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The integrity of the conserved 'WS motif' common to IL-2 and other cytokine receptors is essential for ligand binding and signal transduction

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Recent studies have identified a new family of cytokine receptors, which is primarily characterized by the conservation of periodically interspersed four cysteine residues and the W-S-X-W-S sequence ('WS motif') within the extracellular domain. However, the role of such conserved structures still remains elusive, in particular that of the WS motif. Interleukin-2 (IL-2) is known to play a critical role in the clonal expansion of antigen-stimulated T lymphocytes, and the IL-2 signal is delivered by one of the receptor components, the IL-2 receptor β (IL-2Rβ) chain. The IL-2Rβ chain, unlike the IL-2Rα chain, belongs to this receptor family. In the present study, we analyzed the function of the WS motif of IL-2Rβ (Trp194-Ser195-Pro196-Trp197-Ser198) with the use of site-directed mutagenesis. Our results indicate the critical role of the two Trp residues in the proper folding of the IL-2Rβ extracellular domain and point to the general functional importance of the WS motif in the new cytokine receptor family.

Key words: cytokine receptor family/IL-2 receptor β chain/ligand binding/signal transduction/WS motif

Introduction

In the interleukin-2 (IL-2) system, two distinct IL-2 binding components are known to constitute the high affinity IL-2 receptor; the α chain (IL-2Rα) (Cosman *et al.*, Leonard *et al.*, 1984; Nikaido *et al.*, 1984) and the β chain (IL-2Rβ) (Sharon *et al.*, 1986; Tsudo *et al.*, 1986; Dukovich *et al.*, 1987; Teshigawara *et al.*, 1987; Hatakeyama *et al.*, 1989a,b). Unlike the IL-2Rα chain, the IL-2Rβ chain has a large cytoplasmic domain, the structure of which is conserved between human and mouse (Kono *et al.*, 1990) and lacks an obvious tyrosine kinase domain (Hatakeyama *et al.*, 1989a). Functional analyses of IL-2Rα and IL-2Rβ chains expressed by cDNA transfection have revealed that the latter chain is indeed responsible for delivering the IL-2 induced growth signal intracellularly (Siegel *et al.*, 1987; Tsudo *et al.*, 1987; Okamoto *et al.*, 1990).

Recent advances in the study of cytokine receptors have elucidated that a number of receptor components, including those for interleukin-3 (IL-3) (Itoh *et al.*, 1990), IL-4 (Mosley *et al.*, 1989; Harada *et al.*, 1990; Idzerda *et al.*, 1990), IL-5 (Takaki *et al.*, 1990), IL-6 (Yamasaki *et al.*, 1988), IL-7 (Goodwin *et al.*, 1990), erythropoietin (EPO) (D'Andrea *et al.*, 1989), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gearing *et al.*, 1989), granulocyte colony-stimulating factor (G-CSF) (Fukunaga *et al.*, 1990) and prolactin (PRL) (Boutin *et al.*, 1988) have common structural features with the IL-2Rβ and constitute a new family of receptors (Bazan, 1990, Cosman *et al.*, 1990; Patthy, 1990). Those receptors are characterized by the conservation of the 'cysteine motif'-four periodically interspersed cysteines, $CX_{9-10}CXWX_{26-32}CX_{10-15}C$ (where C represents cysteine; W, tryptophan and X, a non-conserved amino acid) and the 'WS motif' i.e. a WSXWS sequence (W, tryptophan; S, serine; X, a non-conserved amino acid) in the extracellular domain. Usually, the WS motif is located proximal to the cell membrane. Whereas it is very likely that the cysteine motif constitutes a domain(s) for the interaction of receptor with its cognate ligand (Ullrich *et al.*, 1984; Ebina *et al.*, 1985), function of the conserved WS motif is still unknown. Interestingly, this motif has also been detected in non-receptor molecules such as complement components (DiScipio *et al.*, 1984; Stanley *et al.*, 1985; Rao *et al.*, 1987). In order to investigate the role of the WS motif, we have examined the effect of systematic amino acid replacements within the WS motif of IL-2Rβ chain in the IL-2 binding and signal transduction using site-directed mutagenesis. We discuss our experimental data in the light of the conservation of this short sequence in protein folding and intermolecular association.

Results

Construction and expression of mutant cDNAs of IL-2Rβ in lymphocytes and COS cells

In order to study the functional role of the WS motif, we systematically generated mutations in the motif-encoding region (Trp194-Ser195-Pro196-Trp197-Ser198) of the human IL-2Rβ cDNA using oligonucleotide-directed mutagenesis in M13 (Hatakeyama *et al.*, 1986), as depicted in Table I. In mutants M1 and M5, both of the conserved

Table I. Amino acid sequence of the WS motif-encoding region of the wild type (WT) and each mutant (M1-M5) IL-2Rβ chain

