Vav-induced activation of the human IFN-γ gene promoter is mediated by upregulation of AP-1 activity

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Abstract The role of Vav in the transcriptional regulation of the human interferon-γ (IFN-γ) promoter was investigated. Overexpression of Vav in Jurkat-TAg cells enhanced T cell receptor (TCR)-induced activation of a luciferase (Luc) reporter gene construct driven by *cis*-regulatory element of the IFN-γ gene (−346 to +7). Electrophoresis mobility shift and Luc reporter assays demonstrated that the DNA-binding and transcriptional activity of the proximal AP-1-dependent NFAT site (positions −172 to −138), the AP-1/Ying-Yang 1 (YY1)-binding site (−209 to −184), and a consensus AP-1-binding site were upregulated by Vav. Vav enhanced TCR-induced activation of c-Jun N-terminal kinase (JNK) and its upstream regulator, Rho family GTPases. Finally, coexpression of a dominant-negative Rac1 mutant suppressed Vav-mediated upregulation of the transcriptional and DNA-binding activity of the proximal NFAT/AP-1 site and the AP-1/YY1 site, as well as the complete IFN-γ promoter activity. Vav activates the IFN-γ promoter via upregulation of AP-1-binding through a Rac1/JNK pathway.

Key words: Vav; AP-1; Interferon-γ; NFAT; Ying-Yang 1

1. Introduction

The proto-oncogene product, Vav, expressed specifically in hematopoietic and trophoblast cells, has crucial roles in the activation of T cells triggered through the T cell receptor (TCR) [1,2]. Accumulating evidence suggests that Vav, which is rapidly phosphorylated on tyrosine residues in response to TCR stimulation, displays several T cell functions. Overexpression of Vav in T cells enhances basal and TCR-activated transcription of the IL-2 [3–6] or IL-4 [7] genes. Analysis of gene-disrupted mice indicated that T cell development is impaired in the absence of Vav and that Vav-deficient T cells proliferate poorly and produce little IL-2 in response to stimulation through the TCR [8–11]. The enhancement of IL-2 gene transcription by Vav is largely mediated by the activation of the distal NFAT element in the IL-2 gene promoter [3–5,12].

The NFAT family contains five members, NFAT 1–5 [13,14]. NFAT 1–4 are capable of binding to the distal NFAT-binding sequence in the IL-2 promoter, and activating transcription driven from the NFAT sites when they are overexpressed. The NFAT complex is composed of a cytoplasmic subunit whose activity is controlled by subcellular localization. In quiescent T cells, NFAT activity resides in the cytoplasm and, upon activation through the TCR, it translocates into the nucleus [15] in a Ca^{2+}/calcineurin-dependent, and cyclosporin A-sensitive manner [16,17]. In the nucleus, NFAT associates with AP-1, an inducible nuclear component [18–21].

The AP-1 complex is a dimer of members of the Jun, Fos and activation transcription factor (ATF) families [22]. Each of these proteins contains a leucine zipper, which permits its dimerization with other members of the Jun/Fos/ATF family and an adjacent basic region that mediates DNA-binding [22]. Among Jun family proteins, c-Jun is specifically phosphorylated by c-Jun N-terminal kinase (JNK) at two positive regulatory sites residing within its amino-terminal activation domain [23,24]. Activation of JNK by Vav has been demonstrated in non-T cells [25–27]. In addition, Vav displays in vitro guanine nucleotide exchange factor (GEF) activity toward Rho family GTPases, Rac and Cdc42 [2], which are upstream signaling molecules in the pathway leading to JNK activation [28,29]. These findings suggest that Vav may activate AP-1 and thereby contribute to upregulation of NFAT activity. Consistent with this notion, we recently found that transient overexpression of Vav greatly increases AP-1 activity in T cells [6,30], although another recent study reported that Vav does not play a role in AP-1 activation [31].

