Structural domains of interleukin-2 receptor β critical for signal transduction: kinase association and nuclear complex-formation

O. M. Zack HOWARD,*§ Robert A. KIRKEN,† Gonzalo G. GARCIA,* Rebecca H. HACKETT‡ and William L. FARRAR†

*Biological Carcinogenesis and Development Program, Program Resources, Inc./DynCorp. and †Cytokine Molecular Mechanisms Section, Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, MD 21702, U.S.A., and ‡Food and Drug Administration, Center for Biologics Research and Evaluation, Division of Cytokine Biology, 8800 Rockville Pike, Bethesda, MD 20892, U.S.A.

The structural domains of interleukin-2 receptor β (IL-2R β) were examined, characterizing the protein domains, associated phosphoproteins and nuclear complexes of IL-2-induced signal transduction. A series of IL-2R β cytoplasmic deletion mutants were constructed and transfected into a murine pre-B-cell line, Ba/F3. The proliferative response of characterized clones was determined. A minimal linear cytoplasmic sequence required for proliferation and a sequence motif (PQPLXP) needed along with Box1–Box2 for IL-2-induced proliferation were identified. Anti-phosphotyrosine Western-blot analysis of a stimulated biologically active clone showed several IL-2-induced tyrosyl-phosphorylated proteins with molecular masses ranging from 45

to 116 kDa. *In vitro* kinase studies of biologically active clone-receptor complexes showed a 116 kDa protein (p116) to be the major tyrosine-phosphorylated component. The presence of the p116 kinase in the receptor complex correlates with IL-2-induced proliferation. An IL-2-inducible p116 kinase has recently been characterized as a Jak kinase family member and named Jak3. Nuclear complexes were formed with the GRR oligomer only when the IL-2R β mutant supported proliferation. This led us to conclude that Box1–Box2 and PQPLXP motifs associate with Jak3 and that this association is an essential element in the IL-2 signal-transduction pathway culminating in the formation of a nuclear complex.

INTRODUCTION

Interleukin 2 (IL-2) plays a central role in the proliferation and/or differentiation of T-lymphocytes, monocytes, glioma, B-lymphocytes and natural killer cells [1,2]. This haematopoietic growth factor mediates its function by binding to its specific receptor. The human IL-2 receptor is composed of at least three different protein subunits, IL-2 receptor α , p55 (IL-2R α), IL-2 receptor β , p70–75 (IL-2R β) and IL-2 receptor γ , p64 (IL-2R γ) [3]. IL-2R γ is shared with IL-4 and IL-7 and, as such, has recently been named the common γ chain [4,5]. The IL-2–IL-2R complex signal via various receptor forms to increase cytoplasmic alkalization and protein kinase C nuclear translocation, but not activation [1,2], Raf-1 serine kinase activity [6,7] and activation of Ras [8,9]. Perhaps more significant is the IL-2 effect on tyrosine kinases, such as L-Jak/Jak3 [10–12] or the Src family of kinases: Itk [13], Fyn [14, 15], Lyn [14, 16, 17] and Lck [18,19]. The tyrosine kinase signal is one of the earliest biochemical events in IL-2 signal transduction [2,20], indicating that tyrosine kinase activation is a critical event in early IL-2 signal transduction. The tyrosine-phosphorylation kinetics of Jak3 are particularly rapid [11], perhaps indicating that this protein is positioned early in the signal cascade.

Recent advances have been made in elucidating which structural domains are required for interaction of haematopoietic growth factor receptors with downstream cytoplasmic kinases [21–29]. It is known that members of the Jak–Tyk family of kinases associate with cytoplasmic regions of the haematopoietic growth-factor receptors and are the kinases involved in the

earliest steps of receptor-activated signal transduction. In particular, it has been demonstrated that Jak2 associates with the erythropoietin [22], IL-3 [23], growth hormone [24] and prolactin [25,26] receptors. Jak1, Jak2 and Tyk2 associate with the IL-6, leukaemia inhibitory factor, oncostatin M, IL-11 and ciliary neurotrophic factor family of receptors [27,28] and various interferon receptors [29–31]. The Jak–Tyk family of kinases appears to associate with the cytoplasmic domain of these various receptors through the Box1–Box2 homology domain [32]. This domain is also known as the proline-rich motif [33]. IL-2R β contains a region similar to Box1–Box2 but no data exist to demonstrate which regions are required for association with a Jak-like kinase.

This study focuses on the structural features of the IL-2R β cytoplasmic domain that associate with essential signalling proteins. We designed several deletion mutants to address the requirement of Box1–Box2 for IL-2R β signal transduction. We also constructed an internal deletion mutant to aid in the identification of the phosphoproteins that are critical for IL-2-induced proliferation. Using these IL-2R β expression mutants, we characterized the IL-2R β structural features critical for proliferation, phosphoprotein association and nuclear complex-formation.

