

Murine interleukin-2 receptor subunits differentially detected with anti-interleukin-2 monoclonal antibodies

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Received 2 May 1989

Cross-linking of radioiodinated interleukin-2 to murine CTLL-2 cells enabled detection of 70 kDa, 85 kDa and 105 kDa complexes of IL-2 and its binding proteins under the high-affinity binding condition. A series of anti-interleukin-2 monoclonal antibodies (L15, L20, L23, L34, and L61) were tested for their activity to immunoprecipitate these cross-linked complexes. L61, which had strong neutralizing activity, precipitated only the 70 kDa complex. L15, L20, and L34, which also had neutralizing activity, precipitated not only the 70 kDa complex but also the 85 kDa complex. L23, which had practically no neutralizing activity, precipitated the 105 kDa complex as well as the 85 kDa complex. These results suggest that there are at least three distinct binding sites for each receptor subunit on the interleukin-2 molecule, which are discernible by these monoclonal antibodies and that the 105 kDa complex may play a significant role in the formation of the high-affinity receptor complex and the signal transduction.

Monoclonal antibody; Interleukin-2; Interleukin-2 receptor; Crosslinking; Immunoprecipitation

1. INTRODUCTION

Interleukin-2 (IL-2) is a lymphokine produced by helper T cells, which induces proliferation and differentiation of both T [1] and B cells [2]. IL-2 exerts its effects through interaction with a specific receptor present on the surface membrane of responsive cells. There are at least two classes of IL-2 receptor (IL-2R) which differ in their affinities for IL-2 [3]. Tac antigen (p55) was initially identified as the IL-2R using anti-Tac monoclonal antibody (mAb) [4]. However, cloning of the gene which encodes this protein revealed that, in contrast to other known growth factor receptors, p55 had a very short cytoplasmic tail which seemed insufficient for the possession of any enzymatic activity [5,6]. In addition, non-lymphoid cells transfected with p55 cDNA expressed only low-affinity IL-2R and failed to transduce the signal of

IL-2 [7]. Using radioiodinated IL-2 and chemical cross-linking methodology, a non-Tac IL-2 binding protein of 70/75 kDa has been identified [8,9]. The p70/75 IL-2R expressed on LGL cells has an intermediate affinity for IL-2 in itself but is capable of transducing the signal of IL-2 [10]. It has been proposed that the p70/75 IL-2R associates with the p55 IL-2R to form the high-affinity IL-2R complex and has a domain responsible for the signal transduction of IL-2. Recently, the murine IL-2R system has been found to possess a putative third subunit of 90-100 kDa associated with p55 and/or p70/75 [11,12]. However, despite the efforts of many laboratories, little is known at present about the function of p70/75 and p90/100. The same is true for the mechanism of complex formation or interaction of IL-2 with these receptor subunits.

Monoclonal antibodies have proven their usefulness in studies on the relationship between the structure and function of ligands and their receptors. Murine hybridoma cell lines were previously established in our laboratory which produced mAbs (L15, L20, L23, L34, and L61) against

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recombinant human IL-2 (rIL-2) [13]. L61, which has strong neutralizing activity against IL-2 as determined by an inhibition experiment of CTLL-2 cell growth, has strong inhibitory activity for IL-2 binding to the high-affinity IL-2R as demonstrated by Scatchard analysis [14]. L15, L20, and L34 also inhibit high-affinity binding of IL-2 and neutralize IL-2 activity. L23 inhibits only low-affinity binding of IL-2 and has practically no neutralizing activity [14]. In the present investigation, I used chemical cross-linking methodology to analyze the high-affinity IL-2R of murine CTLL-2 cells. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses revealed the presence of p90 IL-2-binding protein as a component of the high-affinity IL-2R. Immunoprecipitation studies with the use of mAbs against rIL-2 offer evidence suggesting that p90 plays a significant role in the signal transduction of IL-2.