The ability of Vav to activate NFAT/AP-1 in the IL-2 promoter [3–5,30], and the presence of a combined NFAT/AP-1-binding site in the promoters of other cytokine genes [13,21,32,33], including the interferon-γ (IFN-γ) gene [13,33–36], suggest that Vav could regulate the expression of a number of cytokine genes having NFAT/AP-1-binding sites in their *cis*-regulatory elements. However, the effects of Vav on cytokines other than IL-2 and IL-4 have not been analyzed. Therefore, we have evaluated the role of Vav in IFN-γ gene transcription. Here we report that Vav activates the IFN-γ gene promoter, map some of the responsive elements and

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Abbreviations: ATF, activation transcription factor; β-gal, β-galactosidase; EMSA, electrophoresis mobility shift assay; HA, hemagglutinin; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; TCR, T cell receptor; YY1, Ying-Yang 1

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examine the mechanism through which Vav activates this promoter.

2. Materials and methods

2.1. Antibodies and reagents

Mouse monoclonal antibodies (mAbs) against Vav or Rac1 were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-CD3 (OKT3) and anti-c-Myc (9E10) mAbs were purified from culture supernatants of the corresponding hybridomas by protein G-Sepharose chromatography. For TCR/CD3 cross-linking, a secondary anti-mouse IgG antibody (Organon Teknica Corp., Durham, NC, USA) was used. The anti-Cdc42 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-hemagglutinin (anti-HA; clone 12CA5) mAb was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Horseradish peroxidase-conjugated $F(ab')_2$ fragments of donkey anti-rabbit IgG or sheep anti-mouse IgG were from Amersham (Piscataway, NJ, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA).

2.2. Plasmids

The cDNAs encoding c-Myc epitope-tagged Vav in the pEF mammalian expression vector [5], HA-JNK1, dominant-negative Rac1 (N17Rac1), or a luciferase (Luc) gene driven by three repeats of the AP-1 site (TGACTCA) in the human metallothionein II_A gene were described [30]. The Luc reporter genes driven by the human IFN-γ promoter (−346 to +7; IFN-γ-Luc), three tandem repeats of the −172/−138 site (−172/−138-Luc) or four repeats of the −209/−184 site (−209/−184-Luc) were cloned in pGL3 basic vector (Promega, Madison, WI, USA). As a control for transfection efficiencies, a β-galactosidase (β-gal) expression plasmid in the pEF vector was used. The correct sequence of all constructs was verified by sequencing.

2.3. Cell culture, transfection and stimulation

Simian virus 40 T antigen-transfected human leukemic Jurkat T (Jurkat-TAg) cells were grown in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Harlan, Indianapolis, IN, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1×MEM non-essential amino acid solution and 100 U/ml each penicillin G and streptomycin. Cells in logarithmic growth phase were transfected with various amounts of plasmid DNAs by electroporation as described previously [5,30]. In each experiment, cells in different groups were transfected with the same total amount of plasmid DNA by supplementing expression vector DNA with the proper amounts of the corresponding empty vector. On average, 35% of the cells expressed the transfected plasmids as determined by FACS analysis of green fluorescence protein-cotransfected cells (data not shown). After 24 h, cells were resuspended ($2–4\times10^7$/ml) in RPMI 1640 medium, equilibrated at 37°C for 5 min and either left unstimulated or stimulated with OKT3 (0.01–1 μg/ml), which had been cross-linked with a secondary anti-mouse IgG antibody (1 μg/ml). This antibody was added 1 min after the addition of OKT3. The secondary antibody alone did not have any effect on the cells (data not shown).

2.4. Reporter assays

After 8 h of stimulation, cells were harvested, washed twice with phosphate-buffered saline, and lysed. Luc or β-gal activities in cell extracts were determined as described previously [5,30]. Luc activity was determined in triplicate by luminometry, and β-gal activity was measured by spectrophotometry (400 nm). Luc activity was expressed as arbitrary units normalized to β-gal activity in the same cells. The standard deviation for triplicates was $<10\%$, and each experiment was repeated at least four times.