EXPERIMENTAL

Cell culture

All cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere. YT cells, a human natural killer cell line, were

Abbreviations used: IL, interleukin; IL-2R β , interleukin-2 receptor β ; GM-CSF, granulocyte–monocyte colony-stimulating factor; IFN- γ , interferon γ ; PVDF, poly(vinylidene difluoride); poly(A)⁺, polyadenylated.

§ To whom correspondence should be addressed.

Figure 1 (a) Schematic diagram of IL-2R β mutants and (b) amino acid sequence of the IL-2R β cytoplasmic domain

(a) Each construct contains an extracellular domain of 214 amino acids (▨), a transmembrane domain of 25 amino acids (■) and a variable cytoplasmic domain (□). Sequence features are also shown: Box1 (▤), Box2 (▥) and the acid-rich domain (▦). The acid-rich domain is composed of amino acids 315–384. The total number of cytoplasmic amino acids is shown above each construct. The deleted internal region of construct AD is noted by a vertical line and the deleted amino acid numbers. (b) The cytoplasmic domain of IL-2R β is shown beginning with amino acid 239 and ending with amino acid 525. The vertical arrows indicate the last amino acid in the IL-2R β mutants. The name of the mutant appears above the arrow. The internal deletion mutant (AD) is marked by two vertical arrows with the name lowered indicating the deleted region. The repeated motif PQPLXP is underlined.

cultured in basal medium [RPMI-1640 (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamine and 20 units/ml penicillin/streptomycin (Mediatech)]. Ba/F3 cells, a murine pre-B-cell line that does not express IL-2R β , were grown in basal medium supplemented with WEHI-3B-conditioned medium. Clonal transfected cells were grown in basal medium supplemented with WEHI-3B-conditioned medium and 200 units/ml hygromycin-B (Calbiochem) or 25 units/ml human IL-2 [BRMP Repository (Hoffman LaRoche)] and 200 units/ml hygromycin-B.

Construction of expression plasmids

Restriction enzymes used in this study were obtained from BRL unless otherwise noted. The IL-2R β expression vectors were constructed by inserting various fragments of IL-2R β generated

by PCR or restriction of pBluIL-2R β into the *Bam*HI, *Hind*III sites of the pREP-10 expression vector (Invitrogen) (see Figure 1). pBluIL-2R β contains the coding region of IL-2R β cloned into the *Eco*RI site of pBluescript II SK⁻ (Stratagene). All IL-2R β constructs are composed of: 132 bp of untranslated 5' sequence, 78 bp (26 amino acids) of translated IL-2R β pre-form, 927 bp which code for the extracellular and transmembrane regions and the variable cytoplasmic sequences. The plasmid, pREP-FL, was constructed by inserting the *Bam*HI–*Hind*III fragment of pBluIL-2R β into pREP-10. The mRNA for IL-2R β expressed by the full-length (pREP-FL) expression vector is 1785 bp plus a 200 bp polyadenylated [poly(A)⁺] tail (provided by the vector) yielding a 1985 bp message. pREP-AD was constructed by ligating two fragments together yielding an internal deletion mutant. The 5' fragment was generated by PCR of linearized pBluIL-2R β with T3 (5'-ATTAACCTCACTAAAG-3') primer and AD5 (5'-

AGGCACCAGGTACCTGCTG-3') primer. The 3' fragment was generated by PCR of linearized pBluIL-2R β with T7 (5'-AATACGACTCACTATAG-3') primer and AD3 (5'-AACCCCTGGTACCTCTGTCTAG-3') primer. These fragments were individually restricted with *KpnI*, combined and ligated together. The resulting fragment was restricted with *BamHI-HindIII* and ligated into *BamHI-HindIII*-restricted pREP-10. The acid deletion (pREP-AD) mutant expression vector expresses an IL-2R β message of 1785 bp; this mutant was constructed from two PCR fragments which delete amino acids 315–384. pREP-BD was constructed by ligating the *BamHI-HindIII*-restricted fragment of a PCR product generated using the T3 primer and BD (5'-TGAAAGCTTACAGAGGCTGCAGGG-3') primer into *BamHI-HindIII*-restricted pREP-10. The β deletion (pREP-BD) mutant expression vector expresses an IL-2R β message of 1565 bp which codes for a deletion mutant containing only 146 amino acids of the cytoplasmic domain. pREP-SD was constructed by inserting the *BamHI-HindIII*-restricted fragment of a PCR product generated using the T3 primer and SD (5'-ACAAAGCTTCAGGAGCAGGTGCGTCA-3') primer into the *BamHI-HindIII*-restricted pREP-10. The signal domain (pREP-SD) mutant expression vector expresses an IL-2R β message of 1349 bp. pREP-LS was constructed by inserting the *BamHI-HindIII*-restricted fragment of a PCR product generated using the T3 primer and LS (5'-TCAAAGCTTCTAGTGGCGAGATCTCA-3') primer into the *BamHI-HindIII*-restricted pREP-10. The LEVL split (pREP-LS) mutant expression vector expresses an IL-2R β message of 1310 bp. pREP-LG was constructed by inserting the *BamHI-HindIII*-restricted fragment of a PCR product generated using the T3 primer and LG (5'-ACAAAGCTTGCCGCGCCAGGGCTGAA-3') primer into the *BamHI-HindIII*-restricted pREP-10. The LEVL gone (pREP-LG) mutant expression vector expresses an IL-2R β message of 1286 bp. pREP-BS was constructed by inserting the *BamHI-PvuII* (NEB)-restricted fragment of pBluIL-2R β into the *BamHI-PvuII*-restricted pREP-10. The β short (pREP-BS) mutant expression vector expresses an IL-2R β message of 1205 bp. The constructs were confirmed by restriction digestion and DNA sequence analysis. Linearized pREP-construct plasmid was generated by *ClaI* digestion.