Name	Amino acid sequence
WT	Trp194-Ser195-Pro196-Trp197-Ser198
M1	**Gly194**-Ser195-Pro196-**Gly197**-Ser198
M2	Trp194-**Gly195**-Pro196-Trp197-**Gly198**
M3	Trp194-Ser195-**Ala196**-Trp197-Ser198
M4	**Gly194-Gly195**-Pro196-Trp197-Ser198
M5	**Ser194**-Ser195-Pro196-**Ser197**-Ser198

Trp residues (194 and 197) are replaced by either Gly or Ser, whereas mutant M2 contains substitutions of the two conserved Ser residues (195 and 198) with Gly. Mutants of IL-2Rβ in which one of the Trp194−Ser195 sequence is converted to Gly−Gly (M4 mutant) or the non-conserved Pro196 residue is converted to Ala (M3 mutant) were also generated (see Materials and methods for details). Mutant IL-2Rβ cDNAs (M1, M2, M3, M4 and M5) were each introduced into an expression vector p1013 (Garvin *et al.*, 1988), and then co-transfected with an expression vector for the neomycin (neo) resistance gene (pST neoB; Hatakeyama *et al.*, 1989b) into EL-4 and BAF-B03 cells. EL-4 is a mouse T lymphoma line which does not express the endogenous IL-2Rα (Kono *et al.*, 1990) and BAF-B03 is a pro-B line which expresses a high level of endogenous IL-2Rα (Hatakeyama *et al.*, 1989b). G418 (neo)-resistant transformant clones were selected for and subjected to FACS analysis for IL-2Rβ expression, using anti-human IL-2Rβ monoclonal antibody, Mik-β1 which recognizes the extracellular domain of the human IL-2Rβ (Tsudo *et al.*, 1989). Although the numbers of established neo-resistant clones were comparable (42−66) for all mutants, clones expressing the Mik-β1 epitope were detectable for M2, M3 and M4, but not for M1 or M5 (Table II) in both EL-4 and BAF-B03 cells. Typical Mik-β1 staining profiles of the transformant clones each expressing the wild type or mutant IL-2Rβ are shown in Figure 1. Furthermore, wild type and mutant IL-2Rβ cDNAs (M1, M2, M3, M4 and M5) were each introduced into expression vector pdKCR giving the plasmids pdKCR-β, pdKCR-M1, pdKCR-M2, pdKCR-M3, pdKCR-M4 and pdKCR-M5 respectively. Cell surface expression of the cDNA-encoded IL-2Rβ was then examined by transfecting these expression plasmids into COS cells by the calcium phosphate method (Turner *et al.*, 1990), followed by FACS analysis as described above. Epitope expression for Mik-β1 was again demonstrable for mutants M2, M3 and M4 but not for M1 or M5 (data not shown).

Collectively, these observations indicate that either the M1 and M5 mutant IL-2Rβs have lost their antigenic epitope for Mik-β1 or they cannot be expressed on the cell surface, or both. The results with BAF-B03 cells also suggest that such a defect of the two mutant IL-2Rβs (M1 and M5) cannot be compensated by the expression of IL-2Rα, with which IL-2Rβ forms a heterodimer otherwise.

Trp194 in the WS motif is essential for ligand binding of IL-2Rβ

We next examined the IL-2 binding properties of the M2, M3 and M4 mutant IL-2Rβ molecules which are expressed in EL-4 cells at a level comparable with that of wild type IL-2Rβ (Hatakeyama *et al.*, 1989a), as monitored by Mik-β1 staining (data not shown). Scatchard plot analyses of IL-2 binding studies using ^{125}I-labeled recombinant human IL-2 revealed that M3 mutant IL-2Rβ has an intermediate affinity for IL-2 with an estimated K_d value similar to that of the wild type IL-2Rβ (3.1 nM). The M2 mutant also had a similar affinity for IL-2, but the K_d was reproducibly somewhat higher (5.0 nM).

On the other hand, no significant IL-2 binding was observed in EL-4 transformant clones expressing M4 mutant IL-2Rβ (Figure 2A). In order to address this issue further, we also carried out [^{125}I]IL-2 chemical cross-linking experiments using the non-cleavable chemical cross-linker, disuccinimidyl suberate (DSS). In the EL-4 transformant clones expressing either the M2 or the M3 mutant IL-2Rβ, SDS−PAGE of the cross-linked products revealed a band broadened from 85 kDa to 90 kDa, as shown previously with Jβ-8 cells which express the wild type human IL-2Rβ (Hatakeyama *et al.*, 1989a). The appearance of this 85−90 kDa band was inhibited by an excess of unlabeled IL-2. However, in the case of EL-4 transformant clones expressing M4 mutant IL-2Rβ, no specific band was detected (data not shown). Collectively, these results demonstrate that both M2 and M3 mutant IL-2Rβ can constitute an intermediate affinity IL-2R, while M4 mutant IL-2Rβ has lost its ability to bind IL-2 by itself.