2.5. Electrophoresis mobility shift assay (EMSA)

Crude nuclear and cytoplasmic extracts were prepared as described [30]. EMSA was performed as described [30,37] using oligonucleotides corresponding to the −172/−138 and −209/−184 sites of the human IFN-γ gene [36] or AP-1 [30].

2.6. Rac1 or Cdc42 activity

After stimulation for various times, cell lysates were prepared by adding 2×Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 40 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10 mM EDTA, 10 mM sodium orthophosphate, 4 mM Na_3VO_4, 20 μg/ml each of aprotinin and leupeptin). Cells were lysed for 10 min at 4°C and insoluble materials were removed by centrifugation ($16\,500\times g$) at 4°C for 10 min. Half of each lysate was mixed with 2 μg of glutathione *S*-transferase (GST) fusion protein expressing the Rac1- or Cdc42-binding domain of mouse p21-activated kinase 3 (GST–PBD; [38] for 1 h, followed by the addition of 40 μl of glutathione-coupled Sepharose 4B beads (Pharmacia Biotech Inc., Piscataway, NJ, USA) for an additional 1 h at 4°C. The beads were washed four times with 1×NP-40 lysis buffer, boiled in 20 μl 2×Laemmli's buffer, subjected to SDS–12% polyacrylamide gel electrophoresis (PAGE) analysis, and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were immunoblotted with anti-Rac1 or anti-Cdc42 antibodies (1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies [30]. The membranes were washed and visualized with an enhanced chemiluminescence detection system (Amersham).

2.7. JNK assay

JNK1 was immunoprecipitated from cell lysates using an anti-HA mAb, subjected to an in vitro kinase reaction GST–c-Jun as a substrate, and kinase activity was determined as described [30]. Following autoradiography to determine kinase activity, the membranes were immunoblotted with an anti-c-Jun mAb as a loading control.

3. Results

3.1. Vav activates the IFN-γ promoter

The transcriptional activity of the IL-2 gene promoter is upregulated by Vav [3–6,12]. However, it has not been known whether Vav also activates the IFN-γ gene. Therefore, we first investigated the effect of Vav on human IFN-γ gene transcription. As shown in Fig. 1, the transcriptional activity of Luc reporter gene plasmid driven by the synthetic IFN-γ promoter (IFN-γ-Luc) was stimulated in a dose-dependent manner by OKT3 stimulation in empty vector-transfected cells. Overex-

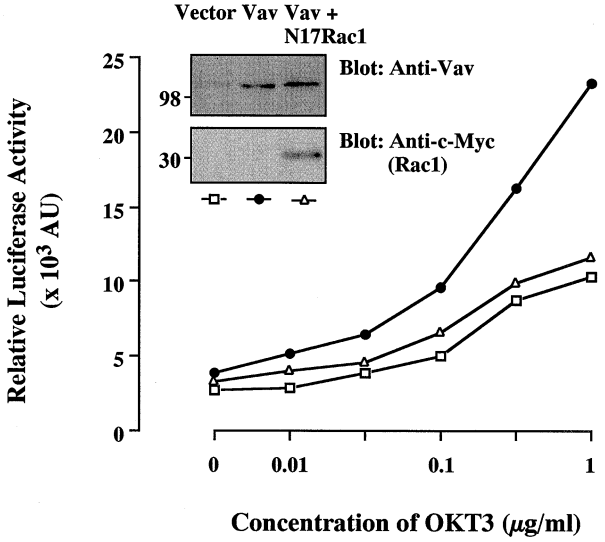

Fig. 1. Effect of Vav on transcriptional activity of the IFN-γ promoter. Cells were cotransfected with an IFN-γ-Luc reporter plasmid (5 μg) plus empty vector, c-Myc-tagged Vav, or Vav plus N17Rac1 (5 μg each). After 24 h, cells were either left unstimulated or stimulated for 8 h with OKT3, which has been linked using an anti-mouse IgG antibody, then lysed for a Luc assay. The data shown are representative of at least four experiments. Samples of the same lysates were analyzed for the expression of Vav and N17Rac1 by immunoblotting with anti-Vav and anti-c-Myc mAbs. Molecular weight standards are shown ($\times10^3$).