Transfection of Ba/F3 cells and selection of transfected clones

Ba/F3 cells in exponential growth phase were washed with BPS and suspended at 5×10^7 cells/ml in PBS. A 200 μ l portion of these cells was placed in a Bio-Rad GenePulser cuvette with 10 μ g of linearized plasmid and chilled for 10 min on ice. The chilled cells were electroporated at 0.2 kV and 500 μ F using a Bio-Rad GenePulser. Cells were cultured for 24–72 h in Ba/F3 medium, cell debris was removed and the medium exchanged for selection medium which consisted of Ba/F3 medium supplemented with 1300 units/ml hygromycin-B. After 2–3 weeks of Hygromycin selection, a stable population was generated, which was serially diluted to generate a clone.

Flow cytometric analysis

Cells in exponential growth phase were washed with PBS and suspended in a 100 μ l volume containing 2 μ g of biotinylated monoclonal antibody. The anti-(human IL-2R β) antibody was biotinylated as previously described [34]. Cells were incubated on ice for 30 min with 2 μ g of biotinylated anti-CD3 (Sigma), then washed with 5 ml of PBS and centrifuged at 1000 *g* for 2 min. These cells were suspended in 100 μ l of PBS followed by a 15 min incubation with ultravidin-R-phycoerythrin (Leinco Technologies). The cells were then washed twice with 5 ml of PBS

followed by centrifugation at 1000 *g* for 2 min. These stained cells were analysed by BRMP-Clinical Services Flow Cytometry utilizing a Coulter Cytometric Epics Profile.

Measurement of cell growth

Cell growth was determined using the cell titer 96 non-radioactive cell proliferation assay as advised by Promega. Cell viability was determined by Trypan Blue exclusion. The proliferation assays were carried out in the following manner. Cells were washed two to three times with PBS and resuspended at 2×10^6 cells/ml in basal medium. Suspended cells (50 μ l) were placed in 96-well plates with 50 μ l of $2 \times$ cytokine and incubated for 48 h, dye reagent was added for an additional 4 h followed by lysis buffer. The developed assay was read using a microplate autoreader at $A_{570-630}$ (Bio-tek EL-309).

Northern-blot analysis

Northern-blot analysis was performed as previously described [35].

Cell lysis and immunoprecipitation

Cells were stimulated and frozen as previously reported [11]. Frozen cell pellets were thawed on ice and solubilized in 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 % Triton X-100 (Sigma), 1 mM phenylmethane sulphonyl fluoride, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A and 2 μ g/ml leupeptin] per 1×10^8 cells, rotated for 1 h at 4 °C followed by centrifugation at 12000 *g* for 30 min. The clarified cell lysate was incubated for 2–3 h at 4 °C with 5 μ g/ml anti-phosphotyrosine. Antibody and cell lysate were incubated with Protein A–Sepharose beads for 30 min, washed three times with lysis buffer and denatured in sample buffer. The samples were then electrophoresed on an SDS/7.5 % polyacrylamide gel [36] and subsequently transferred to poly(vinylidene difluoride) (PVDF) membrane (Millipore). Immunoblot analysis was carried out as described by Kirken et al. [10]. The blots were developed by chemiluminescence (Amersham) and exposed to XAR-5 film.