2. MATERIALS AND METHODS

2.1. Cell culture condition

IL-2-dependent murine CTLL-2 was kindly donated by Professor Kumagai of Tohoku University. CTLL-2 was maintained in RPMI 1640 medium (Nissui & Co.) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin G (Meiji), 100 μ g/ml kanamycin (Meiji), 10 mM Hepes, pH 6.8, and 100 U/ml rIL-2. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ in air.

2.2. mAbs

The five anti-rIL-2 mAbs used, L15, L20, L23, L34, and L61, were previously characterized [13,14]. Subclass-matched control mAbs were produced in my laboratory. All mAbs were purified from ascitic fluid by protein A-agarose affinity chromatography (Bio-Rad, MAPS II Kit).

2.3. Chemical cross-linking

[¹²⁵I]IL-2 (Amersham) was chemically cross-linked to intact CTLL-2 cells as described by Tsudo et al. [9]. Briefly, CTLL-2 cells (2×10^6) were incubated for 1 h at 4°C with 50 pM or 5 nM of [¹²⁵I]IL-2 in a total volume of 3.2 ml of culture medium (as described in section 2.1.) without rIL-2. After centrifugation (600 $\times$ g, 5 min) through a 30-ml layer of 10% sucrose in phosphate-buffered saline (PBS), the cells were suspended in 4 ml of PBS and cross-linked by adding 80 μ l of 25 mM disuccinimidyl suberate (Pierce) in dimethyl sulfoxide for 15 min at room temperature. To the reaction mixture was added 15 ml of 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 0.14 M NaCl. After 5 min, cells were collected by centrifugation (600 $\times$ g, 5 min) and solubilized by incubating for 20 min at 4°C with 1.6 ml of PBS containing 1% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride (PMSF).

2.4. Limited trypsin digestion of IL-2R

After cross-linking of [¹²⁵I]IL-2, CTLL-2 cells were directly subjected to limited proteolysis by trypsin as described elsewhere [15]. Cross-linked cells (1.6×10^6) were resuspended in 150 μ l of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.14 M NaCl. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (2.5 μ g, Worthington) was added in a volume of 25 μ l and the sample was incubated for 10 min to 2 h at 37°C. The reaction was terminated by adding 10- μ l aliquots to 100 μ l of PBS containing 1% Triton X-100 and 1 mM PMSF.

2.5. Immunoprecipitation

Triton X-100 extracts of [¹²⁵I]IL-2 cross-linked CTLL-2 cells were incubated with anti-IL-2 mAbs (final concentration was 100 μ g/ml) for 1 h at 4°C and precipitated with heat-killed formalin-fixed *S. aureus* Cowan I strain cells (SAC, Sigma) for 1 h at 4°C. In some experiments, rabbit anti-mouse IgG subclass-specific antisera (Zymed) were used. The immunoprecipitates were washed three times with MAPS II Kit binding buffer (Bio-Rad).

2.6. SDS-PAGE

SDS-PAGE was performed on cell extracts or immunoprecipitates. The samples were mixed with half the volume of 3 $\times$ concentrated SDS sample buffer (30 mM Tris-HCl, pH 6.8, 20% glycerol, 3% SDS, and 0.05% bromophenol blue) containing 10% 2-mercaptoethanol and incubated in boiling water for 5 min and subjected to SDS-PAGE using 7.5% or 10% acrylamide gel according to Laemmli [16]. [¹⁴C]Methylated standard protein mixture (Amersham) was used as the molecular marker. The gels were dried and autoradiographed at -80°C for 3-21 days on Fuji HR-A film with a Fuji Grenex G-12 intensifying screen.

3. RESULTS AND DISCUSSION

To identify the murine IL-2-binding molecules, cross-linking experiments were performed. SDS-PAGE (10% acrylamide gel) of Triton X-100 extracts was carried out after chemically cross-linking CTLL-2 with [¹²⁵I]IL-2. Under the condition permitting the binding of IL-2 to only high-affinity sites (final concentration of [¹²⁵I]IL-2 was 50 pM) [3,8], radioactivity was detected in the bands corresponding to a molecular mass of 65-70 kDa, 80-85 kDa, 105 kDa, and 170-200 kDa (fig.1A, lane b). In contrast, under the low-affinity-binding condition (final concentration of [¹²⁵I]IL-2 was 5 nM), only one band of 65-70 kDa was detected (fig.1A, lane d). These bands were not detectable in the presence of a 200-fold excess of unlabeled IL-2 (fig.1A, lanes c and e), which indicates that [¹²⁵I]IL-2 bound cell membrane components at sites specific for IL-2. Since 70 kDa and 85-90 kDa complexes observed in human cell line studies have