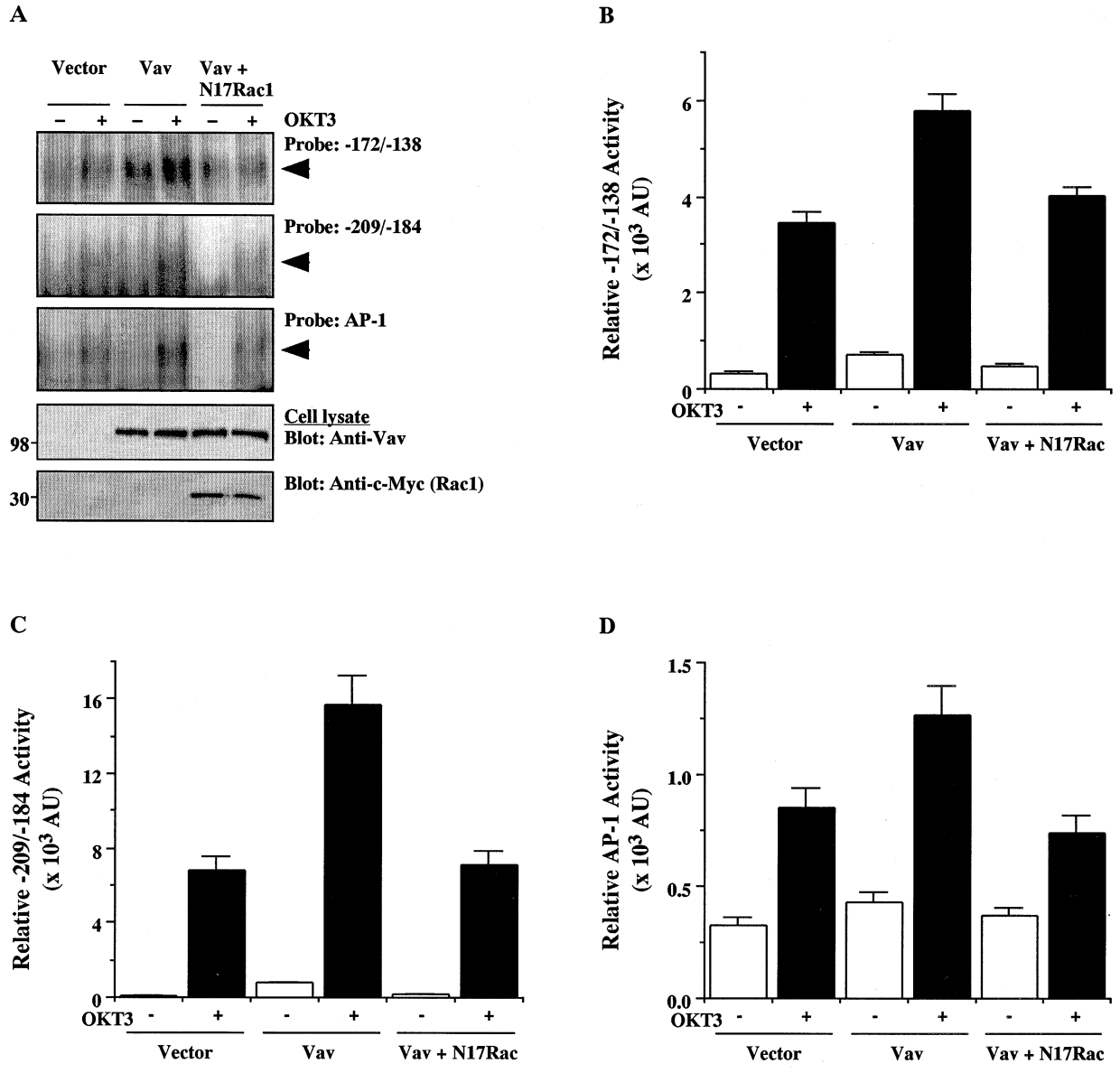

Fig. 2. Effect of Vav on DNA-binding and transcriptional activity of IFN-γ promoter elements and AP-1. A: Cells were transfected with empty vector, Vav, or Vav plus N17Rac1. After 24 h, cells were either left unstimulated or stimulated by cross-linked OKT3 for 2 h. Nuclear extracts were prepared and analyzed by EMSA, using ^{32}P-labeled −172/−138, −209/−184 and AP-1 probes. The membrane was subjected to autoradiography (top three panels), and total cellular extracts from the same transfection groups were immunoblotted with anti-Vav or anti-c-Myc mAbs as indicated. These results are representative of at least four separate experiments. Arrows indicate specific binding complexes. Molecular weight standard are shown (×10^3). B–D: Cells were cotransfected with −172/−138-Luc (B), −209/−184-Luc (C) or AP-1-Luc (D) reporter plasmids plus pEF, Vav, or a combination of Vav and N17Rac1. After 24 h, cells were either left unstimulated or stimulated for 8 h by cross-linked OKT3. Luc activity was determined as in Fig. 1. The data shown are representative of at least four experiments. Proper expression of Vav and N17Rac1 in the cell lysates was confirmed by immunoblotting with anti-Vav and anti-c-Myc mAbs (data not shown).

pression of Vav further enhanced the activity of this reporter gene.

Vav displays guanine nucleotide exchange factor activity toward Rho family GTPases in vitro [2] and this activity is associated with induction of downstream signaling events leading to activation of the IL-2 gene promoter [6,12,39]. Therefore, we next evaluated the role of Rac1 on the activation of IFN-γ promoter by Vav, using dominant-negative Rac1 mutant (N17Rac1). The Vav-mediated increase in the transcriptional activity of IFN-γ-Luc was completely blocked by a coexpressed N17Rac1 (Fig. 1), even though Vav was properly overexpressed, suggesting that Vav activates the IFN-γ gene promoter through a Rac1-dependent pathway.

