3 stimulation induces p62<sup>dok</sup> and Ras GTPase-activating protein recruitment to the plasma membrane. These results suggest that p62<sup>dok</sup> plays a negative role at multiple steps in the CD2 signaling pathway. We propose that p62<sup>dok</sup> may represent an important negative regulator in the modulation of the response mediated by the TCR. *The Journal of Immunology, 2001, 166: 4408–4415.*

Dok proteins belong to a newly identified family. Their overall structure suggests that they might serve as docking proteins for signaling molecules. They contain an amino-terminal pleckstrin homology domain (PH),<sup>3</sup> a central phosphotyrosine binding domain (PTB), and a carboxyl-terminal domain rich in proline and tyrosine residues. The lowest sequence similarity between the members of the family resides in the carboxyl terminus, suggesting that this region is involved in the recruitment of different sets of downstream signaling molecules. The first member identified, p62<sup>dok</sup>, was originally shown to be a target of activated protein tyrosine kinases and, when phosphorylated, to associate with Ras GTPase-activating protein (RasGAP) (1). p62<sup>dok</sup> was subsequently cloned from a p210<sup>bcr-abl</sup>-transformed myeloid cell line (2) and from a v-Ab1<sup>bl</sup>-transformed precursor B cell line (3). Two other members, p56<sup>dok</sup> (4–7) and dok-3 (8, 9), have been characterized.

In T cells, p62<sup>dok</sup> and p56<sup>dok</sup> are expressed (10), whereas dok-3 is absent in thymus and in most T cell lines examined (9). Little information is available on the biological function of these proteins in T cells. Recent evidence suggests that they play a specific role in signal transduction pathways initiated by costimulatory receptors. The phosphorylation of p62<sup>dok</sup> has been reported to occur following CD2 (10) or CD28 stimulation (11), whereas CD3 stimulation does not induce p62<sup>dok</sup> phosphorylation. Phosphorylation of the other Dok member, p56<sup>dok</sup>, seems to be regulated in the same way since we have shown that it is phosphorylated upon CD2 stimulation and not upon CD3 stimulation (10). Several lines of evidence support the conclusion that Src family kinases phosphorylate Dok proteins. Cell adhesion-dependent tyrosine phosphorylation of Dok is mediated by Src tyrosine kinases (12). We have shown that Lck is required for CD2-mediated phosphorylation of Dok proteins (10). In transient transfection assays, Dok proteins are good substrates for Src family kinases (9). Phosphorylation of p56<sup>dok</sup> by Lyn generates binding sites for RasGAP and Nck (7). Depending on the transduction pathway examined, Dok proteins can act as a positive or negative regulator. In B cells, phosphorylation of p62<sup>dok</sup> in involved in the FcyRIIB-mediated inhibition of B cell receptor signaling (13, 14) most likely by negatively regulating the activity of Ras (15), thereby inhibiting the Ras-dependent activation of extracellular signal-regulated kinase 1/2 (Erk1/2) (13, 14). By contrast, transient overexpression of p62<sup>dok</sup> in Jurkat cells, clone 77-6, was grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin. Puromycin at 1 μg/ml was added to the medium when required.

Materials and Methods

Cell lines and Abs

Jurkat cells, clone 77-6, were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin. Puromycin at 1 μg/ml was added to the medium when required.

<sup>1</sup> Institut National de la Recherche Scientifique-Institut Armand-Frappier, Université du Québec, Laval QC, Canada.

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<sup>2</sup> Address correspondence and reprint requests to Dr. Pascale Duplay, Institut National de la Recherche Scientifique-Institut Armand-Frappier, Université du Québec, 531 Boulevard des Prairies, Laval QC, H7V 1B7, Canada. E-mail address: pascale.duplay@irn-sf.ulaval.ca

<sup>3</sup> Abbreviations used in this paper: PH, pleckstrin homology; DAG, diacylglycerol; Erk, extracellular signal-regulated kinase; HA, hemagglutinin; PKC, protein kinase C; PLC, phospholipase C; PTB, phosphotyrosine binding domain; RasGAP, Ras GTPase-activating protein; SH, Src homology; TK, thymidine kinase.
mAbs used included: anti-CD3ε UCHT1 (IgG2a; kindly provided by A. Alcover, Institut Pasteur, Paris, France); anti-CD2 (anti-T11-2 and T11-3, kindly provided by E. Reinherz, Harvard Medical School, Boston, MA); anti-RasGAP (B4F8; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY); and anti-PLCγ1 (a mixture of mAbs; Upstate Biotechnology). Polyclonal Abs used included: anti-p62dok PTB directed against p62dok PTB domain (produced by immunizing rabbits with a GST fusion protein bearing residues 152–259); anti-p62dok directed against aa residues 425–439 of p62dok (kindly provided by B. Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); phospho-specific anti-Erk1/2 (Promega, Madison, WI); and total anti-Erk1/2 (New England Biolabs, Beverly, MA).