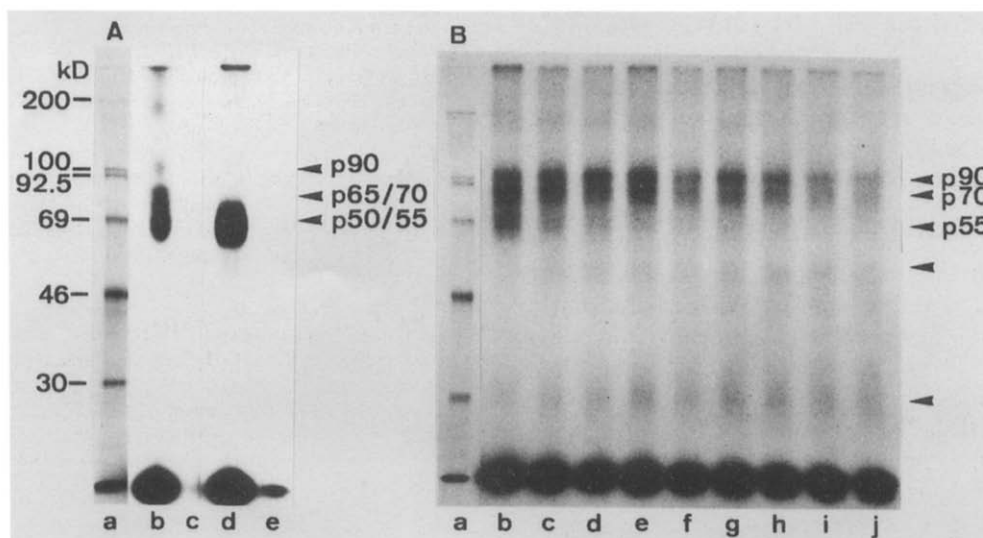


Fig.1. SDS-PAGE (10% acrylamide gel) of $[^{125}\text{I}]\text{IL-2}$ cross-linked to IL-2R. (A) Before cross-linking, CTLL-2 cells were incubated with 50 pM (lanes b and c) or 5 nM (lanes d and e) $[^{125}\text{I}]\text{IL-2}$ in the absence (lanes b and d) or presence (lanes c and e) of an excess amount of unlabeled IL-2. (B) Limited trypsin digestion of cross-linked IL-2R. After cross-linking, CTLL-2 cells were incubated with trypsin at 37°C for 0, 10, 20, 30, 40, 50, 60, 90, and 120 min (lanes b-j).

been ascribed to complexes of a single 15 kDa IL-2 molecule with p55 and p70/75 IL-2R subunit, respectively [8,9], it is very likely that the 65–70 kDa and 80–85 kDa complexes observed in the murine cell system were also complexes between IL-2 with the p50/55 or the p65/70 IL-2R subunit. The 105 kDa complex has not been described for human cell lines, but was recently found in other mouse cell lines as a complex of IL-2 and p90 IL-2R [11,12]. The bands of much higher molecular mass may be aggregates or complexes of receptor subunits as has been suggested for human [8] and mouse cell lines [11,12]. Since the human low-affinity IL-2R, p55 has been reported to be quantitatively cleaved into two fragments by trypsin [15], I tested mouse IL-2R subunits for their sensitivity to trypsin. $[^{125}\text{I}]\text{IL-2}$ cross-linked p55 IL-2R subunit was cleaved into 55 kDa and 30 kDa fragments by trypsin, but the higher molecular mass subunits p70 and p90 IL-2R were much more resistant to trypsin digestion than p55 (fig.1B, lanes b–j). These results rule out the possibility that p70 and p90 are homo- or hetero-dimers of p55. Instead, they support the idea that p70 and p90 are distinct components of IL-2R.