In vitro kinase assay of IL-2R β complex

Cells were stimulated with 100 nM IL-2 or basal medium and lysed in lysis buffer at 1×10^8 cells/ml. The samples were clarified. Non-specific IgG binding was reduced by incubation with anti-mouse IgG prebound to Protein A–Sepharose beads for 6 h at 4 °C, yielding clarified sample. These samples were immunoprecipitated with anti-IL-2R β monoclonal antibody (clone 561) or non-specific anti-mouse IgG for 6 h at 4 °C. After extensive washing with lysis buffer, purified receptor complex components were washed in kinase buffer [150 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 25 mM Hepes (pH 7.2), 0.1 % Nonidet P 40, 10 % glycerol, 100 μ M sodium orthovanadate]. The *in vitro* kinase assay was performed as described previously [37]. The reaction was stopped by the addition of sample buffer; the proteins were resolved by SDS/PAGE (7.5 % gels) and transferred to PVDF membrane as described [38]. After blocking with 1 % BSA, the blot was preincubated with goat anti-mouse IgG for 2 h at 4 °C and extensively washed. This blocking step was included to limit the detection of the murine anti-IL-2R β antibody by the peroxidase-conjugated goat anti-mouse antibody used to develop the anti-phosphotyrosine signal. Tyrosine-phosphorylated proteins were identified by immunoblotting using anti-

phosphotyrosine monoclonal antibody 4G10 (UBI), and the phosphoprotein bands were detected by enzyme-linked chemiluminescence (Amersham) as described by Evans et al. [35].

Analysis of IL-2-induced nuclear complexes

Electrophoretic mobility-shift assays were carried out with a ^{32}P -labelled GRR probe and whole-cell extracts as previously described [39]. The GRR probe is 5'-AGCATGTTTCAAGGATTTGAGATGTATTTCCAGAAAAG-3' and the complement. Antibody complexing of p91 was performed as described previously [39].

RESULTS

Expression of mutant IL-2R β

To investigate the structural requirement for IL-2-induced proliferation and to identify structural elements critical for kinase association, a series of deletion mutants of the IL-2R β were constructed. A schematic diagram of the mutant receptor constructs is shown in Figure 1. These constructs were stably transfected into the Ba/F3 murine cell line. As shown in Figure 2, the parental Ba/F3 cells did not express IL-2R β . Northern-blot analysis confirmed the expression of the appropriately sized IL-2R β mRNA in each cell line. The human natural killer cell line, YT, was used as a control for expression of the naturally occurring IL-2R β mRNA. The native IL-2R β mRNA size is 4 kb, whereas that of the mutants is 2 kb or smaller. The native IL-2R β mRNA contains a large (approx. 2 kb) untranslated region which was not used in these constructs. As seen in Figure 2, all constructs except pREP-BS expressed equivalent amounts of mRNA when standardized against β -actin. Reverse transcriptase PCR was used to confirm the presence of pREP-BS in these cells (results not shown). Cell surface expression of IL-2R β was confirmed in all transfectants by flow cytometry, as shown in Figure 3. The shift in relative fluorescence intensity for all mutant clones, including BS, was similar to that of the IL-2-inducible YT cell line.

Figure 2 Northern-blot analysis

Total RNA was isolated from cellular clones in exponential growth phase and subjected to Northern-blot analysis. The blots were probed for human IL-2R β mRNA. The cellular clones are indicated above the lane. The stripped blots were reprobed with chicken β -actin. These results are shown below the IL-2R β blot.

Figure 3 Flow cytometric analysis

Surface expression of IL-2R β was demonstrated by flow cytometric analysis. All cell lines were incubated with either biotinylated monoclonal anti-IL-2R β antibody or isotype-matched non-specific antibody followed by conjugation with phycoerythrin labelled streptavidin. ----, Fluorescence shift of the non-specific antibody; —, fluorescence shift of the specific antibody.

Proliferative response

The ability of the mutant IL-2R β cellular clones to proliferate in response to murine IL-3 or human IL-2 was evaluated by a non-radioactive cell-proliferation assay. The plotted data are medians of four samples at each concentration and standard deviations were calculated for each point. As demonstrated in Figure 4, the FL, AD and BD clones proliferated in response to both IL-2 and IL-3 whereas the Ba/F3 parental cells, SD, LS, LG and BS clones proliferated in response to IL-3 but not IL-2. The lack of response to IL-2 in the SD, LS, LG and BS clones was therefore not due to an inability to respond to cytokine.

