Identification of a Variable Region within the Cytoplasmic Tail of the IL-2 Receptor β Chain That Is Required for Growth Signal Transduction*

(Received for publication, June 13, 1995, and in revised form, July 18, 1995)

Kathleen D. Liu‡§, Stephen Y. Lai‡§, Mark A. Goldsmith‡, and Warner C. Greene‡¶**

From the ‡Gladstone Institute of Virology and Immunology, Departments of §Medicine and ¶Microbiology and Immunology, School of Medicine, University of California, San Francisco, California 94141

Interleukin-2 (IL-2) regulates numerous biological events, including T lymphocyte proliferation. Interleukin-2 receptor (IL-2R)-mediated signaling is triggered by ligand-induced heterodimerization of the IL-2Rβ and γc subunits, which results in the activation of signaling intermediates that are associated with either IL-2Rα or γc. Previous mutagenesis studies of the IL-2Rβ cytoplasmic tail demonstrated that the partially conserved box 1 and box 2 motifs and specific tyrosine residues are critical for growth signaling. By deletion and alanine scanning mutagenesis, another set of residues that are critical for IL-2R-mediated signaling has now been identified. These residues lie within the divergent 35-amino acid "spacer" region separating box 1 and box 2. The role of this receptor subregion in early phases of IL-2R signaling was evaluated using BA/F3 stable cell lines expressing three functionally impaired mutants from this region. All three cell lines displayed substantially diminished growth responsiveness to IL-2. Receptor-mediated STAT factor activation, IL-2Rβ phosphorylation, and Janus kinase activation were also markedly impaired. These findings indicate that this variable spacer region, which we have termed the V-box, is essential for the initiation of IL-2R-mediated signal transduction.

The interleukin-2 receptor (IL-2R) complex exists in two functional forms: a high affinity heterotrimer comprising the IL-2Rα, IL-2Rβ, and γc subunits, as well as an intermediate affinity heterodimer containing the IL-2Rβ and γc chains. In T lymphocytes, binding of IL-2 to either form of the receptor activates an intracellular signaling cascade that culminates in cellular proliferation (1, 2). The primary role of the IL-2Rα subunit is to confer high affinity binding of the ligand to the receptor complex (3, 4). Consistent with this concept, the cytoplasmic tail of IL-2Rα is only 13 amino acids long (5–7) and no known signaling intermediates associate with this region. Instead, intracellular signal transduction results from ligand-induced heterodimerization of the IL-2Rβ and γc subunits, which have cytoplasmic tails of 385 and 86 amino acids, respectively.

Heterodimerization of these receptor chains results in the concurrent activation of signaling intermediates (8–10), some of which are preassociated with either IL-2Rβ or γc. Specifically, two members of the Janus family of tyrosine kinases, J AK1 and J AK3, are bound to the cytoplasmic regions of the IL-2Rβ and γc chains, respectively, in the absence of IL-2 (11–13). These tyrosine kinases become enzymatically activated following ligand binding, perhaps by cross-phosphorylation. However, the precise molecular details of this inductive event remain unclear. The J AK kinases in turn are thought to activate members of the STAT family of transcription factors ("signal transducers and activators of transcription"). Upon phosphorylation, the STAT factors dimerize and translocate to the nucleus, where they activate the transcription of specific target genes (reviewed in Ref. 14). In the IL-2R system, the activation of STAT5 has recently been demonstrated by a number of laboratories (15–17), and in some cases, the activation of STAT1 and STAT3 has also been observed (16, 18).

The IL-2Rβ and γc chains are both members of the cytokine receptor superfamily (19). Receptors in this family share two extracellular motifs, a set of four cysteines with canonical spacing and a WSXWS sequence. Moreover, these proteins are also characterized by two small, partially conserved, intracellular regions called the box 1 and 2 motifs (20, 21). These motifs are separated by a region often called the "spacer," which varies in length from 13 to 49 amino acids in the various cytokine superfamilies.

