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Two distinct functional high affinity receptors for mouse interleukin-3 (IL-3)

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The human interleukin-3 receptor (IL-3R) is composed of an IL-3 specific α subunit (IL-3Rα) and a common β subunit (β_c) that is shared by IL-3, granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-5 receptors. In contrast to the human, the mouse has two distinct but related genes, AIC2A and AIC2B, both of which are homologous to the human β_c gene. AIC2B has proved to encode a common β subunit between mouse GM-CSF and IL-5 receptors. AIC2A is unique to the mouse and encodes a low affinity IL-3 binding protein. Based on the observation that the AIC2A protein is a component of a high affinity IL-3R, we searched for a cDNA encoding a protein which conferred high affinity IL-3 binding when coexpressed with the AIC2A protein in COS7 cells. We obtained such a cDNA (SUT-1) encoding a mature protein of 70 kDa that has weak homology to the human IL-3Rα. The SUT-1 protein bound IL-3 with low affinity and formed high affinity receptors not only with the AIC2A protein but also with the AIC2B protein. Both high affinity IL-3Rs expressed on a mouse T cell line, CTLL-2, showed similar IL-3 binding properties and transmitted a growth signal in response to IL-3. Thus, the mouse has two distinct functional high affinity IL-3Rs, providing a molecular explanation for the differences observed between mouse and human IL-3Rs.

Key words: cytokine/cytokine receptor/interleukin/hemopoiesis/gene family

Introduction

Hemopoiesis is a complex process of cell proliferation and differentiation that is regulated by a variety of glycoproteins called colony stimulating factors (CSFs) or cytokines (Metcalf, 1986; Arai *et al.*, 1990). Among them, interleukin-3 (IL-3, also known as multi-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF) and interleukin-5 (IL-5, also known as eosinophil CSF) are CSFs produced by activated T cells and appear to have important roles in hemopoiesis associated with inflammatory or immune responses (Arai *et al.*, 1990). IL-3 and GM-CSF bound to their high affinity receptors induce phosphorylation of a similar set of proteins (Isfort and Ihle, 1990; Kanakura *et al.*, 1990) and exhibit similar biological activities on a wide variety of lineage-committed hemopoietic cells as well as more primitive hemopoietic progenitors (Metcalf, 1986; Arai *et al.*, 1990). In human hemopoietic cells exhibiting both receptors on their surfaces, cross-competition of binding between IL-3 and GM-CSF has been well documented (Gesner *et al.*, 1988; Eliott *et al.*, 1989; Park *et al.*, 1989; Budel *et al.*, 1990; Taketazu *et al.*, 1991). Although IL-5 stimulates only B cells and eosinophils (Takatsu *et al.*, 1988), it also induces phosphorylation of a similar set of proteins to those induced by IL-3 or GM-CSF (Murata *et al.*, 1990) and the binding of IL-5 is also inhibited by IL-3 or GM-CSF on some human cells (Lopez *et al.*, 1990). The cross-reactivity between IL-3, GM-CSF and IL-5 occurs at the receptor level: the high affinity receptors for IL-3, GM-CSF and IL-5 are composed of a ligand-specific α subunit and a common β subunit [β_c, originally designated as KH97 (Hayashida *et al.*, 1990)] which cannot bind the cytokines by itself. Cross-competition occurs by competition for β_c by the three distinct ligand-specific α subunits (Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991).

