RESEARCH COMMUNICATION

Interleukin-2 induces tyrosine phosphorylation of the *vav* proto-oncogene product in human T cells: lack of requirement for the tyrosine kinase *lck*

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The haematopoietic protein, *p95^{vav}*, has been shown to be a tyrosine kinase substrate and to have tyrosine kinase-modulated guanine-nucleotide-releasing-factor activity. This implies a function in the control of ras or ras-like proteins. Because ras activation has been shown to be a downstream event following stimulation of the interleukin-2 (IL-2) receptor, we investigated the possibility that *vav* was involved in IL-2 signal transduction pathways, using human T cells as a model. We found rapid tyrosine phosphorylation of *vav* in response to IL-2 within

1 min, with maximum increase of phosphorylation of 5-fold occurring by 5 min after treatment in normal human T cells. IL-2 stimulation of the human T-cell line YT and a subclone of the YT cell line (YT^{*lck*-}) that does not express message for the src-family kinase *p56^{lck}* also results in a rapid rate of tyrosine phosphorylation of *vav* of more than 5-fold by 5 min. These results suggest that *vav* may play an important role in IL-2-stimulated signal transduction and that there is not a strict requirement for the tyrosine kinase *p56^{lck}*.

INTRODUCTION

The product of the proto-oncogene *vav* is a recently identified protein expressed exclusively in cells of haematopoietic origin [1]. While containing SH2 and SH3 domains *vav* shows sequence similarity to proteins which have putative guanine-nucleotide-releasing-factor (GRF) activities; these include yeast CDC24, human CDC24Hs, rodent ras-GRF, human db1, and human bcr [2–4]. Recently, *vav* has been shown to have GRF activity, which is stimulated by tyrosine phosphorylation [5]. This observation suggests a direct role for *vav* in signal transduction pathways involving the activation of ras or ras-like proteins (reviewed in [6]).

Tyrosine phosphorylation is central to the regulation of GRF activity associated with *vav*, and *vav* tyrosine phosphorylation has been observed after activation of several haematopoietic receptor systems. These include stimulation of the T-cell antigen receptor [7], activation of IgM antigen receptors [8], activation of IgE receptors [7], and the activation of c-kit via Steele factor binding [9]. Furthermore, *vav* is tyrosine phosphorylated by epidermal growth factor and platelet-derived growth factor receptor activation in *vav*-transfected fibroblasts [10], and can associate with these receptors through SH2-domain interaction [10].

Signal transduction through the haematopoietin superfamily class of receptors is not well understood. This family is characterized by a lack of an identifiable intrinsic catalytic activity [11–13], and includes receptors for interleukins 2–7, granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, erythropoietin, growth hormone, prolactin, and ciliary neurotrophic factor. Several cytokines, the receptors of which are members of this family, have been shown to activate protein tyrosine and serine kinases rapidly [14–16] with the subsequent activation of ras or ras-like proteins [17–20].

The IL-2 receptor is a member of this family and tyrosine kinase activity has been found associated with this receptor

[21–23] as well as receptors for prolactin [24] and erythropoietin [25], implying that tyrosine kinase activation and substrate phosphorylation is an initial signalling mechanism used by several members of this class of receptors. It was recently reported that the tyrosine kinase *p56^{lck}* (*lck*) directly associates with the IL-2-receptor β chain (IL-2R β) and is the signal transducing tyrosine kinase in the IL-2-receptor system [26]. Additional reports, however, suggest that IL-2R β transfected into myeloid cell lines devoid of *lck* can effectively transduce IL-2-dependent signal [27]. Mills et al. [28] have additionally reported that neither *lck* nor *fyn* tyrosine kinases are obligatory for IL-2-mediated signal transduction in human T-lymphotropic virus type 1-infected T cells. These results raise the possibility that one or more tyrosine kinases, distinct from *lck*, is responsible for IL-2-mediated tyrosine kinase signalling.

