Pleiotropic defects in TCR signaling in a Vav-1-null Jurkat T-cell line

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The Rac/Rho-specific guanine nucleotide exchange factor, Vav-1, is a key component of the T-cell antigen receptor (TCR)-linked signaling machinery. Here we have used somatic cell gene-targeting technology to generate a Vav-1-deficient Jurkat T-cell line. The J.Vav1 cell line exhibits dramatic defects in TCR-dependent interleukin (IL)-2 promoter activation, accompanied by significant reductions in the activities of the NFAT(IL-2), NFκB, AP-1 and REAP transcription factors that bind to the IL-2 promoter region. In contrast, loss of Vav-1 had variable effects on early TCR-stimulated signaling events. J.Vav1 cells display a selective defect in sustained Ca²⁺ signaling during TCR stimulation, and complementation of this abnormality by exogenously introduced Vav-1 is dependent on the Vav-1 calponin homology domain. While JNK activation was severely impaired, the stimulation of Ras, ERK and protein kinase C-θ activities, as well as the mobilization of lipid rafts, appeared normal in the J.Vav1 cells. Finally, evidence is presented to suggest that the alternative Vav family members, Vav-2 and Vav-3, are activated during TCR ligation, and partially compensate for the loss of Vav-1 in Jurkat T cells.

Keywords: protein kinase C-θ/signal transduction/T-cell antigen receptor/Vav

Introduction

The activation of resting T cells is triggered by integrated signals delivered through the T-cell antigen receptor (TCR) and co-stimulatory receptors, such as CD28. A cytoplasmic protein that plays a critical role in this process is Vav-1, a member of the Dbl superfamily of Rac/Rho-specific guanine nucleotide exchange factors (GEFs) (Bustelo, 2000, 2001). The Vav family contains three members, two of which (Vav-2 and Vav-3) are widely expressed in mammalian tissues, whereas Vav-1 expression is restricted to hematopoietic and trophoblastic cells

(Bustelo, 2000). In T cells, Vav-1 undergoes rapid tyrosine phosphorylation in response to TCR ligation, and this response is intensified by CD28 co-stimulation (Nunes *et al.*, 1994; Salojin *et al.*, 1999). The functional importance of Vav-1 is highlighted by the dramatic defects in thymocyte development and mature T-cell functions observed in *Vav-1^{-/-}* mice (Tarakhovsky *et al.*, 1995; Turner *et al.*, 1997; Fischer *et al.*, 1998; Holsinger *et al.*, 1998; Villalba *et al.*, 2001).

The application of mouse genetics to the study of intracellular signaling proteins has provided unparalleled insights into immune system physiology. However, the use of whole-animal model systems presents some significant obstacles, particularly when the goal is to identify biochemical pathways linked to particular proteins, or to establish detailed structure–function relationships for these proteins. A complementary genetic approach involves the random mutagenesis of transformed T-cell lines, with the most popular starting point being the Jurkat T-leukemic cell line (Abraham, 2000). However, this strategy comes with several caveats, including the possibility that the selected cells have fixed multiple mutations in their genomes, with undetermined consequences for cellular behavior in response to various stimuli. Furthermore, the need for a high-throughput selection protocol limits the range of TCR signaling proteins for which effective mutant selection screens can be designed.

An alternative strategy that circumvents many of the disadvantages of random mutagenesis is gene targeting in somatic cells. Recent advances, in particular the development of ‘promoterless’ targeting vectors, have allowed several groups to achieve reasonable rates of homologous integration into specific gene loci in non-lymphoid tissue culture cells (Mateyak *et al.*, 1997; Bunz *et al.*, 1998; Sedivy and Dutriaux, 1999). Based on its structural complexity and functional importance in T cells, we considered *Vav-1* an intriguing candidate for a gene-targeting effort in Jurkat T cells. In this report, we describe the derivation and phenotypic characterization of a Vav-1-null Jurkat T-cell line.

Results

Generation of Vav-1^{-/-} Jurkat T-cell clones

The structure of the human *Vav-1* gene is shown in Figure 1A (Denkinger *et al.*, 2000). To disrupt the *Vav-1* gene in Jurkat cells, we designed a promoterless targeting vector (Sedivy and Dutriaux, 1999) containing a bicistronic selection cassette. Jurkat cells were transfected with the targeting vector, selected for stable G418 resistance, and then sorted by flow cytometry into low, intermediate and high green fluorescent protein (GFP)-positive subpopulations. The rationale for the GFP-based sorting step was to subdivide the bulk transfected population on the

Fig. 1. *Vav-1* gene-targeting strategy. **(A)** The promoterless targeting vector contained a bicistronic selection cassette encoding GFP and Neo^r (open boxes). Two targeting plasmids were generated for sequential disruption of both *Vav-1* alleles. The 5'-flanking region in the first construct spanned exons 2–4 (~1.1 kb) of the human *Vav-1* gene, while the second vector contained a 5'-homologous region derived from exons 5–7 (~2.4 kb) of *Vav-1*. Both targeting vectors contained a 3'-homologous region spanning exons 24–27 (~7 kb) from the *Vav-1* gene. The respective targeted *Vav-1* alleles are depicted in the lower portion of the figure, along with the DNA probe used for Southern blot analyses of the *Vav-1* gene loci. Primers used for clone screening and RT–PCR are indicated with arrowheads. The *Apa*I (A) restriction sites used for Southern analysis are indicated in the figure, along with the predicted size of the genomic restriction fragments from the targeted *Vav-1* alleles. **(B)** Southern blot analysis of genomic DNA isolated from cells containing wild-type *Vav-1* (+/+), a *Vav-1*^{+/-} heterozygous clone (+/-) and three *Vav-1*^{-/-} nullizygotes. Note the presence of a third, non-disrupted *Vav-1*-related genomic sequence in each of the *Vav-1*^{+/-} clones. **(C)** *Vav-1* protein expression. Detergent-soluble proteins were immunoblotted with α -*Vav-1* antibodies, followed by α -ZAP-70 antibodies as a control for protein loading. **(D)** RT–PCR analysis. The amplification products were obtained with primers f and r₃ (see A). The predicted PCR product from the wild-type *Vav-1* cDNA is 2 kb.

basis of the strength of the promoter ‘trapped’ by the targeting vector. In this case, the intermediate GFP⁺ subpopulation was enriched for homologous integration events at the *Vav-1* locus.