Early mutagenesis studies of IL-2Rβ focused on large deletions within the cytoplasmic tail. Analysis of the IL-2Rβ SD mutant, which lacks residues 266–323, revealed that amino acids located within and surrounding the box 2 motif of IL-2Rβ are critical for growth signaling (21). More refined structure-function studies of the cytoplasmic tail of IL-2Rβ have demonstrated that both the proximal box 1 and box 2 motifs, as well as specific tyrosine residues in the distal receptor tail, play a critical role in IL-2R-mediated growth signaling (1, 22). Deletion of either box 1 or box 2 of IL-2Rβ completely abrogated IL-2-induced proliferation. However, alanine scanning mutagenesis unexpectedly revealed that only two residues within boxes 1 and 2 contributed side chains critical for such signaling. These amino acids are Asp-258 and Leu-299 in the box 1 motif (1) and Asp-258 in the box 2 motif (1, 23). Interestingly, Asp-258 is poorly conserved in the box 1 elements of other cytokine superfamily members. This finding raised the possibility that variable rather than conserved residues might play a central role in...
growth signaling through the IL-2R complex. This hypothesis was attractive because it could help explain the specificity of signaling exhibited by various cytokine superfAMILY members despite the presence of partially conserved motifs. Accordingly, we have now investigated the nonconserved 35-residue spacer region separating Asp-258 in the distant portion of box 1 and Leu-299 in the proximal part of box 2. Since this spacer region is highly variable in amino acid composition in different cytokine receptor members, the term variable (V) box will be used to refer to it.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—BA/F3 is an IL-3-dependent murine pro-B cell line described previously (24) and generously provided by Dr. Gordon Mills. These cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum, antibiotics, 0.05 mM l-mercaptoethanol, and 10% WEHI supernatant as a source of IL-3. The COS-7 monkey kidney cell line (ATCC) was maintained in Iscove’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Recombinant human IL-2 was the gift of the Chiron Corp (Emeryville, CA). The anti-IL-2Rβ monoclonal antibody 561 was a generous gift from Dr. Rich Robb. Polyclonal antibodies specific for JAK1 and JAK3 and a mouse monoclonal antibody to phosphotyrosine (4G10) were obtained from Upstate Biotechnology (Lake Placid, NY). Recombinant murine IgG2a UPC10 monoclonal antibody was obtained from Cappell (Gordon Mills). These cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum, antibiotics, 0.05 mM l-mercaptoethanol, and 10% WEHI supernatant. Following stimulation, nuclear extracts were prepared according to the method described in Ref. 26 in the presence of 1 mM NaVO4 and a protease mixture composed of the following inhibitors: 0.5 mM antipain, 0.5 mM aprotinin, 0.75 mM bestatin, 0.5 mM leupeptin, 0.05 mM pepstatin A, 1.4 mg/ml phenylmethylsulfonyl fluoride, and 0.5 mg/ml soybean trypsin inhibitor. Electrophoretic mobility shift assays (EMSA) were performed as described (17).

RESULTS

Replacement or Deletion of the IL-2Rβ V-box Abrogates IL-2-mediated Growth Signaling—To assess the functional role of the IL-2Rβ V-box in IL-2-mediated growth signaling, a mutant IL-2Rβ was created in which this 35-residue region was deleted and replaced with a single methionine residue. In addition, to test the hypothesis that this V-box region could be functionally complemented by an analogous domain from a different cytokine superfamily member, a chimeric IL-2Rβ chain was prepared in which the native V-box was replaced with the corresponding region from the γc receptor chain. The signaling function of each of these mutants was assessed in a transient proliferation assay (1). In this assay, mutant IL-2Rβ chains are transiently transfected into BA/F3, γc-expressing murine pro-B cell line. Growth selection in IL-2 permits the distinction between functional and nonfunctional forms of the IL-2Rβ receptor. This assay appears to be more sensitive to small decrements in function than conventional stable proliferation assays. Both of these mutants failed to display any detectable function in this assay, suggesting that the removal or replacement of the V-box markedly impaired effective growth signaling (Fig. 1A).

To further define V-box sequences needed for growth signaling, a series of smaller deletion mutants was prepared and analyzed. Each of these mutants encoded a IL-2Rβ chain lacking 5–10 amino acids within the V-box. Two of the mutants (IL-2RβΔ261–269 and IL-2RβΔ279–289) failed to proliferate in the transient proliferation assay (Fig. 1A), while a third mutant, corresponding to the most C-terminal deletion, IL-2RβΔ290–294, proliferated in response to IL-2. Expression studies in COS-7 cells confirmed that each of these various V-box mutants was synthesized (Fig. 1B), indicating that the observed lack of function observed was not due to a lack of expression. Together, these results suggest that the IL-2Rβ V-box plays an important role in growth signaling by the IL-2R complex and that this function cannot be simply reconstituted by substitution of a V-box from another cytokine superfamily receptor member.