Next, we asked whether above mutant IL-2Rβ molecules can generate a high affinity IL-2R, using the BAF-B03 transformant clones. BAF-B03 cells express a large number of mouse endogenous IL-2Rα which can form high affinity IL-2Rs in conjunction with the cDNA-directed human IL-2Rβ chain. Furthermore, an IL-2-induced growth signal can be transmitted via the reconstituted IL-2R (Hatakeyama *et al.*, 1989b). We selected BAF-B03 transformant clones expressing either M2, or M3 or M4 mutant IL-2Rβ at a level similar to that of wild type IL-2Rβ in clone F7 (Hatakeyama *et al.*, 1989b) as judged by Mik-β1 staining (data not shown). Using these transformants, we examined the IL-2 binding properties of the receptor as described above. As shown in Figure 2B, Scatchard plot analyses of the IL-2 binding data revealed that BAF-B03 transformant clones which express each M2 and M3 mutant IL-2Rβ displayed both high and low affinity IL-2Rs. In fact, the K_d values for the high affinity IL-2R were very similar among the clones expressing wild type and mutant IL-2Rβ. In contrast, BAF-B03 transformant clones expressing M4 mutant IL-2Rβ displayed only a low affinity IL-2R.

In summary, both M2 and M3 mutant IL-2Rβs have an ability to manifest intermediate and high affinity binding to IL-2 as shown in the wild type IL-2Rβ, but M4 mutant IL-2Rβ itself neither acts as an intermediate affinity IL-2R nor generates a high affinity IL-2 binding site with IL-2Rα.

Table II. Number of EL-4 and BAF-B03 transformants expressing each mutant IL-2Rβ recognized by Mikβ1

Name	Amino acid sequence	EL-4 transformants		BAF-B03 transformants	
		neo-resistant clones	Clones expressing IL-2Rβ	neo-resistant clones	Clones expressing IL2-Rβ
WT	W−S−P−W−S	−	−	−	−
M1	**G**−S−P−**G**−S	48	0	61	0
M2	W−**G**−P−W−**G**	56	6	60	8
M3	W−S−**A**−W−S	42	8	45	5
M4	**G**−**G**−P−W−S	46	7	50	6
M5	**S**−S−P−**S**−S	44	0	66	0

Growth signal transduction by the mutant IL-2Rβs

We next examined the ability of these mutant IL-2Rβs to transduce growth signal using the above BAF-B03 transformant clones. We first examined their IL-2 responsiveness by measuring [^{3}H]thymidine uptake and compared the uptake level with that observed for IL-3, i.e. medium containing 20% WEHI-3B culture supernatant. As shown in Figure 3, BAF-B03 transformant clones which express M3 mutant IL-2Rβ responded to recombinant human IL-2 in a concentration-dependent manner, as well as to F4 and F7 transformants expressing the wild type IL-2Rβ. Interestingly, a notable difference in the IL-2 response was detected for clones expressing M2 mutant IL-2Rβ, which carries substitutions at two conserved Ser residues within the WS motif, despite the comparable expression levels of the high affinity IL-2R (Figure 3). As expected, BAF-B03 transformant clones expressing M4 mutant IL-2Rβ hardly responded to IL-2 at concentrations between 1 pM and 1 nM. Secondly, to test whether these growth signals mediated by the mutant IL-2Rβs could be sufficient to sustain long term cell growth, we examined the increase in the number of each transformants for 10 days induced by 1 nM recombinant IL-2. BAF-B03 transformant clones which express M3 mutant IL-2Rβ (B3-1, -9, -11) could proliferate at almost the same rate as F4 and F7 transformants (Figure 4). The M2 mutant IL-2Rβ (clones B2-8, -38 and -39) also sustained cell proliferation albeit at a lower rate than wild type and M3 mutant IL-2Rβs. In fact, the increase in cell

