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Tyrosine Kinase–Stimulated Guanine Nucleotide Exchange Activity of Vav in T Cell Activation

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The hematopoietically expressed product of the *vav* proto-oncogene, Vav, shares homology with guanine nucleotide releasing factors (GRFs) [also called guanosine diphosphate–dissociation stimulators (GDSs)] that activate Ras-related small guanosine triphosphate (GTP)–binding proteins. Human T cell lysates or Vav immunoprecipitates possessed GRF activity that increased after T cell antigen receptor (TCR)–CD3 triggering; an in vitro–translated Vav fragment that contained the putative GRF domain was also active. Vav-associated GRF stimulation after TCR-CD3 ligation paralleled its tyrosine phosphorylation; both were blocked by a protein tyrosine kinase (PTK) inhibitor. Vav also was a substrate for the p56^{lck} PTK. Thus, Vav is a PTK-regulated GRF that may be important in TCR-CD3–initiated signal transduction through the activation of Ras.

Genes of the *ras*-related superfamily encode small (20 to 29 kD) GTP-binding proteins that act as molecular switches controlling cell growth and differentiation or malignant transformation (*1*). Ras proteins cycle between an inactive, guanosine diphosphate (GDP)–bound state and an active, GTP-bound form (*1, 2*). Hydrolysis of bound GTP, which is accelerated by GTPase-activating proteins (GAPs), inactivates Ras (*3*), whereas GRF-mediated exchange of bound GDP for GTP stimulates Ras (*4*). In contrast to the well-characterized GAP proteins (*5*), relatively little is known about the structure and physiologic function of mammalian GRF. Mammalian cells display exchange activity (*6–8*) that increases in response to differentiation (*7*) or mitogenic (*8*) signals. Two mammalian gene products, Dbl (*9*) and Ras-GRF (*10*), have GRF activity toward distinct Ras-related proteins.

Vav (*11, 12*) is another mammalian GRF candidate (*13*); it shares homology with proven or putative GRF domains found in yeast CDC24; rodent CDC25^{Mm} or Ras-GRF; and human Dbl, Bcr, or CDC24Hs (*4, 10, 14*). Vav is selectively expressed in hematopoietic cells (*11, 12*) and probably participates in signaling pathways, because it contains Src-homology domains 2 and 3 (SH2 and SH3, respectively) (*15, 16*), which are shared by many signaling molecules (*17*), and it is rapidly and transiently phosphorylated on tyrosine after the cross-linking of diverse hematopoietic cell receptors (*15, 16, 18, 19*).

Because Ras becomes activated soon after cross-linking of the TCR-CD3 complex (*20*), we determined whether T cell activation induced by receptor cross-linking was accompanied by increased GRF activity and whether Vav could account for such activity. Exchange activity was examined in whole cell lysates of human leukemic (Jurkat) T cells, which express a functional TCR-CD3 complex, before or after stimulation with OKT3 [a monoclonal antibody (mAb) to CD3]. Recombinant *Escherichia coli*–derived Ha-Ras (*21*) was saturated with excess [^{3}H]GDP, bound to nitrocellulose, and incubated for 0 to 1.5 min with cell lysates in the presence of excess unlabeled GTP plus GDP (Fig. 1A). Jurkat cells contained a basal exchange activity that increased by about 15 times after stimulation. The exchange reaction was nearly linear for the duration of the assay. GRF activity was maximal 1 min after stimulation and declined slowly thereafter, but was still considerably higher than the baseline activity 10 min after stimulation (Fig. 1B). The activation-dependent stimulation of GRF activity was not unique to Jurkat cells: freshly isolated human peripheral blood T lymphocytes had similar time-dependent stimulation after CD3 cross-linking (Fig. 1C). Parallel measurements of [γ-^{32}P]GTP binding to immobilized, unlabeled GDP-Ras complexes revealed that GTP binding occurred only in the presence of cell lysates and increased by four times after stimulation (*22*), thus confirming that the lysates possess a bona fide exchange activity.