Anti-phosphotyrosine immunoblotting

Activation of tyrosine kinases is one of the earliest events in the IL-2-induced signal cascade. To identify the tyrosyl-phosphorylated proteins in the IL-2 proliferative signal, we immunoprecipitated stimulated and unstimulated cell lysates with anti-phosphotyrosine antibody. Treatment of parental Ba/F3 cells with IL-2 did not induce tyrosyl-phosphorylation of any detectable proteins (lane A of Figure 5). However, it did induce tyrosyl-phosphorylation in the FL mutant (lane D of Figure 5). The control lanes were used only for comparison within an individual cell line. IL-2 induced an increase in tyrosyl-phosphorylation of proteins of apparent molecular mass 85, 100 and 116 kDa. We have previously shown a p116 kinase associated with the IL-2R β in human NK cells [10]. It was subsequently identified as L-Jak/Jak3 [11] and was found to be the critical tyrosine kinase responsible for IL-2 signal transduction. Jak3 is

Figure 4 IL-2 and IL-3-induced proliferation

Cells were treated for 48 h with various concentrations of either human IL-2 (●) or murine IL-3 (□), and assayed using a dye-binding assay; the resulting $A_{570-630}$ values were plotted.

also known as Ljk [40], Tk5 [41] and PtK2 [42]. The IL-3 signal was used to demonstrate that the cells were capable of responding to cytokine. In both the Ba/F3 parental cell line and the FL

mutant cell line, IL-3 induced tyrosyl-phosphorylation of proteins of apparent molecular mass 85 and 130 kDa. Previous work has shown that the 130 kDa protein is Jak2 [23]. These data

Figure 5 Anti-phosphotyrosine immunoblotting

Cells were induced with human IL-2 (indicated by 2), murine IL-3 (indicated by 3) or basal medium (indicated by 0), lysed and immunoprecipitated with anti-phosphotyrosine. The precipitated proteins were resolved by PAGE, and immunoblotted. The immunoblot was probed with anti-phosphotyrosine and developed using chemiluminescence. Proteins of particular interest are indicated.

Figure 6 *In vitro* kinase activity of the IL-2R β complex

Cells were induced with either human IL-2 (indicated by +) or basal medium (indicated by -), lysed and immunoprecipitated by the anti-IL-2R β monoclonal antibody clone 561 or an isotype-matched control antibody. An *in vitro* kinase assay was performed, the proteins were resolved by PAGE and immunoblotted. The immunoblot was probed with anti-phosphotyrosine and developed using chemiluminescence. The 50–60 kDa band is IgG heavy chain.

support the conclusion that IL-3 and IL-2 induce distinct but overlapping responses, as predicted by Otani et al. [17].

IL-2R β -associated *in vitro* kinase assay

Identification of the structural domains critical for IL-2-induced proliferation led us to determine the structural domains required for association with the individual phosphoproteins. Clarified lysates from each clone were immunoprecipitated with anti-IL-2R β (clone 561) and subjected to an *in vitro* kinase assay followed by immunoblot detection of tyrosyl-phosphorylated proteins. The results are shown in Figure 6. The strong band in the 50–60 kDa range is IgG heavy chain. When lysates were immunoprecipitated with a non-specific control antibody or when the kinase assay was performed in the absence of ATP, no tyrosyl-phosphorylated proteins were detected. IL-2 treatment of FL, BD and AD cellular clones induced phosphorylation of at least three proteins of apparent molecular mass 75, 97 and 116 kDa. The most abundantly phosphorylated protein was p116. IL-2 treatment of SD and BS did not induce tyrosyl-phosphorylation of any proteins. The same is true of IL-2 treatment of LS and LG (results not shown). These data demonstrate a requirement for p116 (Jak3) in the receptor complex of IL-2-responsive cells.

Nuclear complex-formation

To evaluate the effect of IL-2-induced Jak3 activation on nuclear complex-formation, mobility-shift analysis was performed using the GRR sequence. GRR is in the promoter of the high-affinity IgG Fc receptor gene, and several cytokines induce nuclear complex-formation with this sequence. The complex that binds to this sequence has been previously shown to contain tyrosine-phosphorylated protein p91 (Stat1) when cells were treated with interferon γ (IFN- γ) and IL-10 [39]. Other cytokines, IL-3 and granulocyte-monocyte colony-stimulating factor (GM-CSF), induce nuclear complex-formation but do not contain the phosphorylated p91. Our studies demonstrate that IL-2 induced a GRR nuclear complex-formation in all mutant cells that proliferate, FL, AD and BD. No nuclear complex was formed when the cell did not generate a proliferative signal (Figure 7).

Figure 7 GRR complex-formation

Whole-cell extracts from IL-2R β mutant cell lines were assayed for complex-formation with the GRR probe by electrophoretic mobility-shift assays. Cells were treated with 1 μ g/ml IL-2 (indicated by IL2), 10 ng/ml IFN- γ (indicated by γ) or basal medium (indicated by 0). Specificity of complex formation was demonstrated by competition with 50-fold excess GRR.