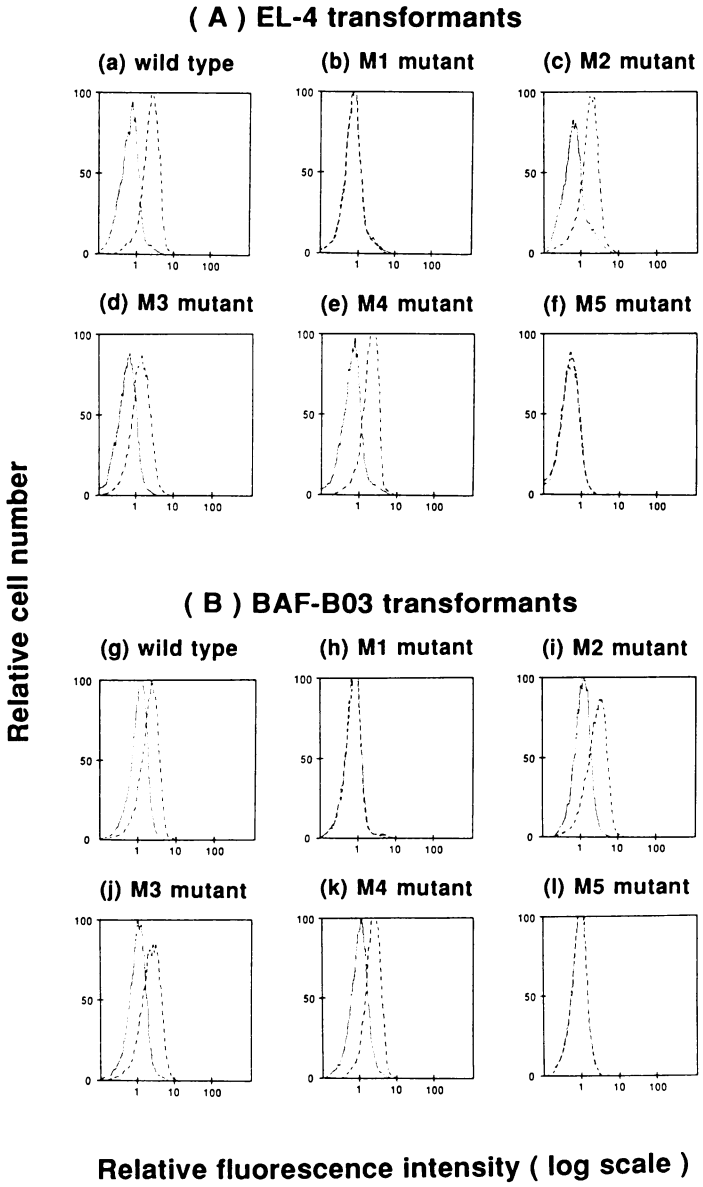

Fig. 1. FACS profile of expression of wild type and each mutant IL-2Rβ on (A) EL-4 and (B) BAF-B03 transformants. (**A**) EL-4 transformant clones expressing (a) the wild type human IL-2Rβ cDNA (clone ELβ-13), (b) M1 mutant clone E1-2, (c) M2 mutant clone E2-18, (d) M3 mutant clone E3-3, (e) M4 mutant clone E4-7 and (f) M5 mutant clone E5-5. (**B**) BAF-B03 transformant clones expressing (g) the wild type human IL-2Rβ cDNA (clone F7), (h) M1 mutant clone B1-3, (i) M2 mutant clone B2-8, (j) M3 mutant clone B3-1, (k) M4 mutant clone B4-2 and (l) M5 mutant clone B5-11. Cells were incubated with the anti-human IL-2Rβ monoclonal antibody Mik-β1 and then with FITC-conjugated goat anti-mouse IgG (dotted lines). Solid lines indicate fluorescence profile of the cells stained with FITC-conjugated goat anti-mouse IgG alone. The corresponding mRNA of each mutant IL-2Rβ was found to be expressed in all these clones at similar levels by RNA blotting analysis (data not shown).