After the tandem drug-GFP selection procedure, clonal cell populations were derived, and screened for homologous integration events by PCR with primer pair f-r₁ (Figure 1A). Two of the 167 clones screened contained one targeted and one intact *Vav-1* allele. The heterozygous cells were transiently transfected with a Cre expression plasmid, and excision of the selection cassette was monitored by PCR (Figure 1A, third row). To avoid re-targeting of the same allele during the subsequent round of transfection, we produced a second targeting construct that contained a different 5'-region of homology to the *Vav-1* gene (Figure 1A, bottom row). Stable clones that had incurred a second targeting event (11/792 clones screened) were isolated as described above, and genomic DNA was analyzed by Southern blotting (Figure 1B). An unexpected finding was that all doubly targeted clones (indicated by the presence of 4.1 and 6.4 kb bands in Figure 1B) retained a 9.5 kb band, which was indicative of an intact *Vav-1* allele. While certain of these clones expressed no detectable Vav-1 protein in immunoblot analyses (Figure 1C, lanes D and E), others (lane F) expressed Vav-1 at the same level as the *Vav-1*^{-/-} heterozygotes. We concluded that our parental Jurkat T-cell line contained a third *Vav-1*-related gene locus, which may be a transcriptionally silent

Vav-1 allele residing on an abnormal chromosome. Disruption of the functional *Vav-1* gene loci in clones D and E was confirmed by RT-PCR of total cellular mRNA (Figure 1D, lanes D and E). Because the two clones exhibited nearly identical phenotypes, we present the results obtained with J.Vav1.D only in this report.

Defective IL-2 promoter activation in J.Vav1 cells

CD4⁺ T cells from *Vav-1*^{-/-} mice display profound defects in interleukin (IL)-2 production after co-stimulation with α -CD3 (OKT3) plus α -CD28 antibodies (Holsinger *et al.*, 1998; Costello *et al.*, 1999; Krawczyk *et al.*, 2000). To determine whether J.Vav1 cells show a similar defect in IL-2 gene transcription, we transiently transfected the cells with an IL-2 promoter-regulated reporter gene (pIL2-Luc), and stimulated these cells with staphylococcal enterotoxin D (SED)-pulsed Raji B cells. IL-2 promoter activation was markedly reduced in J.Vav1 cells, and this response defect was largely corrected by transient re-expression of wild-type Vav-1 (Figure 2). Qualitatively similar results were obtained with OKT3 monoclonal antibody (mAb) plus phorbol myristate acetate (PMA) as the co-stimuli for IL-2 promoter-driven transcription (data not shown).

Role of Vav-1 in NFAT activation

A pivotal event leading to *IL-2* gene transcription in activated T cells is the binding of a NFAT-AP-1 complex to the distal NFAT(IL2) site in the *IL-2* promoter region.

Fig. 2. TCR-mediated IL-2 promoter activation in J.Vav1 cells. Cells were co-transfected with 10 μ g of pIL2-luc DNA, 2 μ g of pRL-TK DNA and, where indicated, 4 μ g of Vav-1-encoding plasmid DNA. After 18 h, the transfected cells were left unstimulated (open bars), or were stimulated with Raji B cells in the absence (horizontal bars) or presence (hatched bars) of superantigen D (SED). The pIL2-Luc (firefly luciferase) activities were measured and were normalized to the pRL-TK-derived *Renilla* luciferase activity in each sample. Data are presented as the mean normalized relative light units (RLU) from triplicate samples.

To examine the role of Vav-1 in this response, we transfected J.Vav1 cells with a luciferase reporter plasmid containing three NFAT(IL2) binding sites. In contrast to the parental Jurkat cells, J.Vav1 cells showed virtually no increase in NFAT-dependent luciferase expression in response to OKT3 mAb stimulation (Figure 3A). This transcriptional defect was attributable to a TCR signaling deficit, as stimulation of the cells with ionomycin plus PMA led to a significant increase in luciferase expression. To confirm that the NFAT(IL2) activation defect was linked to the loss of Vav-1, we stably transfected J.Vav1 cells with a wild-type Vav-1 expression construct. TCR-dependent NFAT(IL2) activation was largely restored in the J.Vav1.WT subline.

Previous studies suggested that the calponin homology (CH) domain of Vav-1 was required for NFAT(IL2)-mediated transcription in Jurkat cells (Billadeau *et al.*, 2000). The availability of the J.Vav1 cell line allowed a more direct assessment of the role of the CH domain in this response. In contrast to wild-type Vav-1, expression of the Vav-1 CH⁻ mutant failed to restore NFAT(IL2)-dependent luciferase expression in OKT3 mAb-stimulated J.Vav1 cells (Figure 3B). Interestingly, expression of Vav-1 CH⁻ complemented the NFAT(IL2) activation defect when the cells were co-stimulated with OKT3 mAb plus ionomycin. These results suggest that the Vav-1 CH⁻ mutant delivers a partial signal(s) for NFAT(IL2) activation, but that this stimulatory function is unmasked only when intracellular Ca²⁺ signaling is augmented with ionomycin.