3.2. *Effect of Vav on DNA-binding and transcriptional activities of AP-1-binding elements in the IFN-γ promoter*

We have recently reported that Vav-induced upregulation of the IL-2 gene transcription is mainly mediated by the enhancement of AP-1 binding to the distal NFAT/AP-1 site in the IL-2 gene promoter [30]. However, it is not clear whether the effect of Vav on transcription of IFN-γ, as well as IL-2, is the result of an increase in DNA–protein binding to the cog-

nate sequence. Therefore, we next evaluated the effect of Vav on the binding and transcriptional activity for several *cis*-regulatory elements in the IFN-γ promoter, including the combined NFAT/AP-1-binding site (−172/−138), and the AP-1/YY1-binding site (−209/−184) [35,36], by comparison with a consensus AP-1-binding site [30]. DNA-binding and transcriptional activities were determined by an EMSA and Luc reporter assay, respectively. Anti-CD3 stimulation increased the binding activity of −172/−138 and −209/−184, as well as AP-1, in empty vector-transfected control cells (Fig. 2A). The binding activity of all three elements was upregulated by overexpression of Vav. These results correlated with the transcriptional activity of the same elements. Thus, the transcriptional activity of all the elements (−172/−138, −209/−184 and AP-1) was stimulated by OKT3 stimulation in empty vector-transfected cells (Fig. 2B–D), and further enhanced by Vav coexpression. The Vav-mediated increase in the binding and transcriptional activity of −172/−138 and −209/−184 was blocked by a coexpressed N17Rac1 mutant (Fig. 2A–C), even though Vav was properly overexpressed (data not shown), suggesting that Vav activates −172/−138 and −209/−184 through a Rac1-dependent pathway. Consistent with our recent report [30], N17Rac1 also abrogated the Vav-mediated increase in the binding and transcriptional activity of a consensus AP-1 site (Fig. 2A,D). We found that Vav also enhanced the TCR-stimulated DNA-binding and transcriptional activities of two other AP-1 sites (data not shown).