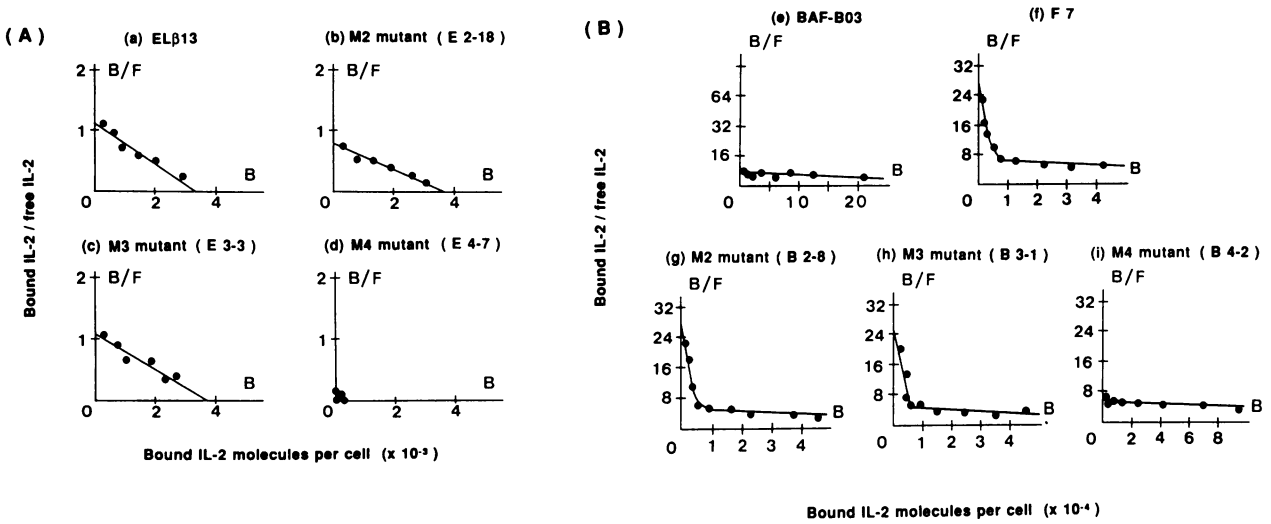

Fig. 2. Scatchard plot analysis of ^{125}I-labeled IL-2 binding to the control cells and EL-4 transformant clones (**A**) and BAF-B03 transformant clones (**B**). The number of IL-2 binding sites per cell and the receptor affinity K_d were determined by computer-assisted analysis of the IL-2 binding data. (A) (a) ELβ-13, 3200 sites of 'intermediate affinity' receptor per cell, (K_d, 3.1 nM); (b) E2-18, 3700 sites of 'intermediate affinity' receptor per cell, (K_d, 5.0 nM); (c) E3-3, 3600 sites of 'intermediate affinity' receptor per cell, (K_d, 3.4 nM); (B) (e) BAF-B03, 1 022 000 sites of 'low affinity' receptor per cell, (K_d, 20.1 nM); (f) F7, 7800 sites of 'high affinity' receptor per cell, (K_d, 27.9 pM); 820 000 sites of 'low affinity' receptor per cell, (K_d, 11.7 nM); (g) B2-8, 8500 sites of 'high affinity' receptor per cell, (K_d, 32.7 pM); 690 000 sites of 'low affinity' receptor per cell, (K_d, 13.8 nM); (h) B3-1, 7100 sites of 'high affinity' receptor per cell, (K_d, 29.6 pM); 930 000 sites of 'low affinity' receptor per cell, (K_d, 18.5 nM); (i) B4-2, 780 000 sites of 'low affinity' receptor per cell, (K_d, 13.0 nM).

number after 10 days is about one to three orders of magnitude lower (Figure 4). As expected, M4 mutant IL-2Rβ could not sustain cell proliferation.

Expression of M1 and M5 mutant IL-2Rβs in COS cells

The observation that M1 and M5 mutant IL-2Rβs were not detected on the cell surface by Mik-β1 provides two different possibilities. One is that Mik-β1 could not recognize M1 and M5 mutant IL-2Rβs, because these mutant IL-2Rβ chains have modified extracellular domains which cannot be recognized by Mik-β1. The alternative possibility is that the IL2Rβ proteins in the M1 and M5 mutants cannot be expressed on the cell surface. Such a situation might happen if the mutation resulted in a decrease of mRNA translation, increase of protein degradation, or a block in protein transport. In order to examine these possibilities, we generated a new monoclonal antibody, designated 54B-1, which specifically recognizes the intracytoplasmic domain of the human IL-2Rβ (see Materials and methods for details). Following transfection of wild type and mutant IL-2Rβ cDNA expression vectors into COS cells by the calcium phosphate method, as described above, cells were labeled with [^{35}S]methionine and [^{3}H]leucine, and then the cell lysate was immunoprecipitated with monoclonal antibody 54B-1 or Mik-β1. As shown in Figure 5A, both M1 and M5 mutant IL-2Rβs were immunoprecipitated as a 70–75 kDa protein (as was the wild type IL-2Rβ) by 54B-1, but not by Mik-β1 (data not shown).

To determine further whether M1 and M5 mutant IL-2Rβs are expressed on the cell surface, COS cells transfected with wild type and mutant IL-2Rβ cDNA expression vectors were surface labeled with ^{125}I and immunoprecipitated with 54B-1. As demonstrated in Figure 5B, radioactive bands diagnostic for M1 and M5 mutant IL-2Rβs were again detected by 54B-1. Collectively, these results suggest that both M1 and M5 mutant IL-2Rβs can be synthesized and expressed on the cell surface equally well as the wild type IL-2Rβ, but that they have lost the epitope(s) for Mik-β1.

Discussion

In this report, we examined the role of the WS motif of the human IL-2Rβ in the IL-2Rβ expression, IL-2–IL-2R interaction and signal transduction with the use of site-directed mutagenesis.

Our experiments have shown that IL-2Rβs, except M3 mutant IL-2Rβ carrying the substitution at the non-conserved Pro196 residue, were either subtly or profoundly different from the wild type IL-2Rβ with respect to ligand binding and subsequent growth signal transduction. In fact, both M1 and M5 mutants carrying the substitutions at two Trp residues (194 and 197) are synthesized and processed on the cell surface, but have lost both the Mik-β1 epitope and IL-2 binding ability, whereas M4 mutant which has one Trp194 and Ser195 substitution still retains the Mik-β1 reactivity but has lost its IL-2 binding ability. Since mutant M2, which has two Ser substitutions (195 and 198), can still bind IL-2, it is likely that the Trp194 (rather than Ser195) substitution is primarily responsible for the observed phenotype of the M4 mutant IL-2Rβ. Presumably, a similar effect could be expected by mutating the Trp197, in view of our observation that this mutation in conjunction with the Trp194 mutation (M1 and M5) gave even more profound effects than the M4 mutant. Although the Ser substitutions at 195 and 198 in the M2 mutant did not result in effects as profound as the above Trp substitutions for IL-2Rβ expression and function in BAF-B03 cells, the IL-2 response in cell growth promotion was significantly lower, indicating a certain disadvantage for such a mutant in the IL-2 signaling process. At present, the