TCR-mediated calcium mobilization in J.Vav1 cells

Previous studies demonstrated moderate to profound defects in TCR-dependent Ca²⁺ mobilization and inositol-1,4,5-trisphosphate (IP₃) production in T cells from *vav-1*^{-/-} mice (Turner *et al.*, 1997; Fischer *et al.*, 1998; Holsinger *et al.*, 1998; Costello *et al.*, 1999). A comparative examination of the [Ca²⁺]_i profiles after TCR cross-linkage revealed a more focal Ca²⁺ signaling defect in J.Vav1 cells. This abnormality was particularly apparent at later times after TCR engagement (Figure 4A), during

Fig. 3. TCR-dependent NFAT activation. (A) Cells were transfected with 5 μ g of pNFAT(IL2)-Luc reporter plasmid, together with the control pRL-TK plasmid. The J.Vav1WT subline was derived by stable transfection of J.Vav1 cells with a wild-type (Wt) Vav-1 expression plasmid. At 18 h post-transfection, the cells were stimulated for 6 h with the indicated agents. The pNFAT(IL2)-Luc activity measured in each sample was normalized to the *Renilla* luciferase activity to control for transfection efficiency. Bars represent the mean $\pm$ standard deviation from triplicate samples. The lower panel indicates the level of Vav-1 protein in each cell population, as determined by immunoblotting. (B) J.Vav1 cells were co-transfected with the pNFAT(IL2)-Luc reporter, plus 10 μ g of either wild-type Vav-1 or Vav-1 CH⁻ plasmid DNA. Bars represent the mean luciferase activities from duplicate samples, after normalization to the maximal response obtained with ionomycin plus PMA. The inset shows the expression levels of the FLAG-tagged Vav proteins.

which the Ca²⁺ elevation is largely attributable to the opening of I_{CRAC} channels in the plasma membrane (Lewis, 2001). Stable re-expression of Vav-1 in J.Vav1 cells restored the plateau increase in [Ca²⁺]_i during TCR stimulation. Consistent with the presence of the normal early Ca²⁺ spike, OKT3-stimulated J.Vav1 cells showed no detectable defects in the tyrosine phosphorylation of PLC γ 1, or in the production of IP₃ (Figure 4B and C, respectively).

Subsequent studies focused on the role of the CH domain in TCR-dependent Ca²⁺ mobilization. J.Vav1 cells were infected with recombinant vaccinia virus encoding either Vav-1 wild-type or Vav-1 CH⁻ proteins. Once again, re-introduction of wild-type Vav-1 fully complemented

Fig. 4. TCR-dependent Ca^{2+} signaling in J.Vav1 cells. (A) TCR-mediated Ca^{2+} mobilization. Jurkat, J.Vav1 and J.Vav1WT cells were loaded with the Ca^{2+} indicator dye Indo-1, and then stimulated with OKT3 mAb cross-linked with G α MiG. Changes in $[\text{Ca}^{2+}]_i$ were monitored by flow cytometry. The data are presented as the fluorescence emission ratio (FER) of the Ca^{2+} -bound and -unbound forms of Indo-1. (B) Tyrosine phosphorylation of PLC γ 1. Cells were stimulated for the indicated times with OKT3 plus G α MiG, and detergent extracts were immunoprecipitated with α -PLC γ 1 antibody. After SDS-PAGE, the samples were sequentially immunoblotted (IB) with α -pTyr mAb (top) and α -PLC γ 1 antibody. (C) IP₃ production. Cells were stimulated as described in (A) and IP₃ levels were quantitated with a radioreceptor assay. Bars indicate the mean $\pm$ standard deviation from triplicate samples. (D) Role of the CH domain in TCR-mediated Ca^{2+} mobilization. Jurkat or J.Vav1 cells were infected with non-recombinant vaccinia virus (WR), or with recombinant virus encoding wild-type Vav-1 or the Vav-1 CH⁻ mutant. The cells were loaded with Indo-1, stimulated with OKT3 mAb and analyzed for $[\text{Ca}^{2+}]_i$ as described in (A).

Fig. 5. Roles of Vav1 in MAP kinase activation. (A) Ras-ERK activation. Jurkat or J.Vav1 cells (2×10^6 cells/sample) were stimulated with OKT3 or PMA for the indicated times, and GTP-bound Ras was precipitated with a GST-RBD fusion protein. The precipitated protein was subjected to SDS-PAGE and immunoblotting with α -Ras mAb (upper panel). Detergent extracts were resolved by SDS-PAGE, and immunoblotted sequentially with α -phospho-ERK (α -p-ERK) and α -ERK 1/2 antibodies (middle and lower panels). (B) JNK activation. Cells (2×10^6 per sample) were exposed for 10 min to the indicated stimuli, and JNK activities were determined in immune complex kinase assays, with GST-c-Jun¹⁻⁷⁹ as the substrate. The incorporation of radioactivity from [γ - ^{32}P]ATP into substrate was visualized by autoradiography (middle panel) and quantitated by phosphorimaging (upper panel). The amount of JNK protein present in each sample is shown in the lower panel.

the Ca^{2+} signaling defect in J.Vav1 cells (Figure 4D). In contrast, the Vav-1 CH⁻ mutant failed to reverse this abnormality, which indicates that the CH domain of Vav-1 is essential for the generation of a sustained Ca^{2+} signal in response TCR ligation.

Ras-dependent signaling events in J.Vav1 cells

In addition to a Ca^{2+} signal, the assembly of active NFAT complexes in activated T cells hinges on triggering of the Ras-dependent signaling cascade. To determine whether Vav1 was required for TCR coupling to the Ras pathway, we examined the activation of Ras and ERK in OKT3-stimulated J.Vav1 cells. Both responses occurred normally in Vav-1-deficient cells (Figure 5A, top and middle panels). In contrast, the J.Vav1 cells displayed significant defects in TCR-dependent CD69 expression, a response that is tightly linked to activation of the Ras signaling cascade in T cells (D'Ambrosio *et al.*, 1994) (Table I). PMA-stimulated CD69 expression was not perturbed in the Vav-1-deficient Jurkat cells. Thus, we conclude that Vav-1 and Ras function in parallel pathways leading to CD69 expression in response to TCR engagement.