In contrast to the human receptors, no such cross-competition of binding among IL-3, GM-CSF and IL-5 has been observed in the mouse (Walker *et al.*, 1985; unpublished observation). Two classes of binding sites for mouse IL-3 (mIL-3) have been found on mouse IL-3-dependent cell lines: one is a high affinity binding site (K_d = 50−500 pM) with a low rate of dissociation (May and Ihle, 1986; Nicola and Peterson, 1986; Park *et al.*, 1986; Schreurs *et al.*, 1990) and the other is a low affinity binding site (K_d = 5−20 nM) with a high rate of dissociation (Schreurs *et al.*, 1990). An anti-mIL-3R monoclonal antibody, anti-Aic2 (Yonehara *et al.*, 1990), led us to clone two highly homologous genes, AIC2A and AIC2B, that encode proteins of 120−130 kDa (Itoh *et al.*, 1990; Gorman *et al.*, 1990). Despite a high degree of sequence homology between them (91% identical at the amino acid level), only the AIC2A protein has a capacity to bind mIL-3 with low affinity. The IL-3 binding characteristics of the AIC2A protein are quite similar to those of the low affinity binding site on mouse hemopoietic cell lines which display a fast rate of dissociation (Itoh *et al.*, 1990; Schreurs *et al.*, 1990). The AIC2B protein does not bind any cytokines, including IL-3, IL-5 and GM-CSF, and does not form a high affinity IL-3R with the AIC2A protein (Gorman *et al.*, 1990). AIC2A and AIC2B are members of the recently identified cytokine receptor family and have two repeats of a conserved cytokine receptor motif in their extracellular domains (Itoh *et al.*, 1990; Gorman *et al.*, 1990). Interestingly, AIC2A and AIC2B have extensive sequence homology to β_c of the human IL-3 (hIL-3), IL-5 (hIL-5) and GM-CSF (hGM-CSF) receptors (Hayashida *et al.*, 1990). In fact, the AIC2B protein has proved to be a common β subunit of both mouse GM-CSF (mGM-CSF) and IL-5 (mIL-5) receptors (Devos *et al.*, 1990; Kitamura *et al.*, 1991a; Shanafelt *et al.*, 1991; Takaki *et al.*, 1991).

Two lines of evidence suggest that the AIC2A protein is a component of a high affinity mIL-3R: (i) the purified high

affinity mIL-3R contains with the AIC2A protein (Schreurs *et al.*, 1991) and (ii) a monoclonal antibody, 9D3, which specifically binds to the AIC2A protein but not to the AIC2B protein, significantly inhibited high affinity mIL-3 binding (Ogorochi *et al.*, 1992). However, these results do not exclude the possibility that the AIC2B protein, as well as the AIC2A protein, is involved in the formation of a high affinity mIL-3R. In contrast to the mouse system, the human system does not seem to have an AIC2A-like IL-3 binding protein, because only one type (β_c) of cDNA or genomic DNA was isolated by hybridization using the mouse AIC2A cDNA (Hayashida *et al.*, 1990) and because human hemopoietic cells have only one type of high affinity hIL-3 binding site consisting of the hIL-3R α subunit (IL-3Rα) and β_c (Kitamura *et al.*, 1991b).

To account for the difference between mouse and human, several possibilities have been suggested (Kitamura *et al.*, 1991b; Nicola and Metcalf, 1991). One possibility is that AIC2A is a unique β subunit for the high affinity mIL-3R and that AIC2B is not involved in the high affinity IL-3R. A second possibility is that both AIC2A and AIC2B independenty form high affinity mIL-3Rs with a putative mIL-3R α subunit. A third possibility is that the high affinity mIL-3R consists of AIC2A, AIC2B and a putative mIL-3Rα. In order to examine these possibilities, we attempted to isolate a putative mIL-3Rα cDNA and to reconstitute a high affinity mIL-3R. Based on the hypothesis that AIC2A is a β subunit of a high affinity mIL-3R, we have designed an expression cloning strategy and isolated a cDNA encoding mIL-3Rα. We demonstrate that mIL-3Rα is capable of forming functional high affinity mIL-3Rs with either AIC2A or AIC2B.

Results

Expression cloning of the murine IL-3 receptor α subunit cDNA

The human high affinity IL-3R has been found to consist of an IL-3 specific α subunit (IL-3Rα) and a β_c which is shared by GM-CSF and IL-5 receptors (Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991), suggesting that the mouse high affinity IL-3R also requires two subunits. As the low affinity mIL-3 binding protein AIC2A appears to be a component of a high affinity mIL-3R (Schreurs *et al.*, 1991; Ogorochi *et al.*, 1992), we considered the possibility that AIC2A and a putative mIL-3Rα form a high affinity IL-3R. We were unable to isolate a mIL-3Rα cDNA by hybridization with the hIL-3Rα cDNA as a probe (unpublished result), so we designed an expression cloning strategy using COS7 cells whereby small pools of a cDNA library were cotransfected together with the AIC2A cDNA and the transfected COS7 cells were screened for high affinity mIL-3 binding. However, because COS7 cells expressing AIC2A alone bound a significant amount of ^{125}I-labeled mIL-3 even at a low concentration (200 pM) of [^{125}I]mIL-3 that detects mainly high affinity binding, we modified the binding assay: after binding of [^{125}I]mIL-3 to COS7 cells at 4°C, cells were incubated with an excess of unlabeled mIL-3 to remove the [^{125}I]mIL-3 bound to the low affinity sites because mIL-3 rapidly dissociates from the low affinity sites (Schreurs *et al.*, 1990, 1991).