3.3. Time course and dose dependence of Vav-mediated activation of Rho family GTPases and JNK

Vav displays in vitro GEF activity for Rho family GTPases, Rac1 and Cdc42 [2], and it also binds these two recombinant GTPases in vitro [40]. Consistent with this GEF activity, Vav has also been reported to activate JNK in non-hematopoietic cells [25–27] and, more recently, also in TCR-stimulated T cells [30]. Moreover, we demonstrated that Vav binds Rac1 and Cdc42 in intact T cells [30]. To gain further insight into the role of the Rac1/JNK pathway in Vav-induced activation of the IFN-γ promoter, we next determined the effect of Vav on Rho family GTPase and JNK activities. The activated, GTP-bound forms of Rac1 or Cdc42 in T cell lysates were captured in vitro by a GST–PBD fusion protein and detected by immunoblotting [30,38]. OKT3 stimulation induced activation of Rac1 and Cdc42 in Vav-transfected cells, reaching a maximum 10–20 min after stimulation (Fig. 3A). This OKT3-induced activation was observed even in empty vector-transfected cells (Fig. 3B). Overexpression of Vav upregulated Rac1/Cdc42 activity in a dose-dependent manner in both unstimulated and OKT3-stimulated T cells (Fig. 3B).

JNK activity was determined in parallel by an in vitro kinase assay using recombinant GST–c-Jun as substrate. Fig. 3A shows that in Vav-transfected cells, OKT3 stimulation induced JNK activation, which reached a maximum after 20 min. The extent of JNK activity in both unstimulated and OKT3-stimulated T cells was dependent on the dose of transfected Vav protein (Fig. 3B).