Figure 8 Composition of the GRR-binding complexes

Cellular extracts from the FL mutant treated with 1 μ g/ml IL-2 (indicated by IL2), 10 ng/ml IFN- γ (indicated by IFN γ) or basal medium (indicated by 0) were incubated with anti-p91 (Stat1) or anti-p113 (Stat2). After incubation with the specific antisera, electrophoretic mobility-shift assays were performed. Specificity of complex-formation was demonstrated by competition with 50-fold excess GRR or ISRE.

Further analysis of the IL-2-induced nuclear complex formed with GRR indicated that only GRR competed for binding of the IL-2-induced complex (Figure 8). The IL-2-induced nuclear complex is distinct from the IFN- γ -induced complex as can be seen in Figures 7 and 8. The IFN- γ -induced GRR complex contains p91 (Stat1), which has previously been shown to be phosphorylated [39]. To demonstrate the presence of p91 in the IL-2-induced complex, extracts were incubated with antiserum specific to p91 and then electrophoretic mobility-shift assays were performed. As can be seen in Figure 8, only the IFN- γ -induced GRR complex was supershifted by anti-p91 antibody. An antibody to p113 (Stat2) did not supershift either the IFN- γ - or IL-2-induced GRR complex. These results indicate that p113 is not a component of either GRR complex whereas p91 is a component of only the IFN- γ -induced complex.

DISCUSSION

The present report identifies four significant properties of IL-2R β : (1) the Box1–Box2 domain of IL-2R β is not sufficient for IL-2-induced cellular proliferation; (2) two regions of the C-terminus are capable of complementing the Box1–Box2 domain to support proliferation; (3) a 116 kDa (Jak3) tyrosyl-phosphorylated protein associates with the IL-2R β mutants and correlates with IL-2-induced proliferation; (4) all IL-2R β mutants that support proliferation also lead to the formation of a GRR nuclear complex. Contrary to the report of Hatakeyama et al. [43] which stated that the 'functional signal transduction domain' also known as Box1 and Box2 was necessary for proliferation whereas the acid-rich region was not essential, our study demonstrates that there is a requirement for Box1–Box2 and an additional region. The Box1–Box2 domain requires either the acid-rich region (amino acids 316–384) or the C-terminal region (amino acids 385–525) to complete the IL-2 proliferation signal (see Figures 4 and 6). Alignment of the amino acids from these regions demonstrates identity between residues 379–384 and 443–448. This alignment identifies a PQPLXP motif (see Figure 1b). Interestingly, this is similar to the proline-rich motif,

PXP, found in the Box1 domain of the haematopoietic receptor family [33]. The requirement for a region other than Box1–Box2 is supported by an earlier observation. Fung et al. [44] constructed an expression vector (β_{88}) that contained Box1–Box2 and 14 additional residues but not the PQPLXP motif; it did not proliferate. IL-2-induced proliferation and complete signal transduction require an IL-2R β that contains Box1–Box2 and one of two PQPLXP-containing domains. These structural features correlate with proliferation and specific interaction with the tyrosyl-phosphorylated protein, p116 (Jak3).

Receptors for the haematopoietic growth factors, prolactin [26], erythropoietin [21, 22], growth hormone [45] granulocyte colony-stimulating factor [46], require a minimal sequence that contains only Box1 and/or Box2 to support cell growth. This is not the case for the IL-2R β . The inability of IL-2R β constructs, which contain only Box1 and/or Box2, to support proliferation demonstrates conclusively that the Box1–Box2 domain of IL-2R β is not sufficient for proliferation (see Figure 1a and 4). In contrast, the interaction of several discontinuous domains is required for the IL-2 proliferative signal.

Both the GM-CSF β and IL-4 receptors have been shown to require discontinuous regions for signal transduction. Two regions of the GM-CSF β subunit are required to induce activation of Ras, Raf-1, c-Fos, c-Jun and c-Myc. Deletion of either region limits the signal transduced by GM-CSF [47]. In addition, internal deletion mutants of the IL-4 receptor indicate that two discontinuous regions are also involved in the IL-4 signal [48,49]. Studies suggest that the IL-4 receptor, like IL-2R β , requires a Box1 homology domain and an acid-rich domain located in the C-terminus. This information in conjunction with our data indicates that there are signal domains contained within the cytoplasmic regions of some cytokine receptors that are not necessarily contiguous.

IL-2-induced proliferation is closely correlated with the interaction of Box1–Box2 and PQPLXP motif with p116 (Jak3). Immunoprecipitation of IL-2-induced tyrosyl-phosphorylated proteins identified several proteins of apparent molecular mass 85, 100 and 116 kDa (Figure 5). Although p85 and p100 were seen in the anti-phosphotyrosine immunoblot, they were not observed in the receptor complex (Figure 6). Increased p116 tyrosyl-phosphorylation in the IL-2R complex is closely correlated with proliferation. Clones that did not contain Box1–Box2 and the PQPLXP motif not only did not proliferate but also did not phosphorylate p116 when stimulated with IL-2. These results indicate that p116 is an integral component of the proliferative signal induced by IL-2. We have previously shown that the 116 kDa protein is a Jak–Tyk2 family member and subsequently identified it as the Jak-like kinase, Jak3/L-Jak. Recent studies have demonstrated that Jak3 is phosphorylated on tyrosine by IL-2 or IL-4 in murine cells [50]. Thus we conclude that p116/Jak3, a unique tyrosyl-phosphoprotein, is an integral component of the proliferative signal of IL-2.