Anti-IL-2 mAbs, L15, L20, L34 and L61 recognized the high-affinity-binding sites of IL-2, while

L23 recognized the low-affinity-binding site of IL-2 [14]. To determine which of the $[^{125}\text{I}]\text{IL-2}$ cross-linked IL-2R subunits is recognized by each mAb, I tested the ability of these mAbs to immunoprecipitate these $[^{125}\text{I}]\text{IL-2}/\text{IL-2R}$ complexes. When SAC were used to recover the immune complex (fig.2, lanes a–e), L15, L20, and L34 precipitated both the p55/IL-2 complex and the p70/IL-2 complex (fig.2, lanes a–c). L61 precipitated only the p55/IL-2 complex (fig. 2, lane d), whereas L23 precipitated only the p70/IL-2 complex (fig.2, lane e). Similar results were obtained when rabbit anti-immunoglobulin subclass-specific antisera (anti-IgG₁ for L61, anti-IgG_{2b} for other mAbs) were used to precipitate the immune complex. Here again, only p55/IL-2 complex was precipitated by L61 (fig.2, lane g) in contrast to L15 (fig.2, lane f; L20 and L34 also precipitated both p55/IL-2 and p70/IL-2 complexes). Interestingly, L23 could precipitate not only the p70/IL-2 complex but also the p90/IL-2 complex. A faint band of p55/IL-2 was also detectable (fig.2, lane h). Control IgG₁ and IgG_{2b} mAbs did not precipitate any complex (fig.2, lanes i and j).

To analyze the higher molecular mass cross-linked protein, immunoprecipitates were run on the 7.5% acrylamide gel SDS-PAGE (fig.3). The 135

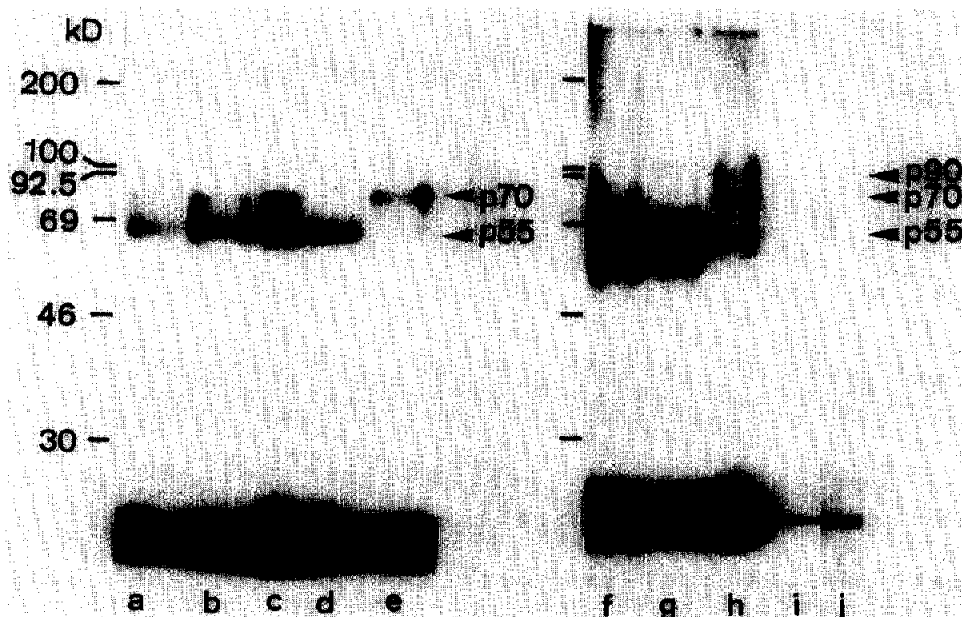


Fig.2. SDS-PAGE (10% acrylamide gel) of immunoprecipitates of cross-linked IL-2Rs by anti-IL-2 mAbs. After cross-linking, cell extracts were immunoprecipitated with mAbs (L15, lanes a and f; L20, lane b; L34, lane c; L61, lanes d and g; L23, lanes e and h; control IgG₁ mAb, lane i; control IgG_{2b} mAb, lane j) using SAC (lanes a-e) or rabbit anti-mouse immunoglobulin subclass-specific antisera (lanes f-j).