Using an SV40-based mammalian expression vector, we constructed a cDNA library from the mouse myeloid cell line B6SUtA which expresses a relatively large number of high affinity mIL-3Rs. The cDNA library was divided into pools of ~100 clones and each cDNA pool (1 μg) was cotransfected with the AIC2A cDNA (0.1 μg) into COS7 cells. After screening 500 pools we identified one pool which reproducibly gave a significantly higher binding (1050 c.p.m.) than COS7 cells transfected with the AIC2A cDNA alone (600 c.p.m.). The positive pool was divided into smaller pools and the binding assays were repeated. After several cycles of rescreening a single positive clone (pSUT-1) was isolated. COS7 cells cotransfected with the AIC2A cDNA (0.5 μg) and pSUT-1 (0.5 μg) bound 19 800 c.p.m., whereas COS7 cells transfected with AIC2A alone (0.5 μg) or pSUT-1 alone (0.5 μg) bound 2084 c.p.m. or 614 c.p.m. of [^{125}I]mIL-3, respectively.

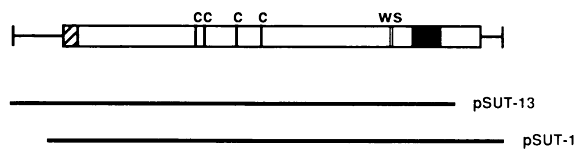
Deduced structure of the SUT-1 protein

We screened 10^6 colonies from the original B6SUtA cDNA library by using the 1.3 kb SUT-1 cDNA as a probe and obtained 13 additional clones. The cDNA inserts of pSUT-1 and one of these additional clones (pSUT-13) were sequenced (Figure 1). The two sequences were found to be identical within an overlapping region and pSUT-13 had 98 more base pairs than the 5′ end of the pSUT-1 insert (Figure 1A). No insertions or deletions were found in the cDNAs of the remaining clones analyzed by polymerase chain reactions using SUT-1 specific oligonucleotides as primers (data not shown). A long open reading frame was followed by the 3′ noncoding region containing a poly(A) addition signal (Figure 1B), although a poly(A) tract was not found in any clone examined.

The open reading frame encodes a polypeptide of 396 amino acids including a putative signal sequence of 16 amino acid residues. The mature protein consists of 380 amino acids with a calculated M_r of 41 215 daltons. The difference between the predicted polypeptide size (41 kDa) and the M_r (60−70 kDa) estimated from the cross-linking experiment (see below and Figure 6, lane 10) is probably due to post-translational modifications which mainly include attachment of sugar chains to some of the five potential *N*-glycosylation sites found in the extracellular domain (Figure 1B). A hydropathy plot (Kyte and Doolittle, 1982) predicts a single membrane-spanning segment of 24 amino acid residues extending from Thr332 to Trp355 (Figure 1B). The extracellular domain (Ser17 to Lys331) contained common features of the recently identified cytokine receptor superfamily that has four conserved cysteine residues and a unique WS motif near the transmembrane domain (Bazan, 1990; Miyajima *et al.*, 1992). The small cytoplasmic domain (Arg356 to Ala396) contains a proline-rich stretch (Pro-Pro-Ile-Pro) which is also found in the α subunits of hIL-3, hGM-CSF, mIL-5 and hIL-5 receptors (Kitamura *et al.*, 1991b; Gearing *et al.*, 1989; Takaki *et al.*, 1990; Tavernier *et al.*, 1991). Any known consensus sequences for known signaling molecules like tyrosine kinases were not identified.

The overall structure of the SUT-1 protein is similar to those of the recently cloned α subunits of human IL-3R (Kitamura *et al.*, 1991b), human GM-CSFR (Gearing *et al.*, 1989) and mouse IL-5R (Takaki *et al.*, 1990). There are several short stretches of conserved amino acid sequences between the mIL-3Rα and the human IL-3Rα including the WS motif as shown in Figure 2. However, the overall sequence identity between hIL-3Rα and SUT-1 was only

A



B

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                         1 GCCGTGGGCG CCACTCAGGA ACCAGAAGCA TCGTGAAGGG GTCGGGATCA GGAGGTGGAG TCGGCTTGCG CCCCGGCCCC GCCCCTCACG   90