Next we determined the contribution of Vav to this exchange activity. A highly specific antiserum to a synthetic Vav peptide that was derived from a region lying outside the putative exchange domain and, thus, had no similarity to potentially related mammalian GRFs (*4, 10, 14*), was used for this purpose (*23*). Vav immunoprecipitates from unstimulated cells had exchange activity comparable to that present in the total cell lysate from the same number of cells (Fig. 2A). This basal activity may reflect the constitutive tyrosine phosphorylation of Vav in resting Jurkat cells (Fig. 2C). In contrast, GRF activity in control immunoprecipitates prepared with normal rabbit immunoglobulin (Ig), or with the Vav antiserum that was blocked by preincubation with the peptide immunogen, was about one-fifth that of the Vav immunoprecipitates (Fig. 2A). A similarly low GRF

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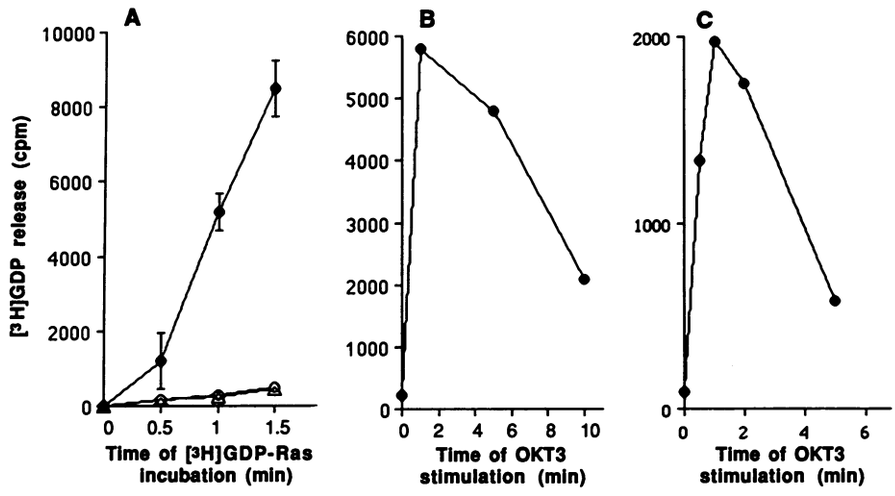

Fig. 1. Activation-induced increase in T cell–derived, Ras-directed GRF activity. (**A**) Recombinant Ras (0.9 μM) was incubated for 90 min at 30°C with 12 μM [^{3}H]GDP (specific activity: 8 Ci/mmol) in 25 mM tris (pH 7.5), 100 mM KCl, 0.01% bovine serum albumin, 0.2 mM dithiothreitol (DTT), and 12 mM EDTA. The [^{3}H]GDP-Ras complex was stabilized by addition of $MgCl_2$ (22 mM) and incubated for an additional 10 min. Complexes were immobilized on 0.45 μm nitrocellulose membranes (Costar, Cambridge, Massachusetts) by filtration and washed with 25 mM tris (pH 7.5), 100 mM KCl, 0.2 mM DTT, and 12 mM $MgCl_2$. The exchange reaction was initiated by adding to the filters buffer alone (△), cell extracts from 2 × 10^6 unstimulated Jurkat cells (○), or extracts from cells stimulated with mAb OKT3 (10 μg/ml) for 1 min (●). Cells were lysed in 100 mM Hepes (pH 7.5), 100 mM NaCl, 2% NP-40, 10 mM each NaF and Na_3VO_4, 12 mM $MgCl_2$, and aprotinin plus leupeptin (20 μg/ml); lysates were cleared of nuclei and cell debris by centrifugation at 15,000 rpm. After incubation at 30°C the amount of released [^{3}H]GDP was determined. The mean ± SD of four independent experiments is shown. (**B**) Jurkat cells (2 × 10^6) were stimulated for the indicated times with OKT3 (10 μg/ml) and extracts were prepared and assayed for GDP-GTP exchange activity. Aliquots were removed after 1 min and released [^{3}H]GDP was counted. (**C**) Human T cells (2 × 10^6) were purified from fresh peripheral blood mononuclear cell preparations by passage over column-immobilized rabbit antibodies to human immunoglobulin (Ig) (Biotex Laboratories Inc., Edmonton, Alberta), treated with OKT3 (5 min on ice), and cross-linked for the indicated times with rabbit antibodies to mouse Ig. Exchange activity in isolated lysates was assayed for 0.5 min.