**Plasmids and transfections**

The plasmid pSRα-dok was generated by cloning an EcoRI fragment corresponding to the human p62dok cDNA (kindly provided by B. Stillman, Cold Spring Harbor, NY) into the plasmid pSRα puromycin. The hemagglutinin (HA)-p62dok construct (pSRα-HA-dok) was generated as follows. The first methionine residue of p62dok was deleted by PCR and cloned into the plasmid MT073 (kindly provided by M. Thome, Institut de Biochimie, Epalinges, Switzerland) in frame with the sequence encoding the HA epitope. The HA-p62dok was then subcloned into the plasmid pSRα puromycin. J.77-6 cell line was transfected with 20 μg of pSRα-HA-dok or pSRα-dok by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA) set at 250 mV and 250 μF. Drug-resistant cells were cloned by limited dilution in puromycin-containing medium. Expression levels of p62dok were evaluated by immunoblotting of cell extracts with anti-p62dok. Expression levels of CD2 and CD3 were evaluated by flow cytometric analysis with an EPICS XL (Coulter Electronics, Hialeah, FL). Clones expressing similar levels of CD3 and CD2 compared with the parental Jurkat cells were kept for further studies. To quantify the amounts of p62dok expressed in these transfectants, we performed serial dilution of p62dok immunoprecipitates. After Western blotting with p62dok Abs, the ECL signal was quantified using Kodak Image Station 440C to acquire the image and the 1D Image Analysis software.

**Measurement of intracellular Ca²⁺**

Cells were washed twice with HBSS and incubated at 10⁷ cells/ml with 3 μM Indo-1 (Molecular Probes, Eugene, OR) and 0.4 mg/ml Pluronic acid F-127 (Roche Diagnostics, Laval, Quebec, Canada) for 15 min at 37°C. Cells were then washed in HBSS and resuspended at 10⁶ cells/ml and Ca²⁺ mobilization studies were conducted on an EPICS ELITE ESP cell sorter (Coulter Electronics, Hialeah, FL). Clones expressing similar levels of CD3 and CD2 compared with the parental Jurkat cells were kept for further studies. To quantify the amounts of p62dok expressed in these transfectants, we performed serial dilution of p62dok immunoprecipitates. After Western blotting with p62dok Abs, the ECL signal was quantified using Kodak Image Station 440C to acquire the image and the 1D Image Analysis software.

**Results**

**Overexpression of p62dok does not affect CD2- or CD3-induced tyrosine phosphorylation**

To evaluate the importance of p62dok in T cell function, we stably transfected Jurkat cells with an expression vector containing an unmodified or an HA-tagged version of p62dok. Drug-resistant clones overexpressing p62dok were identified by immunoblotting of total cell lysates with anti-p62dok Abs (data not shown). Clones were also tested for expression of CD2 and CD3 cell surface molecules, and those expressing levels comparable with that of the Jurkat parental cell line were kept for further study (data not shown). Three representative clones (clones 2, 74, and HA-1), shown in Fig. 1, that express at least 2.5 times more p62dok than the parental Jurkat cells were subsequently characterized for their signaling capacity.

**Luciferase assays**

Jurkat cells (10⁶ cells) were transfected with 2.5 μg NF-AT-firefly luciferase (kindly provided by O. Acuto, Institut Pasteur) and 0.5 μg thymidine kinase (TK)-Renilla luciferase constructs (Promega, Madison, WI) using Fugene transfection assays (Roche Diagnostics, Laval, Quebec, Canada). For transient overexpression of p62dok, 1 μg of pSRα-dok or empty vector was used in combination with 1.5 μg NF-AT-firefly luciferase and 0.5 μg TK-Renilla luciferase constructs. After 24 h, 3 × 10⁶ cells were stimulated with plate-bound anti-CD3 or soluble anti-CD2 Abs for 6 h in a 24-well plate. Maximal stimulation was obtained by a combination of PMA (10 ng/ml) and ionomycin (1 μM). Cells were then lysed and assayed for luciferase activity using the dual luciferase reporter assay system (Promega, Madison, WI) and a luminometer (Berthold, LUMAT LB 9507). The NF-AT-firefly luciferase values were normalized based on the constitutive Renilla luciferase activity.

**IL-2 assays**

A total of 10⁵ Jurkat cells was stimulated by plate-bound anti-CD3ε mAb (50 μg of UCHT1 at 1/100 dilution of ascites in PBS), soluble anti-CD2 (a combination of T11-2 and T11-3 at 1/1000 dilution of ascites), or PMA (10 ng/ml) and ionomycin (1 μM) for 20 h in 96-well plates. Fifty microliters of supernatant were assayed for IL-2 production using the IL-2-dependent cell line CTLL-2, as described previously (17). 3H incorporation was assessed on a liquid scintillation counter (MicroBeta Trilux).