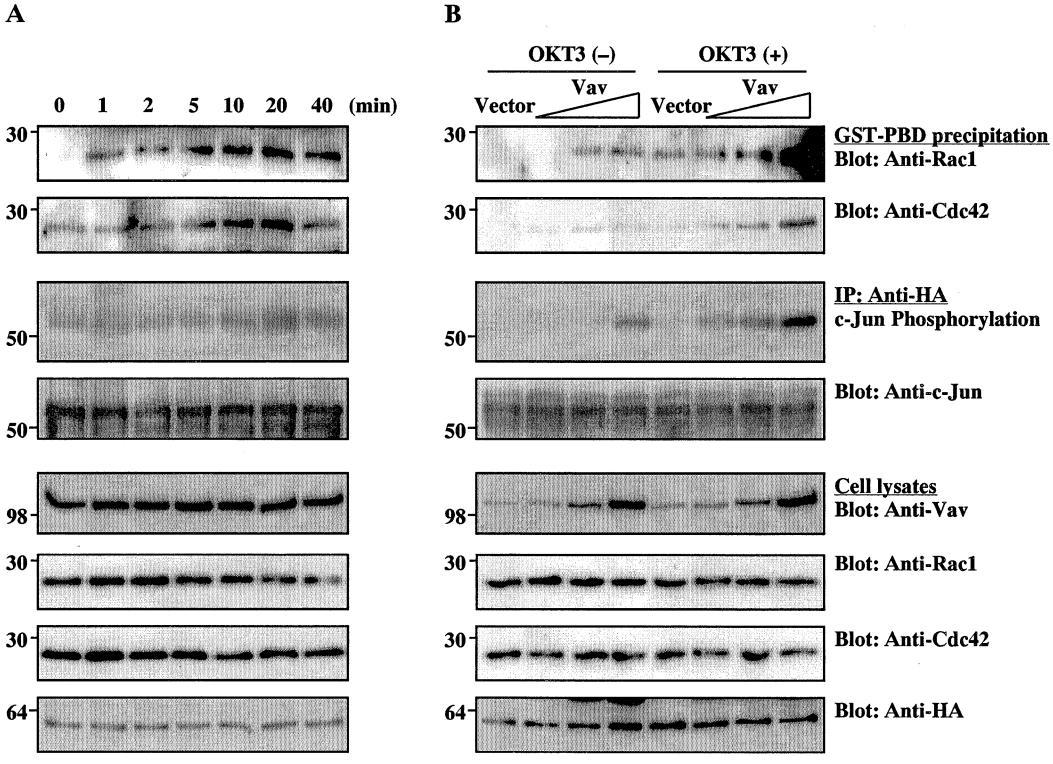

Fig. 3. Effect of Vav on Rho family GTPases and JNK activity. Cells were transfected with 5 μg each of HA-JNK1 plus Vav (5 μg each; A) or increasing amounts of Vav (1, 5 or 10 μg; B) or empty vector (B). After 24 h, cells were either left unstimulated or stimulated by cross-linked OKT3. Cell lysates were prepared at the indicated time points (A) or after 20 min of stimulation (B). Activated Rac1 or Cdc42 in cell lysates were detected by precipitation with GST–PBD and immunoblotting with specific mAbs (top two panels). JNK1 was immunoprecipitated with an anti-HA mAb, and subjected to a radioactive in vitro kinase assay and SDS–PAGE. The membrane was subjected to autoradiography (third panel from the top), followed by immunoblotting with an anti-c-Jun mAb as indicated (fourth panel from the top). Total cellular extracts from the same transfection groups were immunoblotted with anti-Vav, anti-Rac1, anti-Cdc42 or anti-HA mAbs as indicated. The results shown are representative of at least five separate experiments. Molecular weight standards are shown ($\times 10^3$).

4. Discussion

IFN-γ is an important immunoregulatory cytokine responsible for several activities in the immune system, including induction of the Fc receptor, major histocompatibility complex class I and II expression [41], regulation of cytokine genes and activation of immune effector cells [42]. IFN-γ production is largely restricted to activated T cells and large granular lymphocytes [42]. This cytokine belongs to a group of lymphokines whose synthesis parallels that of IL-2 and is inhibited by cyclosporin A [13,33,42]. A wide variety of transcription factors have been implicated in IFN-γ gene transcription [42,43], and the corresponding promoter has several NFAT- and/or AP-1-binding sites [34–36,43]. Since Vav stimulates IL-2 gene transcription by upregulating the distal NFAT/AP-1-binding site [3–5], this raised the unexplored possibility that Vav may also regulate the IFN-γ gene.

Our present findings demonstrate that Vav activates the IFN-γ gene promoter (Fig. 1). Analysis of distinct elements within this promoter revealed that Vav induced Rac1-dependent upregulation of the DNA-binding and transcriptional activity of NFAT/AP-1 (−172/−138)- and AP-1/YY1 (−209/−184)-binding sites, as well as a consensus AP-1 site (Fig. 2). Vav also activated Rac1/Cdc42 and JNK (Fig. 3), suggesting that Vav activates IFN-γ gene promoter through a Rac1/JNK/AP-1 pathway. We reported recently that Vav overexpression failed to exert a detectable effect not only on the DNA-binding and transcriptional activities of the distal NFAT element in the IFN-γ promoter at −285/−257, but also on TCR-stimulated Ca^{2+} mobilization and nuclear translocation of NFAT [30]. This promoter region in the human IFN-γ gene contains a consensus NFAT-binding sequence at position −280/−270, which is necessary for its inducible transcriptional activation. The importance of this site in IFN-γ transcription was demonstrated by mutational analysis, but DNase footprinting or EMSA analysis indicated that this region does not bind AP-1 [30,35,36]. In addition to NFAT, the region of the IFN-γ promoter between nucleotides −284 and −260 was also found to bind nuclear factor κB (NF-κB) [35]. This suggests that NF-κB, which is positively regulated by Vav [44], may be a target for Vav-mediated regulation at this site, even though our earlier study failed to show activation of this site by Vav [30].