activity remained in the Vav-immunodepleted lysate, indicating that Vav accounted for most, if not all, of the unstimulated activity. Vav depletion from the lysate was verified by immunoblotting (*22*). Stimulation of Jurkat cells with OKT3 resulted in a significant increase of the Vav-associated GRF activity (Fig. 2B) with a time course essentially identical to the one observed using whole cell lysates (Fig. 1B). In two experiments, immunoprecipitated Vav accounted for 61 ± 9% of the total OKT3-stimulated GRF activity (*22*).

Because Vav immunoprecipitates contain associated proteins (Fig. 2C), we determined if Vav itself had GRF activity. We used an expression–polymerase chain reaction (PCR) system for in vitro transcription and synthesis of functional proteins (*24*). A human Vav translation product of 455 residues that contained its putative GRF domain had a time-dependent GRF activity that was higher than in a control translation reaction (Fig. 3). Correspondingly, the Vav antiserum reacted with a 55-kD protein present in the Vav, but not in the control, translation reaction mixture (Fig. 3, inset). Thus, Vav itself, and not some associated protein, mediated the exchange activity, and this activity is localized to a segment containing the putative GRF domain.

The kinetics of Vav-associated GRF activity paralleled the activation-induced, time-dependent change in the amount of tyrosine phosphorylation of Vav (Fig. 2C). Phosphorylation increased by five times, as determined by laser densitometry, to a maximum at 1 min after stimulation; similar amounts of Vav were present in the different immunoprecipitates (Fig. 2D). The similar kinetics of the OKT3-induced tyrosine phosphorylation and GRF stimulation of Vav suggested that its phosphorylation by a TCR-CD3–coupled protein tyrosine kinase (PTK) may regulate its GRF activity. We