Defective JNK activation in J.Vav1 cells

Vav-1 has been implicated in the activation of a second MAP kinase signaling module, which leads from Rac/Rho GTPases to the stimulation of JNKs (Crespo *et al.*, 1996; Kaminuma *et al.*, 2001). In line with this model, we

Table 1. CD69 expression in Jurkat and Vav^{-/-} cells

Stimuli	Jurkat		JVav-1.D	
	CD69 ⁺ (%)	MFI	CD69 ⁺ (%)	MFI
5 h				
None	52	26	14	9
OKT3	96	337	83	67
PMA	100	444	99	290
20 h				
None	66	43	31	13
OKT3	99	798	64	80
PMA	100	1411	99	1101

Cells were harvested at 5 or 20 h after stimulation, and incubated with phycoerythrin (PE)-conjugated α -CD69 antibody, or PE-conjugated mouse α -rabbit IgG as the negative control. CD69-positive cells were enumerated by flow cytometry and the mean fluorescence intensity (MFI) of the positive populations was determined as a measure of average CD69 expression level per cell.

observed that J.Vav1 cells displayed a severe defect in TCR-mediated JNK activation, as measured in immune complex kinase assays. Stimulation of Jurkat cells with OKT3 alone increased JNK activity (5.1-fold over basal), and this response was synergistically enhanced by co-stimulation of the cells with PMA, or with PMA and α -CD28 mAb (Figure 5B). In contrast, OKT3 mAb stimulation alone elicited virtually no JNK activation response (1.4-fold over basal) in J.Vav1 cells. Although co-stimulation of these cells with PMA or PMA/ α -CD28 mAb enhanced JNK activity, the response remained significantly lower than that observed with Jurkat cells. Interestingly, JNK activation was also impaired in J.Vav1 cells exposed to PMA only, indicating that this TCR-independent stimulus also requires Vav-1 expression to maximally stimulate the JNK signaling cascade.

Role of Vav-1 in TCR-mediated activation of AP-1 and NF κ B

In addition to NFAT, the IL-2 promoter contains binding sites for the heterodimeric transcription factors AP-1 and NF κ B (Crabtree and Clipstone, 1994). Consistent with the JNK activation defect described above, the transcription of an AP-1-linked reporter gene was profoundly reduced in OKT3 mAb-stimulated J.Vav1 cells, in the presence or absence of PMA (Figure 6A). Co-transfection of the cells with a Vav-1 mutant (L213A) that lacks Rho/Rac-specific GEF activity failed to rescue the AP-1 activation defect in the Vav-1-null cells (Figure 6A). The requirement for Vav-1-associated GEF activity was obviated by transfection of these cells with a constitutively active Rac1 [Rac1 (Q61L)] mutant. On the other hand, the Vav-1 CH⁻ mutant, which had failed to support TCR-mediated NFAT activation (Figure 3B), restored the AP-1-dependent transcriptional response in these cells (Figure 6B). Indeed, Vav-1 CH⁻ expression consistently increased the basal level of AP-1 activity in J.Vav1 cells, which suggests that loss of the CH domain renders Vav-1 a constitutive activator of the AP-1 signaling pathway.

Qualitatively similar results were obtained in reporter assays for TCR-dependent activation of NFAT-, NF κ B- and CD28-response element (REAP)-dependent transcriptional activation. In J.Vav1 cells, introduction of wild-type

Fig. 6. Requirement of Vav-1 for TCR-dependent AP-1 activation. (A) Jurkat cells were co-transfected with 3 μ g of pAP1-Luc reporter construct plus 0.5 μ g of pRL-TK. Where indicated, the JVav1 cells were additionally transfected with pcDNA3 (30 μ g), Rac1 (Q/L) (10 μ g), myc-tagged wild-type Vav-1 (wtVav1; 3 μ g) or Vav-1 Dbl-homology domain mutant (L213A; 10 μ g). After 18 h, the cells were stimulated for 6 h and luciferase activities were determined. The pAP1-Luc-derived luciferase activity was normalized to the *Renilla* luciferase activity in each sample. The results are presented as the mean $\pm$ standard deviation from triplicate samples. Aliquots from representative samples were immunoblotted to indicate expression levels of the various Vav-1 proteins (lower panel). The blot was re-probed with α -ZAP-70 antibodies to control for protein loading. (B) Activation of AP-1-dependent transcription by Vav-1 CH⁻ mutant. Cells were transiently transfected with the pAP1-Luc plus the indicated Vav-1 expression plasmid (10 μ g), and luciferase activities were measured as described in (A). Luciferase activities in each sample were normalized to the maximal levels obtained in cells stimulated with ionomycin plus PMA. Bars represent mean values from duplicate samples. The right panel shows expression levels of the FLAG-tagged Vav proteins.

Vav-1, but not the catalytically inactive Vav-1 (L213A) mutant, rescued all three transcriptional responses, indicating that the Rac/Rho GEF activity of Vav-1 is

Fig. 7. TCR/CD28-mediated PKC- θ translocation in J.Vav1 cells. Jurkat or J.Vav1 cells were stimulated with 1 μ g/ml OKT3 plus 1 μ g/ml α -CD28 antibodies. The cells were subjected to a serial extraction protocol (see Materials and methods) and extracts were immunoblotted with the indicated antibodies. The digitonin-extractable fraction contains mainly cytoplasmic (C) proteins, whereas the NP-40-soluble fraction includes membrane-associated proteins (M).

uniformly required for the transmission of activating signals to NFAT, NF κ B and AP-1.