**FIGURE 1.** Effects of p62dok overexpression on CD2- or CD3-induced tyrosine phosphorylation. A, Total cell lysates from the Jurkat T cell line J.77-6, and from Jurkat cells overexpressing p62dok (clones 2 and 74) or an HA-tagged version of p62dok (HA-1) were analyzed by phosphotyrosine immunoblotting. Cells were left unstimulated (−) or stimulated with an anti-CD2 mAb pair for 3 min or anti-CD3 mAbs for 1 min. B, Positions of molecular mass markers are shown in kilodaltons. B, The indicated Jurkat cells were treated as above. The corresponding lysates were immunoprecipitated with anti-p62dok PTB Abs and analyzed by immunoblotting with either anti-phosphotyrosine mAbs or anti-p62dok Abs, as indicated.
We investigated whether overexpression of p62\textsuperscript{dok} altered the patterns of tyrosine phosphorylation of cellular proteins following either CD3 or CD2 cross-linking (Fig. 1A). Phosphorylation patterns remained unaffected in CD3-stimulated cells in clones overexpressing p62\textsuperscript{dok}, indicating that p62\textsuperscript{dok} overexpression did not noticeably prevent the activation of CD3-mediated tyrosine phosphorylation pathways. CD2 stimulation led to the phosphorylation of a similar set of proteins in all the clones, with the exception of a 62-kDa protein, which is hyperphosphorylated in clones overexpressing p62\textsuperscript{dok}. We have previously shown that CD2 stimulation of Jurkat cells induces tyrosine phosphorylation of p62\textsuperscript{dok} (10). To ascertain that the 62-kDa band corresponded to p62\textsuperscript{dok}, we performed immunoprecipitations from the different Jurkat clones that were left unstimulated, CD2 stimulated, or CD3 stimulated, and were analyzed by anti-phosphotyrosine immunoblotting (Fig. 1B). As expected, CD2 stimulation of the parental Jurkat cell line and clones that overexpress p62\textsuperscript{dok} led to an increased phosphorylation of p62\textsuperscript{dok}. In contrast, CD3 stimulation did not induce p62\textsuperscript{dok} phosphorylation. It should be noted that there is a higher basal level of p62\textsuperscript{dok} phosphorylation in unstimulated clones overexpressing p62\textsuperscript{dok} compared with the parental Jurkat cells. The increase in constitutively phosphorylated p62\textsuperscript{dok} correlates with the levels of p62\textsuperscript{dok} expressed in these clones. Therefore, overexpression of p62\textsuperscript{dok} does not seem to affect the constitutive phosphorylation of p62\textsuperscript{dok}.

**Effect of p62\textsuperscript{dok} overexpression on Ras signaling**

To assess the role of p62\textsuperscript{dok} in the Ras signaling pathway, we examined whether p62\textsuperscript{dok} overexpression interferes with the activation of Erk1/2 following CD2 or CD3 engagement (Fig. 2A). To monitor the activation of Erk1/2, we used Abs specific for the phosphorylated form of Erk1/2. As shown in Fig. 2, phospho-Erk1/2 induction after CD3 stimulation was similar in all clones, indicating that CD3-induced Erk1/2 activation was not inhibited by p62\textsuperscript{dok} overexpression. In contrast, there was a decrease in CD2-mediated Erk1/2 activation in p62\textsuperscript{dok}-overexpressing clones. The intensity of this inhibitory effect correlated with the levels of p62\textsuperscript{dok} overexpression, as evidenced by a greater decrease in CD2-induced Erk1/2 phosphorylation in clone 74 and HA-1 when compared with clone 2. Moreover, the inhibition of CD2-induced Erk1/2 phosphorylation could be rescued by PMA (Fig. 2B). These results show that p62\textsuperscript{dok} overexpression selectively inhibits CD2- but not CD3-mediated activation of Erk1/2.

**Overexpression of p62\textsuperscript{dok} inhibits CD2-induced Ca\textsuperscript{2+} mobilization**

We evaluated the effect of p62\textsuperscript{dok} overexpression on Ca\textsuperscript{2+} mobilization after CD2 and CD3 stimulation. In Fig. 3, we show that Ca\textsuperscript{2+} mobilization after CD3 stimulation was unaltered in all cell lines, regardless of p62\textsuperscript{dok} expression levels. In contrast, the CD2-induced Ca\textsuperscript{2+} response was abolished or greatly diminished in clones overexpressing p62\textsuperscript{dok}. Inositol 3,4,5-triphosphate-mediated mobilization of Ca\textsuperscript{2+} requires the activation of PLC\textsubscript{1}. Since tyrosine phosphorylation of PLC\textsubscript{1} contributes to the activation of the enzymatic activity of PLC\textsubscript{1}, we next investigated whether PLC\textsubscript{1} tyrosine phosphorylation is affected by p62\textsuperscript{dok} overexpression after CD2 or CD3 stimulation (Fig. 4). After CD3 stimulation, PLC\textsubscript{1} was highly phosphorylated in all cell lines tested. However, there was a slight decrease in the phosphorylation levels of PLC\textsubscript{1} after CD2 stimulation when we compared clones 2 and 74 with the control J.77-6 cells. More importantly, PLC\textsubscript{1} phosphorylation was abolished after CD2 stimulation of the HA-1 cell line. The decreased or absence of phosphorylation of PLC\textsubscript{1} in p62\textsuperscript{dok}-overexpressing cells might be, at least in part, responsible for the diminished Ca\textsuperscript{2+} influx following CD2 stimulation.