CCCCGGCCCC GCCCCCAGGC CTCCGAGGCT CCAGCCCCGC CCTCCTATC ATG GCC GCC AAC CTG TGG CTC ATC CTG GGG CTC CTG GCA TCC CAC TCT  187
                                                    1 Met Ala Ala Asn Leu Trp Leu Ile Leu Gly Leu Leu Ala Ser His Ser   16

TCA GAT CTG GCT GCG GTC CGT GAG GCT CCA CCC ACT GCT GTG ACC ACG CCC ATC CAG AAC CTG CAT ATC GAC CCC GCC CAC TAC ACA CTG  277
Ser Asp Leu Ala Ala Val Arg Glu Ala Pro Pro Thr Ala Val Thr Thr Pro Ile Gln Asn Leu His Ile Asp Pro Ala His Tyr Thr Leu   46

AGC TGG GAC CCC GCC CCC GGC GCT GAC ATC ACC ACA GGG GCT TTT TGC AGG AAG GGC AGG GAC ATC TTT GTC TGG GCT GAC CCC GGG CTT  367
Ser Trp Asp Pro Ala Pro Gly Ala Asp Ile Thr Thr Gly Ala Phe Cys Arg Lys Gly Arg Asp Ile Phe Val Trp Ala Asp Pro Gly Leu   76

GCC CGC TGC TCA TTC CAG TCG CTC TCC CTC TGC CAT GTG ACC AAC TTC ACC GTC TTC CTG GGG AAG GAC AGG GCT GTG GCA GGA TCC ATC  457
Ala Arg Cys Ser Phe Gln Ser Leu Ser Leu Cys His Val Thr Asn Phe Thr Val Phe Leu Gly Lys Asp Arg Ala Val Ala Gly Ser Ile  106

CAG TTC CCC CCA GAT GAT GAT GGC GAC CAC GAG GCC GCA GCC CAG GAC CTG CGC TGC TGG GTG CAT GAG GGG CAG CTC AGC TGC CAA TGG  547
Gln Phe Pro Pro Asp Asp Asp Gly Asp His Glu Ala Ala Ala Gln Asp Leu Arg Cys Trp Val His Glu Gly Gln Leu Ser Cys Gln Trp  136

GAG CGG GGT CCG AAA GCA ACG GGT GAC GTG CAC TAC CGG ATG TTC TGG CGT GAT GTG CGG CTC GGT CCT GCC CAC AAC CGC GAG TGT CCA  637
Glu Arg Gly Pro Lys Ala Thr Gly Asp Val His Tyr Arg Met Phe Trp Arg Asp Val Arg Leu Gly Pro Ala His Asn Arg Glu Cys Pro  166

CAC TAC CAC AGC CTT GAT GTC AAC ACC GCT GGC CCC GCC CCT CAT GGT GGC CAT GAA GGC TGC ACC CTT GAC CTC GAC ACT GTC CTT GGC  727
His Tyr His Ser Leu Asp Val Asn Thr Ala Gly Pro Ala Pro His Gly Gly His Glu Gly Cys Thr Leu Asp Leu Asp Thr Val Leu Gly  196

TTC ACC CCC AAC AGC CCT GAC CTT GTC CCC CAG GTC ACC ATC ACC GTC AAC GGC AGT GGC CGC GCT GGC CCC GTG CCC TGC ATG GAC AAC  817
Ser Thr Pro Asn Ser Pro Asp Leu Val Pro Gln Val Thr Ile Thr Val Asn Gly Ser Gly Arg Ala Gly Pro Val Pro Cys Met Asp Asn  226

ACT GTG GAC CTG CAG CGC GCA GAG GTC CTG GCC CCG CCC ACG TTG ACA GTG GAG TGC AAC GGC TCT GAG GCC CAT GCC CGA TGG GTC GCG  907
Thr Val Asp Leu Gln Arg Ala Glu Val Leu Ala Pro Pro Thr Leu Thr Val Glu Cys Asn Gly Ser Glu Ala His Ala Arg Trp Val Ala  256

CGG AAC AGA TTC CAC CAT GGC CTC CTT GGG TAC ACC CTG CAG GTC AAC CAG AGC TCC CGC TCA GAG CCA CAG GAA TAC AAT GTC TCC ATC  997
Arg Asn Arg Phe His His Gly Leu Leu Gly Tyr Thr Leu Gln Val Asn Gln Ser Ser Arg Ser Glu Pro Gln Glu Tyr Asn Val Ser Ile  286