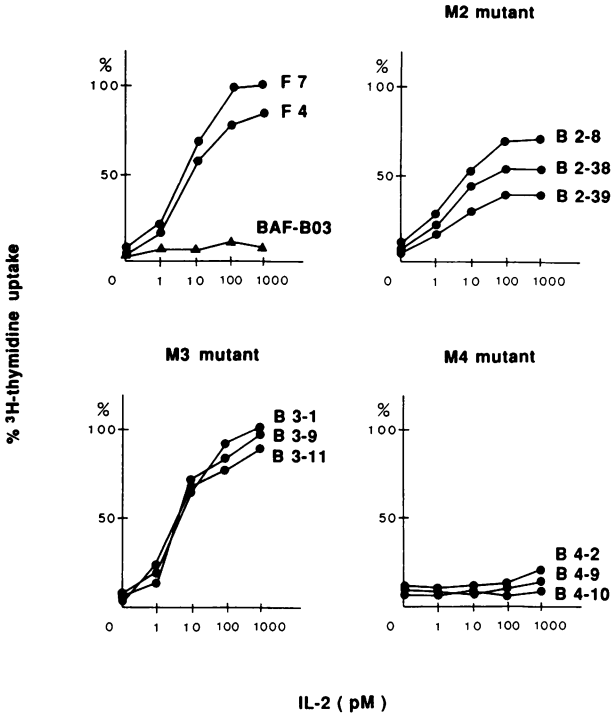

Fig. 3. IL-2 dependent growth of BAF-B03 transformants expressing wild type IL-2Rβ and each mutant IL-2Rβ. BAF-B03 and its transformant clones were independently cultured with various concentrations of human IL-2 and analyzed for their ability to incorporate [^{3}H]thymidine into DNA. The results are expressed as the percentage of incorporation of [^{3}H]thymidine in the same cells incubated with WEHI-3B conditioned medium. Data are the average of triplicate determinations.

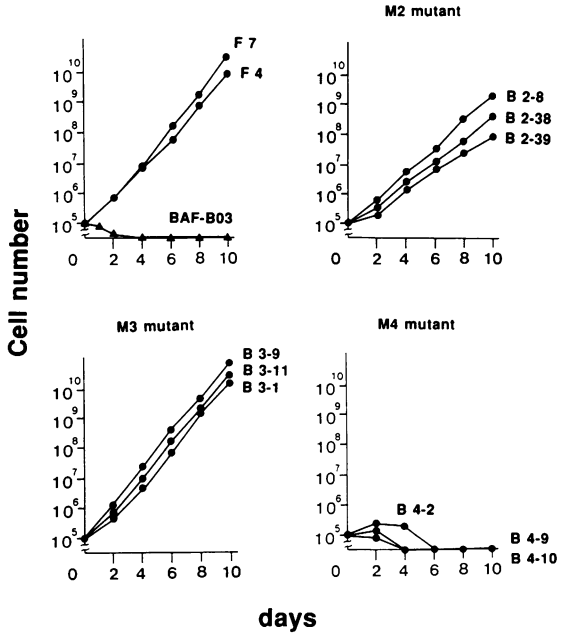

Fig. 4. Increase in cell number of the BAF-B03 transformants expressing wild type IL-2Rβ and each mutant IL-2Rβ. BAF-B03 and its transformant clones were cultured in 25 cm^2 culture flasks at a density of 1 × 10^5/ml (5 ml of culture) in RPMI-1640/10% FCS containing human recombinant IL-2 at a final concentration of 1 nM. Culture media were changed every 2 days. Viable cell counts were determined by trypan blue staining.