Our receptor-associated *in vitro* kinase assay also showed that a 97 kDa protein is associated with the proliferative signal. The presence of a 97 kDa phosphoprotein implied that p91 (Stat 1) could be a component of the IL-2 signal cascade. Evaluation of nuclear complexes induced by INF- γ or IL-2 showed that Stat1 was not a component of the IL-2-induced complex (see Figures 7 and 8). Western-blot analysis also showed that Stat1 was not phosphorylated in response to IL-2 (results not shown). These observations do not rule out other Stat or Stat-like proteins being part of the IL-2 signal. Further research is required to identify the IL-2-induced nuclear proteins.

From these studies we conclude that at least two domains (Box1–Box2 and PQPLXP motifs) are required for the IL-2-

induced proliferative signal, and a 116 kDa tyrosyl-phosphoprotein is intimately associated with the receptor complex. However, activation of p116/Jak3 by IL-2 does not induce the formation of a Stat1-containing nuclear complex. Future studies will focus on the interaction of IL-2R β and common γ chain subunits and their interaction with Jak3.

We thank Dr. James A. Turpin and Dr. Doug Ferris for critical review of this manuscript and Clinical Services for utilization of the flow cytometry services, specifically Refika Turnier for technical services. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the U.S. Government.

REFERENCES

- Mills, G. B., Zhang, N., Schmandt, R., Fung, M., Greene, W., Mellors, A. and Hogg, D. (1991) *Biochem. Soc. Trans.* **19**, 277–285
- Schmandt, R., Fung, M., Arima, N., Zhang, N., Leung, B., May, C., Gibson, S., Hill, M., Green, W. and Mills, G. B. (1992) *Bailliere's Clin. Haematol.* **5**, 551–573
- Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Manakata, H., Nakamura, M. and Sugamura, K. (1992) *Science* **257**, 379–382
- Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I. and Sugamura, K. (1993) *Science* **262**, 1874–1876
- Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X. and Leonard, W. J. (1993) *Science* **262**, 1877–1880
- Maslinski, W., Remillard, B., Tsudo, M. and Strom, T. B. (1992) *J. Biol. Chem.* **267**, 15281–15284
- Turner, B. C., Tonks, N. K., Rapp, U. R. and Reed, J. C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5544–5548
- Satoh, T., Nakafuku, M., Miyajima, A. and Kaziro, Y. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3314–3318
- Izquierdo, M. and Cantrell, D. A. (1993) *Eur. J. Immunol.* **23**, 131–135
- Kirken, R. A., Rui, H., Evans, G. A. and Farrar, W. L. (1993) *J. Biol. Chem.* **268**, 22765–22770
- Kirken, R. A., Rui, H., Malabarba, M. G. and Farrar, W. L. (1994) *J. Biol. Chem.* **269**, 19136–19141
- Johnston, J. A., Kawamura, M., Kirken, R., Chen, Y.-Q., Blake, T. B., Shibuya, K., Ortaldo, J. R., McVicar, D. W. and O'Shea, J. J. (1994) *Nature (London)* **370**, 151–153
- Siliciano, J. D., Morrow, T. A. and Desiderio, S. V. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11194–11198
- Kobayashi, N., Kono, T., Hatakeyama, M., Minami, Y., Miyazaki, T., Perlmutter, R. M. and Taniguchi, T. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4201–4205
- Mills, G. B., Arima, N., May, C., Hill, M., Schmandt, R., Li, J., Miyamoto, N. G. and Greene, W. C. (1992) *Int. Immunol.* **4**, 1233–1243
- Torigoe, T., Saragovi, H. U. and Reed, J. C. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2674–2678
- Otani, H., Siegel, J. P., Erdos, M., Gnarr, J. R., Toledano, M. S., Mostowski, H., Feinberg, M. B., Pierce, J. H. and Leonard, W. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2789–2793
- Hatakeyama, M., Kono, T., Kabayashi, N., Kawahara, A., Levin, S. D., Perlmutter, R. M. and Taniguchi, T. (1991) *Nature (London)* **252**, 1523–1528
- Minami, Y., Kono, T., Yamada, K., Kobayashi, N., Kawahara, A., Perlmutter, R. M. and Taniguchi, T. (1993) *EMBO J.* **12**, 759–768