CCC CAC TTC TGG GTC CCC AAC GCT GGT GCC ATC TCC TTC CGG GTC AAG TCG AGG TCA GAG GTT TAC CCT CGG AAG CTC AGC AGC TGG AGC 1087
Pro His Phe Trp Val Pro Asn Ala Gly Ala Ile Ser Phe Arg Val Lys Ser Arg Ser Glu Val Tyr Pro Arg Lys Leu Ser Ser Trp Ser  316

GAA GCC TGG GGC CTC GTC TGC CCC CCA GAG GTG ATG CCT GTG AAG ACA GCC TTG GTG ACT TCA GTG GCT ACG GTG CTG GGG GCA GGG CTC 1177
Glu Ala Trp Gly Leu Val Cys Pro Pro Glu Val Met Pro Val Lys Thr Ala Leu Val Thr Ser Val Ala Thr Val Leu Gly Ala Gly Leu  346

GTG GCA GCT GGG CTC CTG CTG TGG TGG AGG AAG TCG CTG CTC TAC CGC CTG TGC CCA CCC ATT CCA CGC CTG CGC CTG CCC TTG GCA GGG 1267
Val Ala Ala Gly Leu Leu Leu Trp Trp Arg Lys Ser Leu Leu Tyr Arg Leu Cys Pro Pro Ile Pro Arg Leu Arg Leu Pro Leu Ala Gly  376

GAG ATG GTC GTG TGG GAA CCA GCT CTT GAA GAC TGC GAG GTG ACG CCT GTG ACA GAC GCC TGA GAACTGTGTG GGGGCTGGGG CATAGGGAGC GG 1362
Glu Met Val Val Trp Glu Pro Ala Leu Glu Asp Cys Glu Val Thr Pro Val Thr Asp Ala  *  396

TGGGCGGGGT CAATAAAGTG CGTGGATTCC TG  1394
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Fig. 1. Structure of the SUT-1 cDNA and the SUT-1 protein. (**A**) Schematic representation of two cDNAs for SUT-1. The box shows the open reading frame. The hatched and black boxes represent the signal sequence and the transmembrane region respectively. Positions of the four cysteine residues and the WS motif, which are common features of the cytokine receptor family, are indicated. (**B**) Nucleotide sequence and predicted amino acid sequence of the SUT-1 cDNA. Numbers at the right side indicate positions of nucleotides (above) or amino acids (below). The thick underlining indicates a predicted signal sequence and a putative transmembrane region. The conserved four cysteine residues and the WS sequence are boxed. Five potential *N*-glycosylation sites (Asn-X-Ser/Thr) and a poly(A) addition signal (AATAAA) are underlined.

47% at the nucleotide level and 30% (52% similarity) at the amino acid level, respectively. This low extent of homology between the mouse and human proteins is similar to that of mIL-3 and hIL-3 (29% identity) (Yang *et al.*, 1986). A search of data bases did not reveal any sequence identical to the SUT-1 DNA nor the SUT-1 protein. The amino acid sequence of SUT-1 is of 47% similarity to the mIL-5Rα.

Expression of the SUT-1 mRNA

The high affinity mIL-3 receptors are known to exist on various types of mouse hemopoietic cells including mast cells, myeloid cells and proB cells (Schreurs *et al.*, 1990). To examine the expression of mRNA corresponding to the SUT-1 cDNA in IL-3-dependent cell lines, a Northern hybridization was performed using the 1.3 kb insert of pSUT-1 as a probe. A 1.8 kb mRNA was detected in all IL-3-responsive cell lines examined including mast cells (MC/9 and PT18), a myeloid line (B6SUtA) and a proB line (Ba/F3) (Figure 3). In contrast, the SUT-1 mRNA was not detected in IL-3-nonresponsive cell lines including a B cell line (CH12), a T cell line (CTLL-2) and a fibroblastic line (NIH3T3), although a very small amount of the SUT-1