**p62\textsuperscript{dok} overexpression interferes with IL-2 gene expression**

The activation of signaling pathways initiated by engagement of the CD3/TCR or the CD2 receptor leads to IL-2 production in Jurkat cells. We examined whether p62\textsuperscript{dok} overexpression would influence IL-2 production. Jurkat clones were left unstimulated or stimulated by anti-CD3, anti-CD2 Abs, or by PMA and ionomycin. After 24 h, the culture supernatants were assayed for IL-2 production (Fig. 5A). Activation of the different clones by PMA and ionomycin resulted in equivalent levels of IL-2 production, indicating that p62\textsuperscript{dok} overexpression did not alter the capacity of the cells to produce IL-2. CD2 stimulation of p62\textsuperscript{dok}-overexpressing clones led to significantly reduced levels of IL-2 production compared with the parental cell line. This result was expected given that IL-2 gene expression is dependent on Ca\textsuperscript{2+} mobilization and Erk1/2 activation. The inhibition of CD2-induced IL-2 secretion seems to correlate with the amount of p62\textsuperscript{dok} present in the cells. Surprisingly, PMA restored CD2-induced IL-2 secretion (Fig. 5A). In the clones 74 and HA-1, CD3-induced IL-2 secretion was decreased, whereas CD3-induced IL-2 production in the clone 2 was comparable with that of the parental cell line.
NF-AT activation in response to CD2 and PMA when compared with the parental cell line. The NF-AT activity in clone HA-1 following treatment with anti-CD2 and PMA is similar to the CD3-induced NF-AT activity in wild-type cells. This result might, at least partially, explain why CD2-induced IL-2 secretion is restored in presence of PMA in clones overexpressing p62\textsuperscript{dok}. NF-AT activation requires the cooperative binding between NF-AT and AP-1 to the IL-2 promoter NF-AT site. NF-AT nuclear translocation is mediated by the Ca\textsuperscript{2+}-regulated phosphatase calcineurin, whereas the synthesis and activation of Fos and Jun, components of the AP-1 family of transcription complexes, are mediated by the protein kinase C (PKC)/Ras pathway. Therefore, the inability of PMA treatment to rescue CD2-induced NF-AT activity is most likely due to the absence of Ca\textsuperscript{2+} mobilization in these clones. As expected, in cells overexpressing p62\textsuperscript{dok}, restoration of Ca\textsuperscript{2+} flux by treatment with ionomycin is not sufficient to rescue the CD2-induced NF-AT activation, which is partly dependent on Erk1/2 activation. In contrast, treatment with PMA and ionomycin restored CD2-mediated activation of NF-AT in these cells (Fig. 5E). This indicates that p62\textsuperscript{dok} overexpression does not lead to nonspecific CD2-mediated inhibition of NF-AT activity.

We were surprised to find that p62\textsuperscript{dok} overexpression interfered with CD3-induced NF-AT activation given that p62\textsuperscript{dok} overexpression does not seem to affect Ca\textsuperscript{2+} response and Erk1/2 activation (Fig. 5B). The CD3-induced activation of NF-AT was only slightly reduced in clone 2, whereas there was a marked decrease in CD3-mediated NF-AT activation in clones 74 and HA-1 (2-, 6-, and 28-fold decrease in NF-AT activation for clones 2, 74, and HA-1, respectively). Therefore, as shown for CD2 stimulation, the p62\textsuperscript{dok}-mediated inhibition of CD3-induced NF-AT activation is dependent on the level of Dok overexpression. The inhibition of CD3-induced NF-AT activation can be bypassed by treatment with PMA and calcium ionophore used in combination, but not alone. We have shown that the CD3-mediated Ca\textsuperscript{2+} response is not affected in clones overexpressing p62\textsuperscript{dok} (Fig. 3). These data clearly suggest that there is a difference between anti-CD3 Abs and Ca\textsuperscript{2+} ionophore with respect to Ca\textsuperscript{2+} mobilization. Moreover, CD3-mediated activation of Erk1/2 activation seems normal in clones overexpressing p62\textsuperscript{dok} (Fig. 2). Therefore, in these clones, PMA treatment most likely compensates for a defective component that is required in the NF-AT activation pathway and is downstream or independent of Erk1/2 activation. In addition, in wild-type Jurkat cells, CD3 stimulation or CD2 stimulation further increased NF-AT activity induced by the combination of PMA and ionomycin. Therefore, compared with antireceptor stimuli, the pharmacological stimuli PMA and ionomycin may utilize an additional Ras-independent pathway, which leads to NF-AT activation. These results underline the complexity of the regulation of NF-AT activation. Additional experiments will be required to elucidate the