To analyse the role that *vav* may have in IL-2-directed signal transduction, we tested whether IL-2 could induce tyrosine phosphorylation of *vav* and whether this phosphorylation was linked to the activation of the src-family kinase *lck*. We report here that IL-2 stimulates *vav* tyrosine phosphorylation in normal human T cells, and using *lck*⁺ and *lck*⁻ cell lines show that *lck* is not required for IL-2-dependent tyrosine phosphorylation of *vav*.

EXPERIMENTAL

Reagents

Polyclonal antibody to human *vav* protein and anti-phosphotyrosine monoclonal antibody 4G10 were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Recombinant human IL-2 was provided by Hoffman La Roche. Enzyme-linked chemiluminescence (ECL) development reagents were obtained from Amersham. Immobilon poly(vinylidene difluoride) (PVDF) membranes were obtained from Millipore Corporation. Protein A-conjugated Sepharose was obtained from Pharmacia-LKB. Biotinylated anti-(mouse IgG) and horseradish peroxidase-conjugated streptavidin were obtained from

Abbreviations used: IL-2, interleukin 2; GRF, guanine nucleotide releasing factor; IL-2R β , IL-2-receptor β chain; ECL, enzyme-linked chemiluminescence; PVDF, poly(vinylidene difluoride); PHA, phytohaemagglutinin.

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Kirkegaard and Perry (Gaithersburg, MD, U.S.A.). Phytohaem-agglutinin (PHA) was obtained from Wellcome Diagnostics.

Tissue culture and factor stimulation

Human T cells were obtained from normal donors and isolated by counter-flow centrifugal elutriation as described [29]. Isolated T cells were activated with 1 μ g/ml PHA and cultured for 3 days [30], after which time cells were recovered and G1-enriched as described [30]. MT2, Jurkat, YT and YT^{lck-} cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal-calf serum, glutamine and antibiotics. Before IL-2 stimulations, YT and YT^{lck-} cells were washed with acidified medium as described [30] and incubated for 24 to 48 h in RPMI-1640 medium supplemented with 1% (v/v) fetal-calf serum, glutamine and antibiotics. For IL-2 stimulations, G1-enriched T, YT and YT^{lck-} cells were recovered by centrifugation at 1000 *g* for 10 min, resuspended in RPMI-1640 medium and incubated for 30 min at 37 °C at a cell density of 50 $\times$ 10⁶ cells/ml. IL-2 was added to a final concentration of 150 nM and cells were incubated for periods from 30 s to 30 min. Cells were rapidly recovered by centrifugation for 5 s at 12000 *g* and snap frozen in a solid CO₂ bath.

RNA isolation and Northern-blot analysis

RNA was isolated from log-phase cultures of activated human T, YT, YT^{lck-}, MT2 and Jurkat cells by the guanidinium isothiocyanate/acid phenol method [31]. Aliquots (10 μ g) of total RNA from each sample were electrophoresed on 1%-agarose gels containing 6% formaldehyde and transferred to nylon membranes. DNA probes corresponding to full-length mouse *lck* and full-length chicken *actin* were radiolabelled using random priming (BRL). Prehybridization and hybridization were performed using the method of Maniatis et al. [32]. The hybridized blots were washed in 0.1 $\times$ SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS at 65 °C. Blots were dried and subjected to autoradiography.

Cell lysis, immunoprecipitation and anti-phosphotyrosine immunoblotting

Frozen cell pellets were thawed, lysed by the addition of 1 ml of lysis buffer [10 mM Tris/HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 1% (w/v) Triton-X 100, 1 mM phenyl-methanesulphonyl fluoride, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A and 2 μ g/ml leupeptin] per 5 $\times$ 10⁷ cells, and incubated for 1 h at 4 °C. Lysates were clarified by centrifugation at 12000 *g* for 30 min and the supernatants were subjected to anti-vav immunoprecipitation. Immobilized anti-vav antibody was prepared by incubating 5 μ l of anti-vav polyclonal antiserum resuspended in 0.5 ml of lysis buffer with 20 μ l of hydrated protein A-Sepharose (Pharmacia) for 1 h at 4 °C. Anti-vav-treated protein A-Sepharose was washed three times with lysis buffer and added directly to clarified cell lysates. Immunoprecipitation was performed overnight at 4 °C. Precipitates were washed six times with lysis buffer and proteins were eluted by boiling for 1–2 min in 50 μ l of 2 $\times$ SDS sample buffer [33]. Proteins were resolved on 7.5% (w/v) polyacrylamide gels [33] and transferred to Immobilon PVDF membrane as described [34]. IL-2-stimulated tyrosine phosphorylation was detected by anti-phosphotyrosine immunoblotting using anti-phosphotyrosine monoclonal antibody 4G10, biotinylated anti-(mouse IgG), and