Protein kinase C- θ activation in Vav-1-deficient Jurkat T cells

Previous studies suggested that Vav-1 serves as a critical upstream activator of protein kinase C- θ (PKC- θ) during T-cell activation (Dienz *et al.*, 2000; Villalba *et al.*, 2000). Consequently, we examined the status of the TCR-PKC- θ coupling mechanism in the Vav-1-deficient J.Vav1 cells (Figure 7). Using an *in situ* extraction protocol, we observed that OKT3 plus α -CD28 mAb stimulation induced the translocation of PKC- θ to the digitonin-insoluble fraction in both Jurkat and J.Vav1 cells. This translocation event may mark the movement of PKC- θ into membrane lipid rafts, as we found that a known raft-associated protein, LAT, was constitutively localized in this detergent-resistant fraction (Figure 7). The translocation of PKC- θ in both cell lines was accompanied by an upward shift in electrophoretic mobility, which suggests that the membrane-bound PKC- θ underwent similar post-translational modifications in the presence or absence of Vav-1. Although these findings suggest that PKC- θ translocation and activation are not impaired in the Vav-1-deficient Jurkat cells, we did observe that the level of membrane-bound PKC- θ declined more rapidly in activated J.Vav1 cells (see the 5 min time point in Figure 7). Thus, Vav-1 expression may be necessary for a sustained PKC- θ translocation response under these stimulation conditions.

A recent study demonstrated that antibody-induced coalescence of cholera toxin B (CTxB)-FITC-labeled lipid rafts could be inhibited by overexpression of a dominant-negative Vav-1 mutant, suggesting that this response was Vav-1 dependent (Harder and Simons, 1999; Villalba *et al.*, 2001). However, we found no detectable defect in α -CTxB antibody-stimulated lipid raft clustering when this experiment was performed in J.Vav1 cells (Figure 8). Over four independent experiments, we observed clear raft polarization responses in 58%

(232/400 total cells) of Jurkat and 54% (216/400 total cells) of J.Vav1 cells. Consistent with the biochemical evidence presented in Figure 7, PKC- θ co-localized with polarized lipid rafts to similar extents in Jurkat and J.Vav1 cells.

Functional redundancy among Vav family members during TCR signaling

The finding that TCR-dependent PKC- θ translocation was unimpaired in J.Vav1 cells contradicted recent results obtained through overexpression of dominant-negative Vav1 mutants in Jurkat cells (Villalba *et al.*, 2000, 2001). One plausible explanation for these discrepant results is that PKC- θ activation is redundantly controlled by multiple Vav isoforms expressed in Jurkat T cells, and that overexpression of dominant-negative Vav1 *trans*-inhibits these redundant functions of Vav-2 or Vav-3. As shown in Figure 9A, both Vav-2 and Vav-3 undergo rapid increases in tyrosine phosphorylation in response to TCR cross-linkage, indicating that, like Vav-1, these proteins are biochemically linked to the TCR signaling machinery. In subsequent studies, we examined the abilities of Vav-2 and Vav-3 to complement the IL-2 promoter activation defect in J.Vav1 cells. Transient overexpression of either Vav-2 or Vav-3 effectively restored the increases in IL-2 promoter-dependent transcription triggered by co-stimulation with OKT3 mAb plus either α -CD28 mAb or PMA (Figure 9B). Thus, under these experimental conditions, Vav-2 and Vav-3 show significant functional overlap with Vav-1 during TCR signaling.

Discussion

In this report, we have demonstrated that somatic cell gene targeting is a feasible approach for the generation of 'knockout' Jurkat T-cell lines that lack various components of the TCR-linked signaling machinery. When compared with the random mutagenesis strategies used to derive many of the existing Jurkat somatic mutants, specific gene targeting dramatically broadens the range of proteins that can be scrutinized in this model system. In spite of the well-documented signaling abnormalities in Jurkat cells (Astoul *et al.*, 2001), this cell line continues to represent a mainstay for mechanistic studies of the activation process in T lymphocytes. The successful application of gene-targeting technology in Jurkat cells further extends the usefulness of this model system, and may represent a first step towards the *in vitro* manipulation of the genomes of non-transformed T-cell lines and clones.

In general terms, Vav-1 gene disruption had surprisingly subtle effects on the early biochemical events elicited by TCR cross-linkage in Jurkat cells. In particular, the activation of two key components of the TCR-linked signaling machinery, PLC- γ 1 and the Ras-ERK cascade, was not perturbed in the Vav-1-deficient cells. Perhaps most surprisingly in light of recent findings with wild-type Jurkat cells and *vav-1*^{-/-} murine T cells (Villalba *et al.*, 2001), J.Vav1 cells showed no defects in stimulus-induced lipid raft clustering or PKC- θ translocation. Indeed, TCR cross-linkage induced identical changes in PKC- θ electrophoretic mobility in Jurkat versus J.Vav1 cells, which suggests that the phosphorylation events associated with

Fig. 8. CTxB-induced raft clustering. Jurkat (A–F) or J.Vav1 (G–L) cells were labeled with CTxB–FITC and incubated with goat α -CTxB for 15 min at 37°C. Cells were fixed and stained with a control IgG (A–C, G–I) or with α -PKC- θ (D–F, J–L). PKC- θ (red) was detected with TRITC-conjugated G α Mlg (A, D, G and J), and was analyzed for co-localization with CTxB–FITC (green)-labeled lipid raft clusters (B, E, H and K). Merged images are shown in (C), (F), (I) and (L). Arrows indicate coalesced rafts and PKC- θ . The arrowhead in (J–L) identifies a non-responding J.Vav1 cell.

PKC- θ activation are also not contingent on Vav-1 expression.