To study in greater detail the mechanisms involved in the inhibition of CD3- and CD2-induced IL-2 secretion in clones overexpressing p62\textsuperscript{dok}, we examined the effect of p62\textsuperscript{dok} overexpression on the activation of transcription factors known to regulate IL-2 gene expression. The control Jurkat cell line as well as clones overexpressing p62\textsuperscript{dok} were transiently transfected with luciferase reporter constructs under the control of NF-AT (Fig. 5A). Cells were left unstimulated, or stimulated by anti-CD3 or anti-CD2 Abs or PMA and ionomycin for 6 h. The activation of NF-AT was evaluated by a luciferase activity assay. p62\textsuperscript{dok} overexpression inhibited CD2-induced activation of NF-AT (Fig. 5B). Moreover, the intensity of the inhibition correlates with the levels of p62\textsuperscript{dok} expressed in the cells. We observed a 5- and 15-fold decrease in NF-AT activation for clones 2 and clones 74 and HA-1, respectively. These results were confirmed by performing transient transfection assays with plasmids containing p62\textsuperscript{dok} cDNA and the NF-AT luciferase reporter construct. Transient overexpression of wild-type p62\textsuperscript{dok} or HA-tagged p62\textsuperscript{dok} inhibited (2- to 3-fold) CD2-induced NF-AT activation (Fig. 5C).

As shown in Fig. 5D, PMA increases NF-AT activity upon CD2 stimulation in both wild-type Jurkat cells and cells overexpressing p62\textsuperscript{dok}. However, overexpression of p62\textsuperscript{dok} considerably inhibited
mechanisms by which p62 dok mediates inhibition of CD3-induced activation of NF-AT.

Membrane localization and interaction of p62 dok with RasGAP following CD2 stimulation

p62 dok binding to the Src homology 2 (SH2) domain of RasGAP requires p62 dok phosphorylation, and RasGAP is known to be an important negative regulator of Ras. Therefore, Dok-mediated inhibition of Ras signaling might occur through its interaction with RasGAP. To test this hypothesis, we compared the amount of RasGAP associated with p62 dok in clones overexpressing p62 dok and in the parental cell line. Lysates from cells that were left unstimulated or stimulated with anti-CD2 mAbs for 3 and 10 min were immunoprecipitated with anti-p62 dok Abs and immunoblotted with anti-RasGAP mAbs, anti-phosphotyrosine mAbs, or anti-p62 dok Abs, as indicated. WB, Western blot.

Several pieces of evidence suggest that p62 dok phosphorylation depends on its subcellular localization and occurs at the plasma membrane (12, 14). We therefore tested whether p62 dok overexpression would increase the amount of membrane-bound p62 dok and, consequently, would alter the subcellular localization of RasGAP. We performed subcellular fractionation on the parental Jurkat cells and cells overexpressing p62 dok that were left unstimulated, CD2 stimulated, or CD3 stimulated (Fig. 7). In all the cell lines, the majority of p62 dok was found in the soluble fraction (Fig. 6).

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7A–C). The amount of membrane-bound p62\textsuperscript{dok} in clones overexpressing p62\textsuperscript{dok} was significantly higher than in the parental cell line. However, the percentage of total p62\textsuperscript{dok} present in the membrane fraction in unstimulated cells did not change significantly with varying levels of p62\textsuperscript{dok}. There was a slight increase in the total amount of p62\textsuperscript{dok} in the particulate fraction after CD2 stimulation, whereas CD3 stimulation did not alter the localization of p62\textsuperscript{dok}. The relative proportion of phosphorylated p62\textsuperscript{dok} at each stimulation condition and cytofraction was calculated by normalizing the phosphotyrosine signal to the amount of precipitated protein, and the ratio of these two signals is presented. Ratios in A and B were calculated from the same blots, whereas ratios presented in C were from a different experiment. D, Lysates from clone 2 cells were fractionated, as described above. RasGAP immunoprecipitates were subjected to anti-p62\textsuperscript{dok}, anti-phosphotyrosine, and anti-RasGAP immunoblotting, as indicated. The band indicated by an asterisk corresponds to phosphorylated ZAP-70 associated with CD3 and is due to detecting protein A during the immunoprecipitation. WB, Western blot.