- Farrar, W. L. and Ferris, D. K. (1989) *J. Biol. Chem.* **264**, 12562–12567
- Miura, O., Cleveland, J. L. and Ihle, J. N. (1993) *Mol. Cell Biol.* **13**, 1788–1795
- Whitthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O. and Ihle, J. N. (1993) *Cell* **74**, 227–236
- Silvennoinen, O., Witthuhn, B. A., Quelle, F. W., Cleveland, J. L., Taolin, Y. and Ihle, J. N. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8429–8433
- Argetsinger, L. S., Campbell, G. S., Yang, X., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N. and Carter-Su, C. (1993) *Cell* **74**, 237–244
- Rui, H., Kirken, R. A. and Farrar, W. L. (1994) *J. Biol. Chem.* **269**, 5364–5368
- Dasilva, L., Howard, O. M. Z., Rui, H., Kirken, R. A. and Farrar, W. L. (1994) *J. Biol. Chem.* **269**, 18267–18270
- Lutticken, C., Wegenka, U. M., Yuan, J., Buschmann, J., Schindler, C., Ziemiecki, A., Harpur, A. G., Wilks, A. F., Yasukawa, K., Taga, T., Kisimoto, T., Barbieri, G., Pellegrini, S., Sendtner, M., Heinrich, P. C. and Friedemann, H. (1994) *Science* **263**, 89–92
- Stahl, N., Boulton, T. G., Farrugella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N. and Yancopoulos, G. D. (1994) *Science* **263**, 92–95
- Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A. G., Barbieri, G., Witthuhn, B. A., Schindler, C., Pellegrini, S., Wilks, A. F., Ihle, J. N., Stark, G. R. and Kerr, I. M. (1993) *Nature (London)* **366**, 129–135
- Shuai, K., Ziemiecki, A., Wilks, A. F., Harpur, A. G., Sadowski, H. B., Gilman, M. Z. and Darnell, J. E. (1993) *Nature (London)* **366**, 580–583
- Silvennoinen, O., Ihle, J. N., Schlessinger, J. and Levy, D. E. (1993) *Nature (London)* **366**, 583–585
- Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. and Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11349–11353
- O'Neal, K. D. and Yu-Lee, L.-Y. (1993) *Cytokine* **12**, 309–312
- Holmes, K. and Fowlkes, B. J. (1991) in *Current Protocols in Immunology* p. 5.3.4., Current Protocols, New York
- Evans, G. A., Howard, O. M. Z., Erwin, R. and Farrar, W. L. (1993) *Biochem. J.* **294**, 339–342
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Garcia, G., Evans, G., Michiel, D. and Farrar, W. (1992) *Biochem. J.* **285**, 851–860
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Lerner, A. C., David, M., Feldman, G. M., Igarashi, K.-I., Hackett, R. H., Wev, D. S. A., Sweitzer, S. M., Petricoin, E. F. and Finbloom, D. S. (1993) *Science* **261**, 1730–1733
- Kawamura, M., McVicar, D. W., Johnston, J. A., Blake, T. B., Chen, Y. C., Lal, B. K., Lloyd, A. R., Kelvin, D. J., Staples, J. E. and O'Shea, J. J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6374–6378
- Cance, W. G., Craven, R. J., Weiner, T. M. and Liu, E. T. (1993) *Int. J. Cancer* **54**, 571–577
- Sanchez, M. P., Tapley, P., Saini, S. S., He, B., Pulido, D. and Barbacid, M. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1819–1823
- Hatakeyama, M., Mori, H., Doi, T. and Taniguchi, T. (1989) *Cell* **59**, 837–845
- Fung, M. R., Searce, R. M., Hoffman, J. A., Pfeffer, N. J., Hammes, S. R., Hosking, J. B., Schmandt, R., Kuziel, W. A., Haynes, B. F., Mills, G. B. and Greene, W. C. (1991) *J. Immunol.* **147**, 1253–1260
- Colosi, P., Wong, K., Leong, S. R. and Wood, W. I. (1993) *J. Biol. Chem.* **268**, 12617–12623
- Fukunaga, R., Ishizaka-Ikeda, E. and Nagata, S. (1993) *Cell* **74**, 1079–1087
- Sato, N., Sakamaki, K., Terada, N., Ken-ichi, A. and Miyajima, A. (1993) *EMBO J.* **12**, 4181–4189
- Koettnitz, K. and Kalthoff, F. S. (1993) *Eur. J. Immunol.* **23**, 988–991
- Harada, N., Yang, G., Miyajima, A. and Howard, M. (1993) *J. Biol. Chem.* **267**, 22752–22758
- Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T. and Ihle, J. N. (1994) *Nature (London)* **370**, 153–157