The phenotypic defects displayed by J.Vav1 cells were notably less severe than those reported with T cells from *vav1*^{−/−} mice. Although technical issues (e.g. mode of cellular stimulation) cannot be excluded, a plausible explanation is that murine T-cell development in the absence of Vav-1 leads to persistent abnormalities in the TCR signaling machinery, which manifest as more global defects in TCR function. A more trivial alternative is that the PTEN deficiency in Jurkat cells (Shan *et al.*, 2000) partially alleviates the normal dependence of TCR

signaling pathways on Vav-1. However, this scenario appears unlikely, as pretreatment of J.Vav1 cells with the PI 3-kinase inhibitor wortmannin had no effect on OKT3-stimulated lipid raft coalescence or PKC- θ translocation (our unpublished observations).

Our findings with the J.Vav1 model system also reveal important differences from those obtained via overexpression of dominant-negative Vav-1 mutants in wild-type Jurkat cells (Villalba *et al.*, 2000, 2001). The interpretation of results obtained with dominant-negative Vav-1 mutants is problematic due to possible cross-suppression of the Vav-2 and Vav-3 proteins expressed in these cells. Other

Fig. 9. Functional redundancy among Vav family members in J.Vav1 cells. (A) TCR-induced tyrosine phosphorylation of Vav-2 and Vav-3. Cells were stimulated with OKT3 mAb, and immunoprecipitated Vav proteins were immunoblotted sequentially with α -pTyr mAb (upper panel), followed by either α -Vav-2 or α -Vav-3 antibody (lower panel). (B) Reconstitution of IL-2 promoter activation defect by Vav proteins. J.Vav1 cells were transiently co-transfected with the pIL2-luc reporter plasmid, together with the indicated FLAG-tagged Vav constructs. Cells were stimulated for 16 h and luciferase activities were quantified as RLU. The immunoblot shows the expression levels of the FLAG-tagged Vav protein in each sample.

Dbl superfamily members have been shown to form dimers or higher order complexes through intermolecular interactions involving their Dbl homology domains (Anborgh *et al.*, 1999; Zhu *et al.*, 2001). Hence, it is possible that the expression of dominant-negative forms of Vav-1 leads to *trans*-inhibition of Vav-2 and/or Vav-3 functions in Jurkat cells, through ‘poisoning’ of heterooligomeric Vav protein complexes.

In this study, we demonstrated that Vav-2 and Vav-3 undergo tyrosine phosphorylation in response to TCR cross-linkage in J.Vav1 cells. Furthermore, transient overexpression of either Vav isoform complements the profound defect in TCR plus CD28/PMA-mediated IL-2 promoter activation observed in the Vav-1-deficient Jurkat cells. The ability of overexpressed Vav-2 to complement the transcriptional defects in J.Vav1 cells seems at odds with an earlier report (Doody *et al.*, 2000). This discrepancy is apparently explained by the use of human Vav-2 in the present studies versus its murine counterpart in the earlier report (our unpublished observations). Nonetheless, our findings suggest that the Vav proteins display a considerable level of functional redundancy with respect to TCR signaling in Jurkat cells. Similarly, studies in

single- versus double-knockout mice indicate that Vav-1 and Vav-2 reciprocally compensate for one another to support antigen receptor-induced Ca^{2+} mobilization in B cells (Doody *et al.*, 2000; Tedford *et al.*, 2001). More rigorous analyses of the contributions of Vav-2 and Vav-3 to TCR signaling are clearly warranted.

The TCR-dependent Ca^{2+} signaling defect in J.Vav1 cells was also less severe than previously reported with *vav-1*^{-/-} murine T cells (Fischer *et al.*, 1995, 1998; Turner *et al.*, 1997; Holsinger *et al.*, 1998; Costello *et al.*, 1999). In J.Vav1 cells, the initial $[\text{Ca}^{2+}]_i$ spike provoked by TCR cross-linkage was relatively normal, but the cells failed to sustain the $[\text{Ca}^{2+}]_i$ elevation at later times after receptor stimulation. This relatively selective defect in Ca^{2+} mobilization could not be attributed to a defect in TCR-dependent IP_3 generation. In light of these results, we suggest that Vav-1 deficiency significantly impairs the coupling between IP_3 -dependent intracellular Ca^{2+} release and opening of CRAC channels in the T-cell plasma membrane (Lewis, 2001).

Our results corroborate earlier evidence that the CH domain of Vav-1 plays a key role in TCR-dependent Ca^{2+} signaling (Billadeau *et al.*, 2000). Expression of a Vav-1 mutant lacking the CH domain reversed the AP-1 activation defect in J.Vav1 cells, but failed to rescue the defect in sustained Ca^{2+} signaling observed at later times after TCR triggering. The exact contribution of Vav-1 and, in particular, of the Vav-1 CH domain to store-operated Ca^{2+} influx in T cells represents an important area for future investigation. In order to understand the linkage between the CH domain and the Ca^{2+} signaling pathway in T cells, it will be important to identify the proteins that interact with this region of Vav-1.

Although many of the early signaling events triggered by TCR engagement were relatively unperturbed in J.Vav1 cells, the loss of Vav-1 led to much more global and profound defects in the transmission of TCR-dependent signals to the nucleus. Given the relatively modest Ca^{2+} signaling defect in J.Vav1 cells, it was particularly noteworthy that the NFAT(IL2)-dependent transcriptional response was virtually abrogated in these cells. The NFAT(IL2) site is a composite transcriptional element that binds both a NFAT family member and a Fos-Jun heterodimer (Crabtree and Clipstone, 1994). Consistent with the partial defect in TCR-mediated Ca^{2+} mobilization, we found that activation of an AP-1-independent NFAT reporter gene [derived from the human interferon (IFN)- γ enhancer region] was reduced by ~50% in OKT3 mAb-stimulated J.Vav1 cells (our unpublished observation). Complementation of either the NFAT(IL2)- or NFAT(IFN- γ)-dependent transcriptional defect in J.Vav1 cells required the CH domain of Vav-1, which further substantiates the critical contribution of the CH domain to the TCR-linked Ca^{2+} signaling pathway. Interestingly, while the CH domain serves a positive role in TCR-mediated Ca^{2+} mobilization, studies with the Vav-1 CH⁻ mutant indicate that this domain simultaneously functions as a negative regulator of the signaling pathway leading to AP-1 activation. In J.Vav1 cells, Vav-1 CH⁻ expression triggered a substantial increase in the basal level of AP-1-dependent transcription, which indicates that loss of the CH domain renders Vav-1 a constitutive activator of the AP-1 signaling pathway. Dereglated activation of AP-1

probably contributes to the enhanced oncogenic activities of N-terminally truncated forms of Vav-1 in rodent fibroblasts (Bustelo, 2000).