FIGURE 7. Phospho-p62\textsuperscript{dok} and RasGAP are recruited to the membrane following CD2 stimulation. Jurkat J.77-6 cells (A) or clone HA-1 cells (B) or clone 2 cells (C) were left unstimulated (−) or stimulated with an anti-CD2 mAbs pair for 3 min or anti-CD3 mAbs for 1 min. Cells were lysed in a hypotonic buffer using a Dounce homogenizer. Lysates were fractionated into cytosolic and membrane fractions and were immunoprecipitated with anti-p62\textsuperscript{dok} PTB. p62\textsuperscript{dok} immunoprecipitates were immunoblotted with either anti-phosphotyrosine mAbs or anti-p62 Abs, as indicated. The relative proportion of phosphorylated p62\textsuperscript{dok} at each stimulation condition and cytofraction was calculated by normalizing the phosphotyrosine signal to the amount of precipitated protein, and the ratio of these two signals is presented. Ratios in A and B were calculated from the same blots, whereas ratios presented in C were from a different experiment. D, Lysates from clone 2 cells were fractionated, as described above. RasGAP immunoprecipitates were subjected to anti-p62\textsuperscript{dok}, anti-phosphotyrosine, and anti-RasGAP immunoblotting, as indicated. The band indicated by an asterisk corresponds to phosphorylated ZAP-70 associated with CD3 and is due to detecting protein A during the immunoprecipitation. WB, Western blot.

Discussion

In this study, we analyzed the effect of p62\textsuperscript{dok} overexpression on the signal transduction pathways initiated by CD2 or CD3 cross-linking. We showed that p62\textsuperscript{dok} overexpression interferes with CD2- and CD3-induced IL-2 expression in Jurkat cells. In contrast, overexpression of p62\textsuperscript{dok} specifically inhibits CD2-mediated PLC\textsubscript{γ1} phosphorylation, Erk1/2 activation, and Ca\textsuperscript{2+} mobilization, whereas these events remain unaffected when initiated via the CD3/TCR complex.

Our finding that overexpression of Dok affects PLC\textsubscript{γ1} phosphorylation upon CD2 stimulation indicates that p62\textsuperscript{dok} functions early in the signal transduction cascade initiated via CD2. Activation of PLC\textsubscript{γ1} requires several targeting signals for its recruitment to the plasma membrane, where its substrate, phosphatidylinositol 4,5-biphosphate, is found. SH2-mediated interaction of PLC\textsubscript{γ1} with linker for activation of T cells is required for complete stimulation of its enzymatic activity and is most likely mediated by the tyrosine kinase Itk (21, 22). The Gads/SLP-76 complex recruited by linker for activation of T cells also plays an important role for PLC\textsubscript{γ1} activation (23, 24). p62\textsuperscript{dok} overexpression may interfere with the formation of any of these signaling complexes. Additional experiments are required to define the molecular basis of p62\textsuperscript{dok} involvement in PLC\textsubscript{γ1} activation.

Importantly, we found that Dok-mediated inhibition of PLC\textsubscript{γ1} phosphorylation, Erk1/2 activation, and Ca\textsuperscript{2+} mobilization correlates with phosphorylation of specific tyrosine residues that are phosphorylated upon CD2 cross-linking. Indeed, although the amount of tyrosine-phosphorylated p62\textsuperscript{dok} is higher in unstimulated or CD3-stimulated clones 74 and HA-1 than in CD2-stimulated clone 2, there is no inhibition of CD3-mediated PLC\textsubscript{γ1}
phosphorylation, Erk1/2 activation, and Ca\(^{2+}\) mobilization in these clones. Therefore, if phosphotyrosine residues are involved in CD2-mediated inhibition of PLC\(\gamma\)1, Erk1/2, and/or Ca\(^{2+}\) mobilization, these tyrosine residues are specifically phosphorylated upon CD2 stimulation and are different from those that are constitutively phosphorylated.

Since PLC\(\gamma\)1 is a critical regulator of Ca\(^{2+}\) mobilization, defect in PLC\(\gamma\)1 activation is a likely cause of the impaired Ca\(^{2+}\) response in clones overexpressing p62\(^{dok}\). However, we cannot exclude the possibility that overexpression of Dok may directly interfere with Ca\(^{2+}\) mobilization downstream of inositol 3,4,5-triphosphate production.

The inhibition of Erk1/2 activation following CD2 stimulation in clones overexpressing p62\(^{dok}\) may also be secondary to defective induction of PLC\(\gamma\)1 activation and diacylglycerol (DAG) production. DAG, a product of PLC\(\gamma\)1, activates PKC, which in turn stimulates Erk1/2 via the Ras/Raf pathways (25). Consistent with a role of PKC-mediated Erk1/2 activation, PMA rescued CD2-induced IL-2 secretion and Erk1/2 activation in clones overexpressing Dok (Figs. 5A and 2A). Since the contribution of PKC activation in the CD2 regulation of Erk1/2 activity has not been studied, it is not possible to discriminate whether p62\(^{dok}\) acts in CD2 signaling upstream of PKC or acts on a CD2-induced Erk1/2-activating pathway independent of PKC.