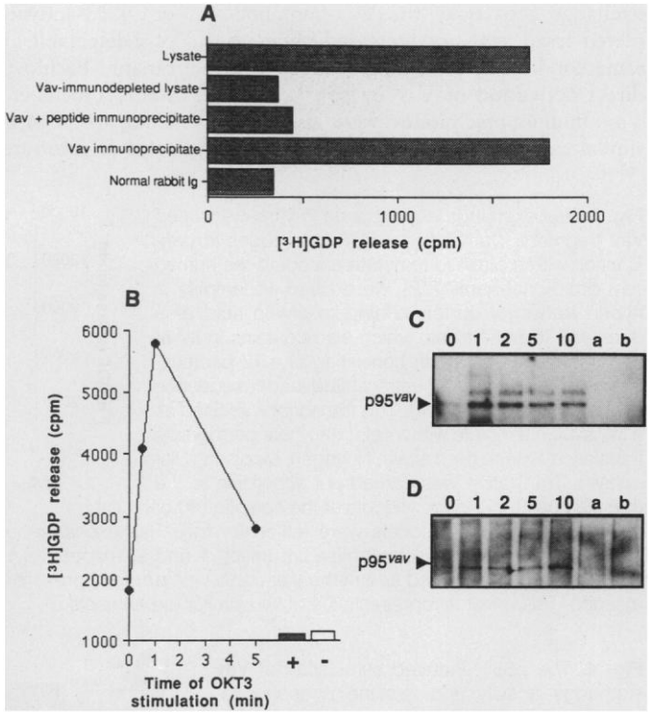

Fig. 2. Activation-induced stimulation of GDP-GTP exchange activity and tyrosine phosphorylation of T cell–derived Vav. (**A**) Jurkat T cells (10 × 10^6) were lysed, subjected to immunoprecipitation with normal rabbit Ig or a rabbit anti–Vav peptide serum (*12*) in the presence or absence of the peptide immunogen, and incubated overnight at 4°C, after which fixed *Staphlococcus aureus* (Pansorbin, Calbiochem, San Diego, California) was added. Washed immunoprecipitates or lysates were assessed for exchange activity after 1 min as described in Fig. 1. (**B**) Exchange activity (1 min) in Vav immunoprecipitates from unstimulated Jurkat cells (10 × 10^6) or from cells stimulated with OKT3 for the indicated times. The bars represent [^{3}H]GDP release caused by Vav immunoprecipitates from unstimulated (−) or OKT3-stimulated (+; 10 μg/ml, 1 min) Jurkat cells that were first incubated for 12 hours with herbimycin A (10 μM). Vav immunoprecipitates from unstimulated Jurkat cells (20 × 10^6) or cells stimulated with OKT3 (10 μg/ml) for the indicated times were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to Immobilon-P membranes and immunoblotted with (**C**) a mAb to phosphotyrosine (4G10; Upstate Biotechnology, Inc., Lake Placid, New York) or with (**D**) Vav antiserum. Lanes a and b represent immunoprecipitates with normal rabbit IgG or Vav antiserum that was preblocked with the peptide immunogen, respectively. All experiments were performed twice.

therefore incubated Jurkat cells with a specific PTK inhibitor, herbimycin A (*25, 26*), and then determined the OKT3-induced stimulation of Vav-associated GRF activity. Drug treatment reduced the GRF activity of Vav from unstimulated cells and completely prevented the OKT3-induced stimulation of this activity (Fig. 2B). Control experiments verified that herbimycin A blocked in parallel the OKT3-induced increase in tyrosine phosphorylation of Vav (*22*).

Members of the *src* family of PTKs, including $p56^{lck}$, participate in T cell activation (*27*), and $p56^{lck}$ is essential for TCR-CD3-initiated signaling (*28*). We determined, therefore, whether Vav can serve as a substrate for $p56^{lck}$. Cell lysates or Vav immunoprecipitates from unstimulated Jurkat cells were incubated in vitro in the absence or presence of recombinant $p56^{lck}$ (*29*); GRF activity and tyrosine phosphorylation of Vav were measured. Incubation with $p56^{lck}$ increased the exchange activity of the whole cell lysate by about ten times (Fig. 4) and was accompanied by tyrosine phosphorylation of a 95-kD protein comigrating with Vav (*22*). In contrast, the low exchange activity of the Vav-immunodepleted lysate was not increased under the same conditions, indicating a direct or indirect activation of Vav by $p56^{lck}$. When Vav immunoprecipitates were used in a similar experiment, $p56^{lck}$ caused a fourfold increase in the exchange activity (Fig. 4). The activity of the in vitro–phosphorylated Vav was similar to that present in the Vav immunoprecipitate from OKT3-stimulated cells (Fig. 4, group 4 versus group 6). Incubation of Vav immunoprecipitates with recombinant $p56^{lck}$ increased its tyrosine phosphorylation (Fig. 4, inset). Thus, the GRF activity of Vav is probably regulated by TCR-CD3–coupled PTKs, and Vav may be an additional site for the inhibitory effect of PTK inhibitors on T cell activation (*26, 30*).

The presence of a putative GRF domain in Vav (*4, 13, 14*) and its selective expression in hematopoietic cells (*11, 12*) suggest that Vav may represent a hematopoietic cell-specific GRF, by analogy with Ras-GRF, which is expressed only in brain (*10*). The homology between Vav and other proteins possessing proven or putative GRF activity toward products of the *ras*-related gene superfamily (*4, 14*) suggested that Vav may regulate guanine nucleotide exchange on *rho*-, rather than *ras*-encoded proteins (*4*). However, preliminary results indicated that Vav immunoprecipitates from resting or OKT3-activated Jurkat cells did not display detectable GRF activity against recombinant baculovirus-produced (processed) Rac1, a member of the *rho* subfamily (*22*). Comparison of additional Ras-related proteins as substrates for Vav is necessary to establish its relative affinity toward distinct members of this G protein superfamily.