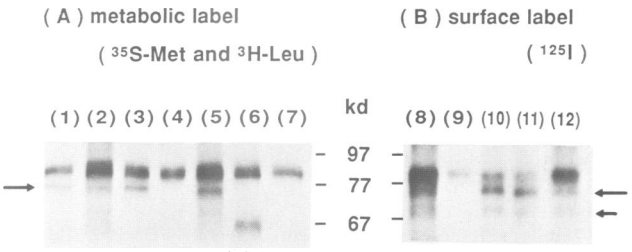

Fig. 5. Detection of wild type and mutant IL-2Rβs in (**A**) metabolically labeled COS cells and (**B**) surface labeled COS cells by immunoprecipitation with monoclonal antibody 54B-1. (A) COS cells transfected with pdKCR-M1 (lane 1), pdKCR-M3 (lane 2), pdKCR-M5 (lane 3), pdKCR vector only (lane 4) and pdKCR-β (lane 5) were metabolically labeled, and then the cell lysate was immunoprecipitated with anti IL-2Rβ monoclonal antibody 54B-1. Positions of the wild type and each mutant IL-2Rβ corresponding to 70−75 kDa, are indicated by an arrow. The slower moving band of 85−90 kDa is apparently a non-specific signal common to all samples. In fact, this band is also detected in mock-transfected COS cells (data not shown). In order to confirm that the bands indicated by an arrow correspond to IL-2Rβ, we tried to detect another set of IL-2Rβ mutants; i.e. A and ST mutant IL-2Rβs (Hatakeyama *et al.*, 1989b) in metabolically labeled COS cells with 54B-1 (lanes 6 and 7). As expected, A mutant IL-2Rβ is detected as a 62−67 kDa protein (lane 6) and ST mutant IL-2Rβ in which most of the cytoplasmic region was deleted (therefore the 54B-1 epitope must be lost), did not give any specific band (lane 7). Since the upper band is detectable again, these data support further the above view. (B) COS cells transfected with pdKCR-β (lane 8), pdKCR vector only (lane 9), pdKCR-M1 (lane 10), pdKCR-M5 (lane 11) and pdKCR-M3 (lane 12) were surface labeled, and then the cell lysate was immunoprecipitated with 54B-1. Wild type and each mutant IL-2Rβ are indicated by an arrow as described for (A). The faster moving band in (B), indicated by a shorter arrow, is likely to represent a degraded form of the IL-2Rβ, since it is not seen in the control sample (lane 9).

molecular basis for the difference is not clear. Since the receptor expression levels are not significantly lower, it is presumably not due to receptor density differences. In this regard, the result of Scatchard plot analysis in Figure 2A might be taken as an indication that the M2 mutant shows a slightly lower affinity for IL-2, and this may be reflected in the observed difference in the IL-2 signaling.

Recently the WS motif was found to be conserved in a family of cytokine receptors, which contained IL-2Rβ, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, EPO-R, GM-CSF-R, G-CSF-R and PRL-R. The modeling of secondary and tertiary structures for this cytokine receptor superfamily was reported using the sequence and structural pattern-matching methods (Bazan, 1990). The WS motif was predicted to lie on a loop between two β strands presumed to be common in these cytokine receptors, helping to form the floor of the binding crevice. Accordingly, it was suggested that the WS motif fulfils this function by constituting the main ligand binding site of these cytokine receptors. This prediction was consistent with our results that the WS motif had an important role in the formation of ligand binding site.

The WS motif is also observed in complement components C7, C8a, C8b and C9 and human thrombospondin, although its function has not yet been elucidated. In the case of human thrombospondin (Lawler and Hynes, 1986), the WS motif is located at three spots in the type I repeating sequences, which is known to be the binding region for several matrix proteins, such as type V collagen, laminin, fibronectin, fibrinogen and plasminogen. Therefore the WS motif in these molecules may be an important part in the binding or

recognition between several matrix proteins, and this mechanism might be utilized for the binding of several ligands for IL-2 and other cytokine receptors.

In several proteins, Trp residues have been demonstrated to be critical for the binding of proteins and amino acids (Hochschwender and Laursen, 1981; Blackburn *et al.*, 1984; Mogi *et al.*, 1989) or to be important for maintaining the structure of the native enzyme (Huang *et al.*, 1989). In this regard, an intriguing possibility is that the Trp residues within the IL-2Rβ WS motif may be involved in the interaction with the conjectured third chain of the IL-2R complex (Takeshita *et al.*, 1990).

In conclusion, we have shown that the conserved WS motif located just outside the membrane-spanning domain plays a critical role for the formation of ligand binding site in IL-2Rβ and the cell growth signal transduction, and this may be true for other members of the new cytokine receptor family.

Materials and methods

Plasmid construction

We first subcloned a 0.6 kb *Sac*I−*Hin*dII fragment, corresponding to the IL-2Rβ chain extracellular and transmembrane regions including the WS motif site, from pIL-2Rβ30 (Hatakeyama *et al.*, 1989a) into M13 mp18 vector. Using single stranded DNA from this recombinant vector and five synthetic oligonucleotides [(1) 5′-GAGTTCACGACCGGGAGCCCCGGGAGCCAGCCCC-3′; (2) 5′-TTCACGACCTGGGGCCCCTGGGGCCAGCCCCTGG-3′; (3) 5′-ACGACCTGGAGCGCCTGGAGCCAGC-3′; (4) 5′-GAGTTCACGACCGGGGGGCCCTGGAGCC-3′; and (5) 5′-AGTTCACGACCTCGAGCCCCTCGAGCCAGCCCCT-3′], a series of site directed mutations were introduced. The mutations were confirmed by nucleotide sequencing and the *Hin*dII−*Sac*I fragment was excised from each of the recombinant M13 vectors. Each fragment containing a specific mutation was then replaced with a *Hin*dII−*Sac*I fragment from wild type IL-2Rβ cDNA which had been inserted into the *Hin*dII−*Sac*I site of pUC19. The mutated IL-2Rβ cDNAs were excised from the pUC19 vector with *Bam*HI and inserted into the *Bam*HI site of pdKCR (Fukunaga *et al.*, 1984) and p1013 expression vectors.