Alanine Scanning Mutagenesis Identifies Several Specific Residues Within the V-box That Are Critical For Growth Signaling—To delineate specific amino acids within the IL-2Rβ V-box that participate in growth signaling, alanine scanning mutagenesis of this region was performed. Glycine, alanine, and valine residues were replaced with arginine residues, while all other amino acids were substituted with alanine. One to three contiguous amino acids were mutated in each construct. The effects of these substitution mutations in the transient proliferation assay are shown in Fig. 2A. Three of these
point mutants, IL-2R β261–263A, IL-2R β270A 271–2R, and IL-2R β278–280A, displayed essentially no proliferation in the transient assay. Expression studies in COS-7 cells confirmed that all three of these constructs encoded appropriately sized transient assay. Expression studies in COS-7 cells confirmed that all three of these constructs encoded appropriately sized transfection assay. [3H]Thymidine incorporation (shown in counts/min) was measured on days 7–14 post transfection. βWT, wild-type IL-2R β; βΔV, IL-2R β, deleted of residues 261–295 and replaced with a single methionine residue; βγV, IL-2R β, deleted of residues 261–293 and replaced with residues 284–318 from the γc receptor chain; βΔ261–269, IL-2R β deleted of residues 261–269; βΔ279–289, IL-2R β deleted of residues 279–289; βΔ290–294, IL-2R β deleted of residues 290–294. B, IL-2R β mutants were transfected into COS-7 cells to demonstrate that IL-2R β chains of appropriate lengths are synthesized. Cell lysates were subjected to SDS-PAGE, followed by immunoblotting with an anti-IL-2R β antibody, 561. The mutants analyzed are described in panel A.

Fig. 1. Substitution and deletion analysis of the IL-2R β V-box. A, mutant IL-2R β chains were screened for proliferation function in a transient transfection assay. [3H]Thymidine incorporation (shown in counts/min) was measured on days 7–14 post transfection. βWT, wild-type IL-2R β; βΔV, IL-2R β, deleted of residues 261–295 and replaced with a single methionine residue; βγV, IL-2R β, deleted of residues 261–293 and replaced with residues 284–318 from the γc receptor chain; βΔ261–269, IL-2R β deleted of residues 261–269; βΔ279–289, IL-2R β deleted of residues 279–289; βΔ290–294, IL-2R β deleted of residues 290–294. B, IL-2R β mutants were transfected into COS-7 cells to demonstrate that IL-2R β chains of appropriate lengths are synthesized. Cell lysates were subjected to SDS-PAGE, followed by immunoblotting with an anti-IL-2R β antibody, 561. The mutants analyzed are described in panel A.

IL-2R-mediated growth signaling was first assessed in these stable cell lines by measuring [3H]thymidine incorporation in response to varying doses of IL-2. The IL-2R β261–263A, IL-2R β270A 271–272R, and IL-2R β278–280A mutations all displayed markedly reduced responsiveness to IL-2 in the assay. As expected, the IL-2R β264–266A mutant proliferated normally in the dose-response curve, while the IL-2R β261–263A mutant produced a more marked shift in response to IL-2. The IL-2R β270A 271–272R mutant conferred the smallest shift in the dose-response curve, while the IL-2R β278–280A mutant produced a more marked shift in response to IL-2. The IL-2R β261–263A mutant was essentially unresponsive to IL-2, even at very high doses. Hence, these data confirmed the results obtained with the transient proliferation assay and revealed more subtle differences between these three mutants.

V-box Mutants Fail to Activate STAT Factors—Recently, a number of STAT factors have been implicated in IL-2R signaling (15–18). Moreover, activation of one or more of these factors may play a role in proliferation signaling by the IL-2R complex, since many mutations in the receptor chains that abrogate STAT induction affect proliferation as well. Consequently, the V-box mutants described above were evaluated by EMSA for STAT induction affecting proliferation as well. Consequently, the V-box mutants described above were evaluated by EMSA for STAT induction affecting proliferation as well.
abnormalities in STAT induction. The STAT factor induced by IL-2 in the BA/F3 cell line was identified as STAT5, by supershifting of the STAT/DNA complex with an anti-STAT5 antibody (data not shown). As shown in Fig. 5, activation of STAT5 was detectable but markedly diminished in the cell lines expressing the IL-2Rβ270A 271–2R and IL-2Rβ278–280A mutants (lanes 9 and 12), as compared to the receptor-matched control cell line. STAT activation was undetectable in the cell line expressing the IL-2Rβ261–263A mutant (lane 3). Despite these differences in STAT DNA binding activity, all of the nuclear extracts contained equivalent amounts of SP1 binding activity, as demonstrated by EMSA (data not shown), indicating that the diminished STAT factor detection in the V-box mutant cell lines was not due to inadvertent degradation of nuclear proteins during extract preparation. Thus, STAT activation is significantly decreased in the BA/F3 stable cell lines expressing two of the three V-box mutants (IL-2Rβ270A 271–2R and IL-2Rβ278–280A) and is undetectable in the third cell line (IL-2Rβ261–263A).