The defect in AP-1 activation is probably related to the dramatic reductions in TCR-dependent JNK activation in the J.Vav1 cells. The failure of the Vav-1 (L213A) mutant to rescue the JNK and AP-1 activation defects in J.Vav1 cells indicates that these responses require the Rho/Rac GEF activity of Vav-1. Accordingly, Vav-1 (L213A) expression also failed to reconstitute TCR-dependent NFAT activation in J.Vav1 cells (our unpublished observations). These results are consistent with those obtained in several studies (Hehner *et al.*, 2000; Kaminuma *et al.*, 2001; Moller *et al.*, 2001), and argue against the notion that a GEF-independent function of Vav-1 mediates the formation of the transcriptionally active NFAT-AP-1 complex in T cells (Kuhne *et al.*, 2000). While Vav-1 may well serve as a scaffolding protein during T-cell activation (Bustelo, 2001), the transmission of signals for *IL-2* gene transcription appears to require the concomitant activation of Rho/Rac GTPases. Additional studies with the J.Vav1 model system should provide a more detailed view of the pleiotropic functions of Vav-1 in downstream signaling from the TCR.

Materials and methods

Reagents and plasmids

The α -CD3 mAb OKT3 was purified from mouse ascites by protein G-Sepharose chromatography. Anti-human CD28 mAb (clone 15E8) was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). The α -phosphotyrosine mAb (4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phycoerythrin-conjugated α -CD69 mAb was obtained from Becton-Dickinson (San Jose, CA). Rabbit polyclonal α -Vav-1, α -Vav-2 and anti-ZAP-70 antibodies were described previously (Williams *et al.*, 1998; Billadeau *et al.*, 2000). The α -Vav-3 antibodies were generated by immunization of rabbits with a keyhole limpet hemocyanin-coupled peptide corresponding to amino acids 566–591 of human Vav-3. Polyclonal α -JNK antibody (sc-571) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and α -PKC- θ mAb was obtained from BD Biosciences (Palo Alto, CA). The pan Ras-specific mAb, and polyclonal α -phospho-MAPK and α -ERK1/2 antibodies were purchased from Promega (Madison, WI). Goat α -mouse IgG (G α Mlg) was obtained from Pierce Chemical Co. (Rockford, IL). FITC-conjugated CTxB was obtained from Sigma (St Louis, MO), goat α -CTxB was from Calbiochem (San Diego, CA) and tetramethylrhodamine-5-isothiocyanate (TRITC)-labeled G α Mlg was obtained from Molecular Probes (Eugene, OR).

The expression plasmids for Vav-1 and the constitutively active form of PKC- θ (PKC- θ A/E) were described previously (Villalba *et al.*, 1999; Billadeau *et al.*, 2000). FLAG-tagged human Vav2 and Vav3 expression constructs were prepared by subcloning the human Vav-2 and Vav-3 cDNAs into the pcDNA3.FLAG vector as *HindIII*–*NotI* restriction fragments. The plasmid vector encoding constitutively active Rac1 [Rac1(Q61L)] was provided by Dr Silvio Gutkind (NIH, Bethesda, MD). The *IL-2* promoter-, NF κ B- and NFAT-linked luciferase reporter plasmids (pIL2-luc, pNF κ B-luc and pNFAT-luc, respectively) were provided by Dr David McKean (Mayo Clinic, Rochester, MN), and the pCD28RE-luc reporter was provided by Dr Arthur Weiss (University of California-San Francisco). The pNFAT(IFN- γ)-Luc reporter plasmid was constructed by cloning three tandem repeats of the –280 NFAT site (Sweetser *et al.*, 1998) from the human IFN- γ promoter into the *XhoI*–*HindIII* site of the pT8.Luc reporter plasmid (Promega).

Cell culture, transfection and stimulation

Jurkat T cells (clone E6-1) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Transient transfections were performed with 1×10^7 cells/sample and a total of 30 μ g of plasmid DNA. The cell-DNA mixture (total volume 300 μ l) was pulsed with a BTX Electro-square Porator model T820 (BTX Inc., San Diego, CA) at

field settings of 310 V, 10 ms. For luciferase reporter assays, 100 μ l aliquots (1×10^5 cells) were distributed into 96-well culture plates. After 18 h in culture, the cells were stimulated for 6 h with the indicated agents. Unless stated otherwise, the concentrations of the stimuli used in both reporter gene and biochemical assays were 1 μ g/ml OKT3 (cross-linked with 1 μ g/ml G α Mlg), 1 μ g/ml α -CD28 mAb, 20 ng/ml PMA and 20 nM ionomycin. Samples were harvested and prepared for luciferase assays according to the manufacturer's protocol (Promega, Madison, WI). Where indicated, the cells were co-transfected with a pRL-TK reporter plasmid (Promega, Madison, WI) to control for variations in transfection efficiency. Firefly and pRL-TK-derived *Renilla* luciferase activities were measured in each sample with a Dual Luciferase Assay kit (Promega). All reporter gene assays were repeated a minimum of three times, and representative results from a single assay are shown in the figures.