kDa complex was precipitated by L15, L20, and L34, but not by L61 or L23. Since L15, L20, and L34 were able to bind both p55/IL-2 and p70/IL-2, the most plausible explanation was that the 135

kDa band was due to the ternary complex of p55, p70, and IL-2. the 125 kDa complex was precipitated by L61, and consisted presumably of IL-2 and p55 homodimer. The reason that L15, L20, and L34 could not precipitate the 125 kDa complex is not clear at present, but it is possible that the homodimer formation conceals the binding sites for these mAbs. Further studies are needed to clarify these points.

The results obtained from the immunoprecipitation experiments suggest the following models of action for these mAbs: L61 does not affect the binding of IL-2 to p55 but inhibits the binding of IL-2 to p70 and p90; L23 does not affect the binding of IL-2 to p70 and p90 but inhibits the binding of IL-2 to p55; L15, L20, and L34 permit not only the binding of IL-2 to p55 and p70 but also the ternary complex formation between IL-2, p55 and p70. However these mAbs inhibit the binding of IL-2 to p90.

These results on the activity pattern of anti-IL-2 mAbs for binding the IL-2/IL-2R complex, coupled with our previous studies on the activity of these mAbs to inhibit both IL-2 binding to IL-2R and IL-2 dependent growth of CTLL-2 cells, reveal

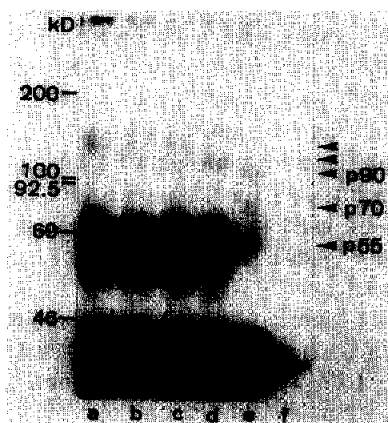


Fig.3. SDS-PAGE (7.5% acrylamide gel) of immunoprecipitates of cross-linked IL-2Rs by mAbs. After cross-linking, cell extracts were immunoprecipitated with mAbs (L15, lane a; L20, lane b; L34, lane c; L61, lane d; L23, lane e; control mAb, lane f) using rabbit anti-mouse immunoglobulin subclass-specific antisera).

the following mechanism of mAb-IL-2-2R interaction: L61, mAb with a strong neutralizing activity [13], binds IL-2 at its high-affinity-binding site [14], thereby preventing IL-2 from binding to p70 and p90, while leaving the p55-binding site intact. The unavailability of IL-2 to p70 and/or p90 leads to cell growth inhibition. L23, which has practically no neutralizing activity, binds IL-2 at its low-affinity-binding site [14], thereby preventing IL-2 from binding to p55. Since p70 and p90 are accessible to the IL-2-L23 complex, cells can grow in the presence of L23. L15, L20, and L34 bind IL-2 in such a way as to hinder its binding to p90. The resulting effect is the inhibition of high-affinity binding as revealed by Scatchard analyses [14]. p55 and p70 are freely accessible to IL-2 in the presence of these mAbs, nevertheless, they inhibit IL-2-dependent cell growth [13].

These results suggest that CTLL-2 cell growth is dependent upon the accessibility of IL-2 to p90 rather than p70. The p90/100 was recently reported as a possible high-affinity component in the murine IL-2R system [11,12]. The present results are consistent with these findings and offer further evidence for the possible role of p90 in signal transduction of IL-2 in the murine system. Recent experiments revealed that IL-2-induced phosphorylation occurred at 85–100 kDa protein [17]. It is tempting to speculate that p90 is a preferential cellular substrate for receptor-associated kinase involved in cell growth and proliferation.

Acknowledgements: I thank Dr Misao Ide for his helpful advice.

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