Others reported a high basal rate of guanine nucleotide exchange on Ras in permeabilized T cells, which was not increased upon stimulation with a protein kinase C-activating phorbol ester or TCR-CD3 ligands (*20*). This high basal activity, which may have resulted from the permeabilization procedure, could have masked any anti-CD3–induced increase in guanine nucleotide exchange. However, the time course of Ras activation in T cells (*20*) closely paralleled the stimulation of T cell GRF activity associated with both whole lysates or Vav immunoprecipitates (Figs. 1 and 2, respectively).

Vav may mediate TCR-CD3–initiated signal transduction by coupling receptor-initiated signals to the activation of small GTP-binding proteins. The presence of a phosphotyrosine-binding SH2 domain in Vav provides a potentially direct coupling mechanism between antigen receptor-activated PTKs and Ras-related proteins. Mammalian GRF activity can thus be regulated by PTKs during the physiological process of cellular activation. Future studies aimed at identifying the regulators and targets of Vav will aid in elucidating the exact function of this proto-oncogene product in signaling pathways that activate physiological genetic programs of differentiation and growth in T and other hematopoietic cells. Furthermore, we found that lysates from NIH 3T3 fibroblasts transformed by a *vav* oncogene (*12*) contain an anti-Vav precipitable GRF activity that was five to ten times higher than in control cells (*22*), which suggests that unregulated, abnormally elevated GRF activity of Vav may underlie its transforming activity.

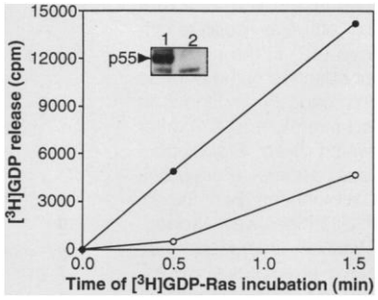

Fig. 3. Ras exchange activity of an in vitro–translated Vav fragment. Conventional PCR techniques in conjunction with a plasmid template encoding the human *vav* proto-oncogene (*12*) were used to amplify a hybrid transcript corresponding to amino acid residues 143 to 597 of Vav, which also contains in its 5′ end a universal promoter consisting of a T7 bacteriophage promoter and an untranslated leader sequence of alfalfa mosaic virus (*24*). This transcript was used as a translation template with a cell-free wheat germ lysate translation system (Red Nova, Novagen, Madison, Wisconsin). Translation was carried out according to the manufacturer's protocol. Aliquots of the specific (●) or control (○) reaction products were tested for their Ras exchange activity and immunoblotted in parallel with the Vav-specific antiserum (inset; 1 and 2, respectively). The arrowhead indicates a 55-kD band (the expected size of the Vav translation product) present only in the specific translation reaction. This result is representative of two similar experiments.

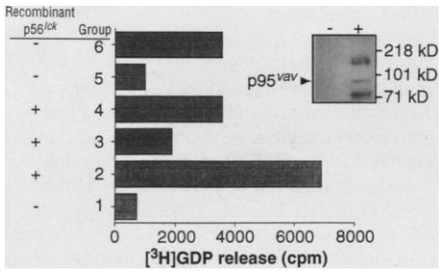

Fig. 4. The $p56^{lck}$-induced stimulation of Vav exchange activity and tyrosine phosphorylation. Whole cell lysates (groups 1 and 2), Vav immunoprecipitates (groups 4, 5, and 6), Vav-immunodepleted cell lysates (group 3) from 5×10^6 unstimulated Jurkat cells (groups 1 to 5), or cells stimulated for 1 min with 10 μg/ml OKT3 (group 6) were incubated (10 min, 30°C) with (+) or without (−) 200 ng of recombinant $p56^{lck}$ in the lysis buffer described in Fig. 1 plus 10 μM adenosine triphosphate. Exchange activity of the samples was measured as described in Fig. 1. (**Inset**) Vav immunoprecipitates incubated in the absence (−) or presence (+) of $p56^{lck}$ were resolved by SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with mAbs to phosphotyrosine. The arrow indicates a tyrosine-phosphorylated protein comigrating with Vav as detected by anti-Vav immunoblots (*22*). The experiments were repeated at least twice.