In contrast to the above distal NFAT site, the *cis*-element at −163/−155 is similar to composite NFAT/AP-1 elements found in other cytokine gene promoters [13], and was found to bind an NFAT/AP-1 complex [36]. The importance of this site is evident from findings that point mutation in it decreased IFN-γ gene transcription and, conversely, NFAT overexpression enhanced gene expression [36]. The effects of Vav on transcriptional and DNA-binding activities of the −172/−138 were concordant (Fig. 2), suggesting that Vav stimulates IFN-γ gene transcription by upregulating the DNA-binding activity of the combined NFAT/AP-1-binding site at −172/−138 (Fig. 2A).

An additional AP-1-binding site in the human IFN-γ promoter at positions −209/−184 binds a complex of AP-1 and Ying-Yang 1 (YY1; [36]). Two constitutive YY1-binding sites have been identified in this region at positions Y1 (−203/−199) and Y2 (−221/−217) [45,46], but the role of YY1 in IFN-γ transcription is controversial [36,45,46]. The AP-1 site at −196/−183 contributes to mitogen- or IL-18-induced IFN-γ transcription, and it also plays a positive role in IL-12-dependent activation of the IFN-γ promoter in primary $CD4^+$ T cells [47,48]. Here we demonstrate that the DNA-binding and transcriptional activities of the AP-1/YY1-binding site (−284/−209) were also upregulated by Vav (Fig. 2A,C). However, it remains to be determined whether Vav affects the binding of AP-1 and/or YY1 to this site.

Taken together, our previous [30] and current findings demonstrate that Vav overexpression enhances the DNA-binding and transcriptional activities of reporter genes containing AP-1-binding sites, i.e. −172/−138 and −284/−209 (as well as a consensus AP-1 site), but has no detectable effect on an AP-1-independent NFAT site (−285/−257). These results suggest that Vav does not directly affect NFAT-binding but, rather, acts primarily on AP-1. Nevertheless, others reported reduced TCR-induced Ca^{2+} mobilization in Vav-deficient T cells [11,44,49,50], but the biological significance of this reduction is unclear since NFAT2 nuclear translocation remained intact in Vav-deficient T cells [50].

The TCR-induced activation of Rac1 and Cdc42 slightly preceded JNK activation (Fig. 3A). This result and the dose-dependent upregulation of Rac1/Cdc42 by Vav further support a role for Vav in the JNK/AP-1 pathway. This is consistent with findings using several other cell types [25–27] and with our recent report [30]. However, the physiological role of a Vav in JNK activation is not completely understood, since Vav-deficient T cells display intact JNK activation [49,50]. It is possible that a compensatory mechanism leading to JNK activation (e.g. expression of other Vav family members) might be upregulated in the chronic absence of Vav, consistent with the results of a recent study [51]. In addition, JNK activation is not required for TCR/CD28-costimulated IL-2 production by primary T cells [52]. Nevertheless, Vav could play a physiological role in JNK activation in memory or effector T cells.

In summary, our findings lend further support to the notion that upregulation of AP-1 activity via the Rac1/JNK pathway is the primary physiological mechanism through which Vav activates various cytokine genes, including the IL-2 [3–6], IL-4 [7] and IFN-γ ([30] and herein) genes. Additional studies are required in order to identify the intermediates and the relevant interactions in this signaling pathway leading from Vav to AP-1 activation.

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