horseradish peroxidase-conjugated streptavidin as described [24]. Blots were developed using ECL (Amersham).

RESULTS AND DISCUSSION

Because vav has been shown to be involved in tyrosine kinase signal transduction pathways in several haematopoietic receptor models, we investigated the possibility that vav may function in signal transduction pathways stimulated by IL-2. For this study we isolated normal human T cells, activated the T cells with PHA to render them IL-2-responsive and treated the T cells with IL-2 for up to 30 min. We then assayed for vav tyrosine phosphorylation using anti-vav immunoprecipitation and anti-phosphotyrosine immunoblotting. A representative experiment is shown in Figure 1 and demonstrates that IL-2 induces tyrosine phosphorylation of vav (Figure 1a). Increased tyrosine phosphorylation of vav is detected in normal T cells within 1 min of treatment with IL-2 and approaches a maximum 3- to 5-fold increase within 5 min (Figure 1b). Vav phosphorylation in response to IL-2 is rapid and dose-dependent (Figure 2), being detectable with 150 pM IL-2. The ability to observe vav phosphorylation at physiological concentrations of IL-2 suggests a role in IL-2 signal transduction. We can also detect tyrosine phosphorylation of a 75–80 kDa protein which co-precipitates with vav (see Figures 1 and 4). This protein has been called vav-1, for vav-associated protein, by Bustelo and Barbacid [8] and its identity has yet to be determined.

The rapid phosphorylation of vav implies that it is a substrate for IL-2-activated tyrosine kinases. The best-characterized tyrosine kinase which is modulated in response to IL-2 in T cells is the src-family kinase *lck* [35]. The activity of *lck* increases rapidly

Figure 1 IL-2 induces rapid tyrosine phosphorylation of the vav proto-oncogene product in normal human T cells

Normal human T cells were PHA-activated for 3 days and G1-enriched as described. Cells were treated with 150 nM human recombinant IL-2 for periods of from 30 s to 30 min, then lysed and immunoprecipitated with vav-specific polyclonal antibody. vav immunoprecipitates were analysed for tyrosine phosphorylation by anti-phosphotyrosine immunoblot. (a) Anti-phosphotyrosine immunoblot of vav immunoprecipitates taken at 0 and 10 min after IL-2 treatment. The positions of vav and immunoglobulin are indicated on the right. (b) Densitometric analysis of vav tyrosine phosphorylation from 0 to 30 min after IL-2 treatment. Relative phosphorylation is expressed as the percentage of maximum phosphorylation observed within 30 min of treatment with IL-2. Error bars indicate the S.E.M. of three experiments.

Figure 2 vav phosphorylation in response to IL-2 is dose-dependent

Normal human T cells were activated, G1-enriched and treated with from 0 to 300 pM IL-2 for 10 min. The cells were then analysed for vav tyrosine phosphorylation by lysis, immunoprecipitation and anti-phosphotyrosine immunoblotting as described in the text. Relative phosphorylation is expressed as the percentage of the maximal IL-2-induced phosphorylation. Error bars indicate the S.E.M. for three experiments.