The recently described Ras-guanine nucleotide exchange factor, RasGRP, has been shown to be involved in CD3-induced Ras activation (26, 27). DAG binding to RasGRP can recruit RasGRP to the membrane and thereby promote Ras signaling. Although the importance of RasGRP in the regulation of CD2-induced Ras activation is unknown, impaired production of DAG in clones overexpressing p62\(^{dok}\) might be in part responsible for the decrease in Ras-mediated Erk1/2 activation.

Ras function is negatively regulated by RasGAP, which stimulates the GTPase activity of Ras (28). In addition, RasGAP binds to tyrosine-phosphorylated p62\(^{dok}\) via an SH2-mediated interaction (2, 3). Therefore, RasGAP was a good candidate for mediating the negative effect conducted by p62\(^{dok}\) in the Ras/Erk1/2 signaling pathway. We have shown that there is a specific recruitment of RasGAP to the plasma membrane following CD2 stimulation and not CD3 stimulation. Moreover, the relocalization of RasGAP is mediated by phosphorylated p62\(^{dok}\). Although it was recently reported using an in vitro assay that p62\(^{dok}\) binding to RasGAP diminished its catalytic activity (29), recruitment of RasGAP in the proximity of Ras is likely to have a global negative effect on Ras function. In addition, binding of p62\(^{dok}\) to RasGAP might induce a conformational change that exposes domains of RasGAP (such as SH3) to binding partners. This would allow RasGAP to undergo additional protein interactions that might be important in Dok-mediated functions.

Surprisingly, we find that IL-2 secretion and NF-AT activation induced by CD3 cross-linking are inhibited by p62\(^{dok}\) overexpression. The level of inhibition correlates with the amount of p62\(^{dok}\) overexpression. The inhibition of CD3-induced IL-2 expression might be related to the increased amount of tyrosine-phosphorylated p62\(^{dok}\) present in clones overexpressing p62\(^{dok}\). Alternatively, but not exclusively, other structural elements of p62\(^{dok}\) might be responsible for Dok-mediated effects on CD3-induced IL-2 secretion. Activation of Erk1/2 is required, but not sufficient for NF-AT induction (30). Since we have shown that CD3-induced Erk1/2 activation is not affected by overexpression of p62\(^{dok}\), p62\(^{dok}\) may inhibit the Rac-regulated pathway involved in the regulation of NF-AT induction (31). In unstimulated cells, the amount of membrane-bound p62\(^{dok}\) in clones overexpressing p62\(^{dok}\) is significantly higher than in the parental cell line. Our current data do not allow us to distinguish whether CD3-mediated inhibition of NF-AT activation takes place in the cytosol or and in the membrane. In any case, it is important to point out that CD2 stimulation, and not CD3 stimulation, specifically increases tyrosine phosphorylation and induces membrane translocation of p62\(^{dok}\). Therefore, the involvement of p62\(^{dok}\) in the signaling cascade initiated by CD2 is likely to be different from that initiated by CD3.

Our data support a model in which phosphorylation of p62\(^{dok}\) following CD2 engagement enables its translocation from the cytosolic to the membrane compartment. Activation of phosphoinositide 3-kinase upon CD2 stimulation (32, 33) is likely to be involved in the PH domain-dependent recruitment of p62\(^{dok}\) to the membrane (12). Recently identified CD2-binding proteins (34–36) might also regulate CD2-mediated p62\(^{dok}\) membrane localization. Once at the membrane, p62\(^{dok}\) is in the proximity of various protein tyrosine kinases, such as Lck, thereby allowing phosphorylation of tyrosine sites that act as SH2 acceptor sites for downstream signaling molecules such as RasGAP. p62\(^{dok}\)-mediated RasGAP recruitment to the proximity of Ras leads to the accumulation of RasGDP and consequently to the down-regulation of CD2-mediated Erk1/2 activation. Although this model takes into account the p62\(^{dok}\)-mediated Ras inhibition, other molecules are likely to be recruited by p62\(^{dok}\) and involved in Dok-mediated inhibition of PLC\(\gamma\)1. To identify such molecules, we have tested the co-association of p62\(^{dok}\) with inhibitory molecules such as Csk, SH2-containing inositol 5'-phosphatase-1, or -2, but were not able to detect any significant interactions (data not shown).

Maximal phosphorylation of p62\(^{dok}\) occurred 10 min following CD2 stimulation and remained high for 30 min (data not shown). This slow time course supports a model in which p62\(^{dok}\) could be considered a hinge molecule that comes into play to down-regulate CD2-mediated activation signals. Although originally described as a receptor delivering stimulatory signals to the cell upon ligand binding (37), CD2 has also been described as a molecule capable of transducing inhibitory signals (38–40). Since p62\(^{dok}\) is clearly involved in the negative regulation of B cell signaling mediated by FcγRIIB (13, 14), p62\(^{dok}\) might be considered as a key molecule involved in the modulation of the response mediated by the TCR and the B cell receptor.