V-box Mutations Abrogate IL-2Rβ Tyrosine Phosphorylation following IL-2 Binding—Studies were next performed to assess the effects of these V-box mutations on early IL-2R-mediated signaling events. STAT factors are thought to interact with specific phosphotyrosine residues within cytoplasmic receptor tails through SH2-mediated interactions (14). Moreover, the rapid tyrosine phosphorylation of the IL-2Rβ chain has been shown to be critical for effective growth signaling (22, 27–30). Therefore, it was of interest to determine whether or not IL-2Rβ chain tyrosines were phosphorylated in cell lines stably expressing the various IL-2Rβ V-box mutants. In all three cases, IL-2Rβ tyrosine phosphorylation was markedly reduced, as compared with the functional receptor-matched control cell line (Fig. 6, lanes 4, 6, 8, and 10). The IL-2Rβ270A 271–272R mutant displayed the highest level of IL-2Rβ phosphorylation, consistent with its moderately shifted dose-response curve for proliferation. In contrast, tyrosine phosphorylation of IL-2Rβ was undetectable in stable cell lines expressing either the
IL-2Rβ261–263A mutant or the IL-2Rβ278–280A mutant.

**V-box Mutations Abrogate JAK1 and JAK3 Induction**—Having observed impaired STAT activation and reduced tyrosine phosphorylation of the IL-2Rβ chain, the effects of these V-box mutations on an even earlier step in the signaling pathway, JAK activation, were investigated. Following the addition of IL-2, the JAK1 and JAK3 kinases undergo tyrosine phosphorylation within 5 min (31, 32). This phosphorylation correlates with enzymatic activation of the J anus kinases in vitro. (31, 32). Following IL-2 stimulation of the mutant V-box cell lines, tyrosine phosphorylation of JAK1 and JAK3 was assessed by serial immunoprecipitation and immunoblotting with an antiphosphotyrosine antibody. In all three of the nonfunctional V-box mutant cell lines assessed, IL-2-induced phosphorylation of JAK1 and JAK3 was undetectable (Fig. 7, A and B, respectively: lanes 2, 4, 6, and 8). Hence, these IL-2Rβ V-box mutations compromise one of the earliest signaling events mediated through the IL-2R complex.

**DISCUSSION**

Previous work from this and other laboratories has demonstrated that membrane-proximal regions of the cytoplasmic tail of IL-2Rβ are critical for signaling by the IL-2R complex (for a review, see Ref. 33). Surprisingly, only a single residue in each of the box 1 and box 2 appears essential for growth signaling. Furthermore, the critical residue within the box 1 motif (Asp-258) is not well conserved among other cytokine receptor superfamilly members. This result led us to consider the hypothesis that variable amino acids, rather than conserved residues, play an important role in signaling by IL-2Rβ. Therefore, we focused on the spacer region positioned between the box 1 and box 2 motifs that contains predominantly nonconserved amino acids. Previous work had suggested that this region, which we have termed the V-box, plays a role in IL-2Rβ-mediated growth signaling. The critical residues identified in the box 1 and box 2 motifs directly flank the intervening V-box, i.e. Asp-258 is positioned at the C-terminal end of box 1, whereas Leu-299 is at the N-terminal end of box 2. Therefore, these motifs might serve to expose the critical intervening variable region, which could function as a binding platform for one or more signaling intermediates.

The mutagenesis approaches presented in this paper further support the hypothesis that the IL-2Rβ V-box contains elements critical for growth signaling. A gross deletion of this region completely abrogated function in a transient assay of receptor function. Furthermore, a mutant in which the IL-2Rβ V-box was replaced with an analogous portion of the γc receptor also failed to support growth signaling. This result suggested that V-box elements are not readily interchangeable, although additional experimentation is required to prove the generality of this observation. It should be noted that the analogous region for substitution was defined on the basis of sequence homology, not function, and these artificial sequence boundaries might in fact interrupt functional domains. Thus, replacement of only the V-box region of this domain could result in a hybrid domain that cannot support growth signaling.