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TECHNICAL COMMENT

Postglacial Offset Along the Seattle Fault

Five reports in the 4 December issue of *Science* discuss the geologic evidence that a large earthquake struck the Seattle area about 1000 years ago (*1, 2*). Although this earthquake was accompanied by at least 7 m of uplift along the Seattle fault, there is a lack of evidence of ground rupture or shaking. Seismic turbidites in Lake Washington could have been produced by motion along other faults in the region. The differential uplift of wave-cut platforms and estuarine deposits used to reconstruct fault movement near Seattle are late Holocene in age. Thus, the location of the fault trace beyond the immediate Seattle area, the long-term rate of uplift, and the frequency of fault motion are not well known.

There is additional evidence that bears directly on the trend and offset rate along the Seattle fault (*3*). With records of the glacial deltas that were part of ice-dammed lakes at the site of Puget Sound about 16,000 years ago (*4*), I reconstructed a domal pattern of postglacial uplift dominated by isostatic uplift. A striking local anomaly in the uplift trend coincides with the crustal discontinuity now named the Seattle fault, and suggests that the southern block was uplifted approximately 9 m relative to the north. Apparently, the minimum fault offset (7 m) for the well-documented earthquake of about 1,000 to 1,100 years ago is broadly comparable to the total uplift for the last 16,000 years. This comparison suggests that episodes of comparable fault movement are either a recent development or that their repeat times are extremely long. This interpretation is consistent with the stratigraphic evidence containing the record of only one earthquake in the past 2000 years (*2*).

I replotted the widely dispersed population of glaciolacustrine control points in order to resolve the net vertical offset (Fig. 1). I chose a line of projection parallel to the maximum slope of the isostatic anomaly and nearly perpendicular to the fault trace. I then subdivided the data set into three geographically restricted clusters, each of which could be projected separately to a line of section crossing the fault.

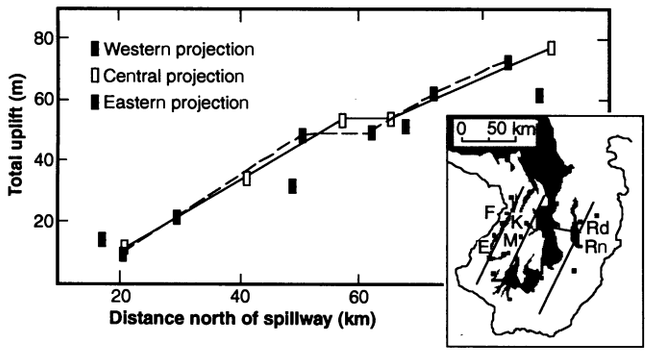

Fig. 1. Net uplift of glaciolacustrine deltas relative to the lake outlet for three parallel projections. Location of deltas (F, Fulton Creek; E, Eldon; K, Kitsap Lake; M, McKenna Falls; Rd, Redmond; and Rn, Renton) and the trace of the Seattle fault (northwest-trending line) are shown on inset map.

The amount of net postglacial deformation along the western projection relative to the Black Lake Spillway decreases from 72.7 ± 2 m in the north to about 11.5 ± 2 m in the south, with a nearly uniform regression slope of 0.95 m/km (*5*). The Eldon delta, located near the middle of the projection, lies 9.9 ± 2 m above the regression line and is responsible for most of the residual error. Although linear regression provides a valid first approximation, the control points define a true but irregular paleosurface. The plot is more accurately described as a single line composed of three individual segments, each with a different slope.

Although located as much as 45 km from their counterparts along Hood Canal, glacial deltas from central Puget Sound also define a southward-inclined paleoshoreline with an identical regression slope and with three distinct segments whose locations and slopes are similar. On the basis of the mean regression slope of the central projection, the McKenna Falls delta lies 7.5 m ± 2 m above its expected position. Deltas from the eastern part of the Puget lowland project to a line with a more gentle southerly slope, and one that is less obviously segmented. This difference probably reflects a combination of factors: neotectonic deformation, larger projection errors, and a more complex pattern of glacier loading.

The Seattle fault must lie north of the

Table 1. Net offset on the Seattle fault.

Projection	Estimated vertical offset	
	Maximum (m)	Minimum (m)
West	14.70	9.10
Central	9.90	7.10
East	21.50	No data