In conclusion, p62\(^{dok}\) is a multifunctional adaptor protein that most likely plays a negative role at multiple steps in the CD2 signaling pathway. Better insight into the molecules recruited by p62\(^{dok}\) will help to identify the molecular mechanisms involved in Dok-mediated inhibition of T cell signaling.

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References
19. Falasca, M., S. K. Logan, V. P. Lehto, G. Baccante, M. A. Lemmon, and
19. Falasca, M., S. K. Logan, V. P. Lehto, G. Baccante, M. A. Lemmon, and 
linker protein SLP-76.
A. C. Chan. 1998. Regulation of PAK activation and the T cell cytoskeleton by 
PI 3-kinase-induced PH domains of activated T cells signaling to Ras.

J. Biol. Chem. 268:25728.

Park, S., and R. Jove. 1993. Tyrosine phosphorylation of Ras GTPase-activating 
protein stabilizes its association with p62 at membranes of v-Src transformed 

Complete dissection of the Hb(64–76) determinant using T helper 1, T helper 2 

Bubeck Wardenburg, J., R. Pappu, J. Y. Bu, B. Mayer, J. Chernoff, D. Straus, and 
A. C. Chan. 1998. Regulation of PAK activation and the T cell cytoskeleton by 

Falasca, M., S. K. Logan, V. P. Lehto, G. Baccante, M. A. Lemmon, and 

1998. Activation of phospholipase Cγ by phosphatidylinositol 3,4,5-trisphos-

1997. Iκκ, a T cell-specific tyrosine kinase, is required for CD2-mediated inter-
27:834.

Schaeffer, E. M., J. Debnath, G. Yap, D. McVicar, X. C. Liao, D. R. Littman, 
A. Sher, H. E. Varma, M. J. Lenardo, and P. L. Schwartzberg. 1999. Require-
ment for Tec kinases Rlk and Iκκ in T cell receptor signaling and immunity. 
Science 284:638.

of T cell receptor-dependent signaling pathways by adapter proteins. Annu. Rev. 
Immunol. 17:89.

Liu, S. K., N. Fang, G. A. Koretzky, and C. J. McGlade. 1999. The hematopoiet-
ic-specific adaptor protein gads functions in T-cell signaling via interactions 


Ebina, J., O. S., L. Chang, C. Teixeira, D. A. Botto, J. Hooton, P. M. Blumberg, 
T-cell receptor signaling to Ras. Blood 95:3199.

1998. RasGRF, a Ras guanyl nucleotide-releasing protein with calcium- and 

Henning, S. W., and D. A. Cantrell. 1998. GTPases in antigen receptor signalling. 

Kashige, N., N. Carnino, and R. Kobayashi. 2000. Tyrosine phosphorylation of 
p62(dok) by p210bcr-abl inhibits RasGRF activity. Proc. Natl. Acad. Sci. USA 97: 
2093.

Genot, E., S. Cleverley, S. Henning, and D. Cantrell. 1996. Multiple p210bcr-
mediator pathways regulate nuclear factor of activated T cells. EMBO J. 15:3925.


Uncoupling activation-dependent HS1 phosphorylation from nuclear factor of 
activated T cells transcriptional activation in Jurkat T cells: differential signaling 
161:4506.

phosphatidylinositol 3-kinase in the regulation of β2 integrin activity by the CD2 

Nishizawa, K., C. Freund, J. Li, G. Wagner, and E. L. Reinherz. 1998. Identifi-
cation of a proline-binding motif regulating CD2-triggered T lymphocyte activa-

Li, K., K. Nishizawa, W. An, R. E. Hussey, F. E. Lalias, R. Salgia, P. R. Sunder, 
and E. L. Reinherz. 1998. A cdc15-like adaptor protein (CD2BP1) interacts with 
the CD2 cytoplasmic domain and regulates CD2-triggered adhesion. EMBO J. 
17:7320.

Dusten, M. L., M. W. Olszowy, A. D. Holdorf, J. Li, S. Bromley, N. Desai, 
P. Widdler, F. Rosenberger, P. A. van der Merwe, P. M. Allen, and A. S. Shaw. 
1998. A novel adaptor protein orchestrates receptor patterning and cytoskeletal 

Meuer, S. C., J. E. Hussey, M. Fabbri, D. Fox, O. Acuto, K. A. Fitzgerald, 
alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep 

Miller, G. T., P. S. Hochman, W. Meier, R. Tizard, S. A. Bixler, M. D. Rosa, and 
B. P. Wallner. 1993. Specific interaction of lymphocyte function-associated an-
tigen 3 with CD2 can inhibit T cell responses. J. Exp. Med. 178:211.

receptor and anti-CD2 ligand (CD48) antibodies synergize to prolong allograft 

174:937.