Finer mutagenesis studies, including alanine scanning of the IL-2Rβ V-box region, identified a number of residues that are important for growth signaling in the transient proliferation assay. Many of these critical amino acids do not have side chains that can be phosphorylated or covalently modified in another way following ligand binding. Consequently, it seems unlikely that this region serves as an inducible binding site for a signaling intermediate; rather, this V-box region might serve as a constitutive docking site for one or more signaling molecules. Of note, replacement of Trp-277 with alanine does not affect receptor function. Previous mutagenesis studies had demonstrated that whereas a W277G mutation results in impaired IL-2Rβ function (1), a W277R mutation does not affect signaling by the receptor complex (34). Consequently, Trp-277 may play an important role in the secondary or tertiary structure of this region, rather than contributing a specific side chain that participates directly in receptor-signaling intermediate interactions.

In studies designed to dissect where the V-box mutations exert their inhibitory effects within the signaling cascade, we examined a relatively distal effect of ligand-induced signaling, STAT activation. In the IL-2R system, mutants that display impaired STAT activation also fail to proliferate normally in response to IL-2, suggesting that STAT factors may play a role in growth signaling. In the stable cell lines expressing various V-box mutants, STAT activation is only barely detectable in the cell lines expressing IL-2Rβ270A 271–2R or IL-2Rβ278–280A and is undetectable in the cell line expressing IL-2Rβ261–263A. Consequently, the V-box mutations must affect a signaling step proximal to STAT factor activation but distal to ligand binding.

Subsequent studies focused on earlier steps in the signaling cascade, in order to define more precisely the contribution of these critical V-box residues to IL-2R-mediated signaling. Phosphorylation of IL-2Rβ tyrosines is thought to allow additional signaling intermediates to be recruited into the activated receptor complex, probably through SH2-mediated interactions. This phosphorylation is rapid and may contribute to
Signaling Function of the IL-2R β V-box

4 K. D. Liu, W. Xu, M. A. Goldsmith, and W. C. Greene, unpublished observations.

effective growth signaling (22). Since STAT activation is likewise thought to depend upon receptor-based phosphotyrosine residues, the integrity of IL-2R β tyrosine phosphorylation was examined in these mutant cell lines. Importantly, ligand-induced tyrosine phosphorylation of IL-2R β was inhibited in cell lines expressing each of the three nonfunctional V-box mutant receptors. Notably, a minor degree of IL-2R β phosphorylation was detected in the IL-2R β270A 271–2R line, but phosphorylation was undetectable in the IL-2R β278–280A cell line. In contrast, as discussed above, a small degree of STAT activation was detectable in both the IL-2R β270A 271–2R and IL-2R β278–280A cell lines. These results may simply reflect a small difference in sensitivity of these two assays, with the gel shift analysis of STAT activation being the more sensitive of the two. Nonetheless, in both cases, IL-2R β phosphorylation and STAT activation are minimal, as compared to the receptor-matched positive control cell line.

The diminution of IL-2R β phosphorylation was particularly intriguing because it suggested that membrane-proximal mutations can affect signaling events occurring at more distal sites within the IL-2R β chain. Moreover, this result suggests that these mutations affect an early signaling event known to be important for a full mitogenic response. The V-box therefore might be critical for the association of the cytoplasmic receptor tail with either a kinase that phosphorylates the IL-2R β tail directly or a protein that activates such a kinase. In the erythropoietin receptor system, Aoki and co-workers (35) have recently shown that the association of JAK2 with the erythropoietin receptor is abrogated when a tryptophan residue within the β chain, including the SD region.4 It is of interest that JAK3, with JAK1 in both COS-7 and CV-1 cells and have been unable to map the JAK1 binding site to any one domain of the receptor chain, including the SD region.4 It is of interest that JAK3, which is generally thought of as a γc-associated molecule, fails to be activated in the context of IL-2R β mutations. This finding strongly argues for cross-talk between the IL-2R β and γc through the two associated JAK kinases. Alternatively, we and others have detected an association between IL-2R β and JAK3 in overexpression systems (13).4 This association may simply be the result of an intrinsic affinity of the JAK1 binding site for all kinases within the JAK family, and may be enhanced by the concomitant overexpression of both the receptor chain (containing the JAK1 binding site) and a JAK kinase. This result may suggest a more complex kinase activation mechanism in which γc donates JAK3 to the IL-2R β chain following ligand-induced heterodimerization of IL-2R β and γc. This event might in turn permit the simultaneous activation of the JAK1 and JAK3 kinases.

In summary, these studies suggest that the IL-2R β V-box region contains determinants that are critical for a very proximal step in the growth signaling cascade initiated by the IL-2R complex. Our findings further support the hypothesis that vari-