Figure 3 Northern-blot analysis verifies that YT^{lck-} cells do not express lck

RNA from MT2, Jurkat, YT, YT^{lck-}, and activated human T cells (Act. T cell) was isolated and 10 μ g from each was subjected to Northern-blot analysis using either a full-length mouse *lck* probe or full-length chicken *actin*. Cell-specific RNA is noted above and the positions of *lck* and *actin* are shown on the right.

on IL-2 treatment [35], and phosphorylation of key substrates by lck may act as a major signal transduction pathway. To investigate this, we used the cell line YT, which is a large granular

Figure 4 IL-2-dependent tyrosine phosphorylation of vav does not require the tyrosine kinase lck

YT and YT^{lck-} cells were stimulated with 150 nM IL-2 for from 30 s to 30 min, lysed and immunoprecipitated with vav-specific polyclonal antibody. Vav immunoprecipitates were analysed for tyrosine phosphorylation by anti-phosphotyrosine immunoblot. The positions of vav and immunoglobulin are indicated on the right-hand side. The experiment was repeated three times with equivalent results.

lymphocyte-like cell line derived from a human thymic lymphoma [36] that expresses IL-2R β in high numbers [37]. From this cell line we have isolated a subclone which does not express message for p56^{lck}, termed YT^{lck-}. We verified the absence of *lck* transcript in these cells by Northern blot using full-length mouse *lck* as a probe and comparison with three *lck*⁺ cell lines (Jurkat, activated T cells and YT) and a well-described *lck*⁻ cell line, MT2 (Figure 3). Additional experiments using reverse transcriptase PCR also revealed a lack of *lck* transcript in the YT^{lck-} cells (results not shown). IL-2 has been shown to stimulate tyrosine phosphorylation rapidly in YT cells [38]. Analysis of YT and YT^{lck-} cells shows that IL-2 can induce tyrosine kinase substrate phosphorylation in a similar manner in both cell lines (results not shown). To determine whether lck activation is an obligatory step leading to the tyrosine phosphorylation of vav in response to IL-2, we treated YT and YT^{lck-} cells with IL-2 and assayed for vav tyrosine phosphorylation by anti-vav immunoprecipitation, followed by anti-phosphotyrosine immunoblotting. This analysis shows that IL-2 stimulates vav tyrosine phosphorylation in both *lck*⁺ and *lck*⁻ YT cells (Figure 4). The response shows kinetics very similar to that seen in normal human T cells, with initial phosphorylation detected as early as 30 s and maximum phosphorylation of greater than 5-fold occurring within 5 to 10 min after IL-2 stimulation (Figure 4 and densitometric analysis which is not shown).

These results clearly show that IL-2 is capable of inducing vav phosphorylation, and that there is no strict requirement for the src-family kinase lck. Otani et al. [27] suggest that one or more tyrosine kinases other than lck are responsible for mediating an IL-2-induced mitogenic response in IL-2R β -transfected myeloid cells. Additionally, we see no qualitative difference in the substrates phosphorylated in response to IL-2 in normal T cells, YT, YT^{lck-}, or the *lck*⁻ cell lines HUT102 or MT2 (results not shown), suggesting that there is a common tyrosine kinase activation pathway in these cells that does not involve lck. In contrast, Gulbins et al. [5] have reported the tyrosine phosphorylation of vav by lck *in vitro*. However, this may not be representative of events *in vivo*.

Several scenarios can be envisioned that may explain the lack of requirement for *lck* in the IL-2-induced tyrosine phosphorylation of *vav*. Our results do not rule out the possibility that another *src*-family kinase may functionally replace *lck* in the *lck*⁻ cell. This may be supported by recent observations showing that *fyn* and *lyn* are also activated upon IL-2 stimulation and that *fyn* may associate with the IL-2 receptor [39]. Alternatively, IL-2 interaction with receptor may activate several distinct tyrosine kinase pathways, with the observed activation of *lck* or other *src*-family kinases [35,39] being a peripheral event following stimulation. Within this model, *vav* phosphorylation would be more closely associated with tyrosine kinase activation that is directly relevant to the salient transduction of IL-2 signal leading to changes in *ras* activity, and not necessarily linked to *src*-family kinase activation. Our results do not support the contention that *lck* is the tyrosine kinase activated as a first mechanism in IL-2 signal transduction.

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