Vav-1 gene targeting

The promoterless *Vav-1* gene-targeting vector was constructed with two selectable markers, GFP and neomycin phosphotransferase (Neo^r), each containing a 5'-internal ribosome entry site (IRES). The IRES-GFP sequence was PCR amplified from pIRES2-EGFP (Clontech), while the IRES-Neo^r-poly(A) fragment was obtained by PCR amplification of a DNA template provided by Dr John Sedivy (Brown University, Providence, RI). This selection cassette was flanked with *loxP* sites to allow for Cre-mediated excision of the cassette from the first targeted *Vav-1* allele, and recycling of the targeting vector during disruption of the second *Vav-1* allele. The entire cassette was cloned into pCR 2.1 (Invitrogen) and the resulting construct was termed pCST-GN. Details regarding the preparation of the base vector and the *Vav-1* targeting construct are available on request.

The targeting vector was digested with *SpeI* and *Bsu36I* to remove non-essential, vector-derived sequences, and transfected into Jurkat T cells by electroporation as described above. After 24 h, the medium was supplemented with 1 mg/ml G418 and the cells were cultured in the presence of drug for 5–7 days. The surviving cell population was then subjected to fluorescence-activated cell sorting (FACS), and arbitrarily gated low, intermediate and bright fluorescent cells were sorted into 96-well culture plates (1 cell per well) with an automated cell-depositing unit. Clonal cell populations were duplicate plated into 96-well plates. One aliquot ($\sim 1 \times 10^5$ cells) of each clonal population was lysed in PCR lysis buffer [10 mM Tris-HCl, 5 mM EDTA pH 8.0, 0.2% (w/v) Triton X-100 and 400 μ g/ml proteinase K] with sequential incubations at 50°C for 30 min and at 94°C for 10 min. The cleared extract was then chilled on ice and added to PCR reactions with the primers depicted in Figure 1A. The amplification products were resolved by agarose gel electrophoresis and positive clones (*Vav-1*^{+/+}) were selected for a second round of *Vav-1* gene targeting. The heterozygous clones were transiently transfected with a Cre expression vector, and re-cloned by FACS as described above. Cre-mediated excision of the *loxP*-flanked selection cassette was detected by PCR and confirmed by re-acquisition of cellular sensitivity to G418. Selected clones were then subjected to a second round of *Vav-1* gene targeting by transfection with the second targeting construct (Figure 1A legend). Targeted disruption of both *Vav-1* alleles was confirmed by Southern blot analysis.

IP₃ production

Cells were stimulated at 37°C in 0.4 ml of solution 2 (Hanks' balanced salt solution buffered to pH 7.4 with 10 mM HEPES and supplemented with 5 mM glucose) containing OKT3 mAb (1 μ g/ml) cross-linked with G α Mlg (10 μ g/ml). The cells were extracted with trichloroacetic acid and soluble IP₃ levels were determined with a radioreceptor assay kit (NEN, Boston, MA).

Calcium mobilization

Cells were loaded with Indo-1 acetoxy-methyl ester (Molecular Probes) and stimulated with 100 ng/ml OKT3 mAb. Changes in [Ca²⁺]_i were determined by flow cytometry as described previously (Williams *et al.*, 1998; Billadeau *et al.*, 2000). Where indicated, the cells were infected with either non-recombinant vaccinia virus (WR) or recombinant vaccinia virus expressing the wild-type Vav-1 or the Vav-1 CH⁺ mutant prior to loading with Indo-1.

Ras activation and MAPK phosphorylation

The accumulation of GTP-bound Ras in stimulated cells was assayed by precipitation with immobilized glutathione S-transferase (GST) fused to the Ras-binding domain of Raf (GST-RBD) (Williams *et al.*, 1999). For analysis of MAPK activation, the cells were lysed in buffer A (Williams *et al.*, 1998), cleared extracts were separated by SDS-PAGE, and

immunoblotted sequentially with anti-phospho-MAPK and anti-ERK1/2 antibodies.

JNK kinase assays

The protein kinase activity of JNK was determined in immune complex kinase assays with 1 µg of GST-c-Jun(1–79) as the substrate (Kaminuma *et al.*, 2001). Incorporation of radiolabeled phosphate into the substrate was quantitated with a phosphoimaging system.

Cell fractionation

Jurkat or JVav1 cells (1×10^7 cells/sample) were stimulated, washed in ice-cold PBS and resuspended in 1 ml of hypotonic digitonin extraction buffer (5 mM Tris pH 7.5, 10 mM NaCl, 0.5 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 40 µg/ml digitonin, 5 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and 1 mM Na₃VO₄). After 15 min on ice, the cells were pelleted for 5 min at 1000 g, and the supernatant was collected and assayed for total protein content (Bio-Rad). The insoluble pellet was washed with PBS, and then re-extracted with 0.5 ml of NP-40 lysis buffer (10 mM Tris pH 7.5, 40 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 0.2% NP-40, 5 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and 1 mM Na₃VO₄). The samples were vortexed and incubated on ice for 10 min. Cell lysates were cleared of insoluble material by centrifugation at 18 000 g and soluble proteins were quantitated as described above. Aliquots (25 µg protein/sample) of the digitonin- and NP-40-extractable fractions from each sample were resolved by SDS-PAGE and analyzed by immunoblotting.

Raft polarization and immunofluorescence

Raft polarization was carried out as described previously (Harder and Simons, 1999; Bi *et al.*, 2001; Villalba *et al.*, 2001). Immunolocalization of PKC-θ was performed as described previously (Lou *et al.*, 2001) with 0.5 µg/ml α-PKC-θ or control IgG followed by TRITC-labeled GαMig (1:500). Images were analyzed with a confocal microscope and the LSM510 software package (Carl Zeiss).

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