Cells

COS-7 cells were maintained in Iscove's modified-DMEM (IMEM) supplemented with 10% FCS. The mouse T lymphoma line EL-4 cells were cultured in RPMI-1640 medium supplemented with 10% FCS. The mouse pro-B BAF-B03 cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 20% WEHI-3B culture supernatant as a source of IL-3.

Isolation of stable transformants expressing mutant IL-2Rβ

Each cDNA expression vector and neo-resistance gene (pST neoB) were co-transfected into EL-4 and BAF-B03 cells by electroporation as previously described (Hatakeyama *et al.*, 1989b). After selection with G418 (2 mg/ml), mutant IL-2Rβ positive cells were analyzed by flow cytometry using the mouse anti-human IL-2Rβ monoclonal antibody, Mik-β1.

DNA transfection into COS cells

Transfection of plasmids into COS cells was performed according to the calcium phosphate method as described previously (Turner *et al.*, 1990). Briefly, 1.5×10^6 COS-7 cells were plated in a 10 cm dish in IMEM supplemented with 10% FCS one day before the transfection. For each dish, 1 ml of calcium phosphate−DNA coprecipitates (20 μg of wild type and each mutant IL-2Rβ expression vector) were added together with chloroquine (final concentration 100 μM). Cells were incubated for 16 h and, after washing, incubated further for 56 h at 37°C.

IL-2 binding assay

IL-2 binding assay was performed as described previously (Hatakeyama *et al.*, 1989a). Cells (5×10^6/ml) were incubated with ^{125}I-labeled IL-2 in the presence or absence of a 250-fold excess of cold IL-2. After incubation at 4°C, for 30 min, bound and free IL-2 were isolated by centrifugation through an oil cushion and specifically bound IL-2 was determined using a γ-counter.

Cell growth assay

Cells (1×10^4) were cultured in RPMI-1640 medium with various concentrations of IL-2 for 24 h in a 96-well microculture plate. [^{3}H]thymidine (1 μCi) was then added to each well during the last 4 h of incubation and the cells were harvested. The [^{3}H]thymidine uptake was measured and compared with that of cultured cells in RPMI-1640 medium supplemented with 20% of WEHI-3B supernatant.

Antibodies

Mouse monoclonal antibody Mik-β1 (kindly provided by Dr M.Tsudo, Yunichika Chuo Hospital, Uji, Japan) recognizes human IL-2Rβ. Mouse monoclonal antibody 54B-1 was purified from the ascitic fluid of isolated hybridoma clone 54B-1 which was produced with the use of recombinant human IL-2Rβ cytoplasmic region protein. A 1.05 kb cDNA insert of the cytoplasmic region was excised from pIL-2Rβ30 by *Sac*I and *Bam*HI, inserted into *Bam*HI cleaved expression vector pET-3a using two synthetic oligonucleotide linkers (5′-GATCCAAGTTCTTTTCCCAGCTGAGCT-3′ and 5′-CAGCTGGGAAAAGAACTTG-3′). After recombinant protein was synthesized in bacteria BL-21 by T7 expression system and purified from an SDS−PAGE gel, this purified protein was injected into BALB/c mice using pristane. A murine hybridoma cell line 54B-1 was established by ELISA screening using the purified protein.

Immunoprecipitation

COS cells (2×10^7) harvested with PBS/1 mM EDTA were washed in PBS and lysed in 800 μl lysate buffer (20 mM Tris−HCl pH 8.0, 3% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 30 min at 4°C as described (Barber *et al.*, 1989). After centrifugation at 12 000 *g* at 4°C for 20 min, the cell lysate was precleared with protein A−Sepharose CL-4B which had been preincubated with normal mouse serum for 2 h at 4°C. The lysate was then incubated with antibodies (1:200 dilution of ascitic fluid) and then 20 μl protein A−Sepharose CL-4B at 4°C for 12 h. The immunoprecipitates were washed seven times with the lysate buffer and was subjected to 10% SDS−PAGE under reducing condition.

Metabolic labeling of COS cells

COS cells (2×10^7) harvested with PBS/1 mM EDTA were washed and incubated in 2 ml methionine- and leucine-free MEM supplemented with 10% dialyzed FCS for 20 min at 37°C. Then cells were resuspended in 1 ml of methionine-free MEM/10% FCS containing 0.5 mCi L-[^{35}S]methionine (EXPRE^{35}S^{35}S; NEN) and 0.5 mCi [^{3}H]leucine and incubated for 5 h at 37°C, 5% CO_2. Cells were then harvested and lysed as described above and utilized for immunoprecipitation.

Surface labeling of COS cells

COS cells (2×10^7) harvested with PBS/1 mM EDTA were washed and suspended with 50 μl PBS. Two beads of Iodo-Beads (Pierce Chem.) were washed with PBS and mixed with 20 μl PBS, 5 μl 1 M Tris−HCl pH 7.5 and 1 mCi Na^{125}I at room temperature for 5 min in the reaction vial. Then the cell suspension was added to this reaction vial and mixed occasionally at room temperature for 30 min. Cells were then harvested and lysed as described above and utilized for immunoprecipitation.

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