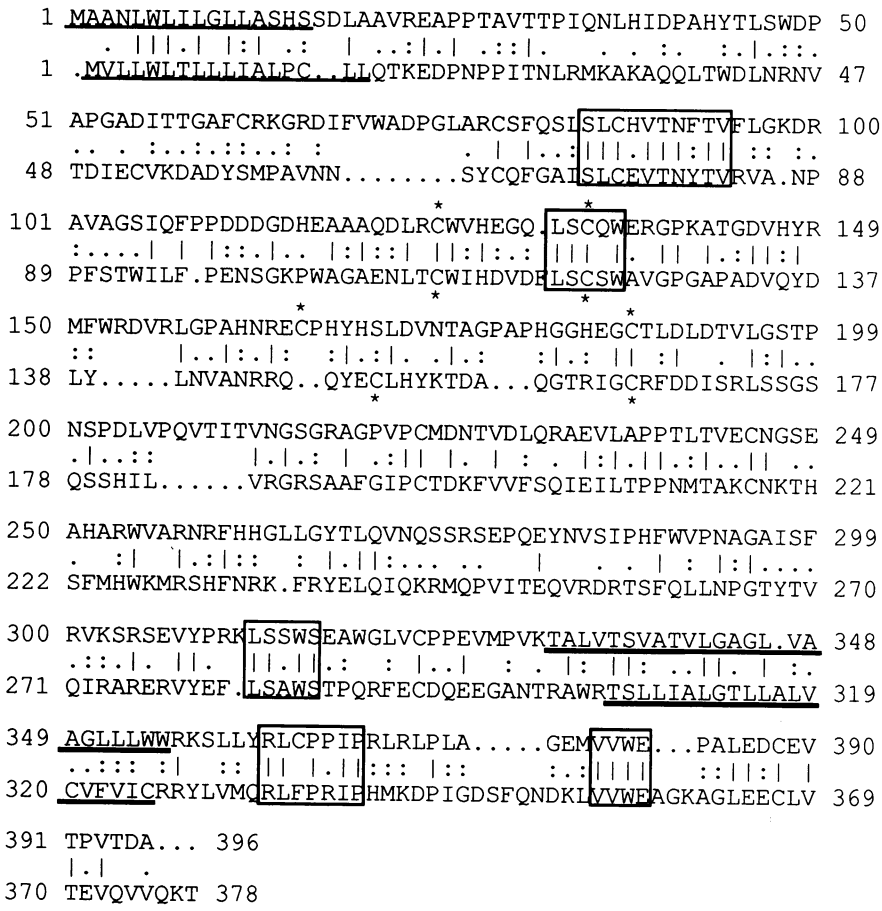

Fig. 2. Sequence comparison of the SUT-1 (mIL-3Rα) (upper) and the hIL-3Rα (lower). The numbers in the left and right columns indicate the positions of amino acids. Sequences which appear to be conserved between the two are boxed. The signal sequences (thin lines) and the transmembrane regions (thick lines) are underlined. The four cysteine residues characteristic among the cytokine receptor family are marked (*). The extent of amino acid similarity between the two proteins is shown as vertical lines (identical), two dots (conservative) and one dot (neutral) according to Gibskov and Burgess (1986). The sequence data reported here have been deposited in the EMBL Data Library under the accession number X64534.

transcript was detected in a macrophage cell line (P388D1). This result indicated that expression of the SUT-1 mRNA seems to be restricted to IL-3-responsive cells. Expression of the β subunits (AIC2A and AIC2B) mRNAs was also detected in all IL-3-responsive cells examined (Itoh *et al.*, 1990; Gorman *et al.*, 1990), as well as in IL-3 nonresponsive cells such as CH12 and P388 (Gorman *et al.*, 1990).

Formation of high affinity mIL-3Rs by coexpression of SUT-1 with either AIC2A or AIC2B in COS7 cells

As human β_c is involved in the formation of high affinity receptors for IL-3, GM-CSF and IL-5, and as AIC2B has proved to be a common β subunit between mGM-CSF and mIL-5 receptors (Kitamura *et al.*, 1991a; Shanafelt *et al.*, 1991; Takaki *et al.*, 1991; Devos *et al.*, 1991), we examined the possibility that AIC2B also forms a high affinity mIL-3R. COS7 cells were transiently transfected with SUT-1, AIC2A and AIC2B cDNAs and binding of [^{125}I]mIL-3 was examined (Table I). Scatchard analysis of the binding data showed that COS7 cells transfected with the AIC2A cDNA alone bound [^{125}I]labeled mIL-3 with low affinity (K_d = 7 nM) as previously described (Itoh *et al.*, 1990). Cells transfected with pSUT-1 alone exhibited a second low affinity binding site (K_d = 45 nM) for mIL-3 which has lower affinity than AIC2A. Coexpression of AIC2A and SUT-1 resulted in both high (K_d = 270 nM) and low affinity (K_d = 32 nM) binding sites for mIL-3 (Table I). The low affinity site observed in these cells may be a mixture of the two low affinity binding sites, AIC2A and SUT-1. Interestingly, coexpression of AIC2B and SUT-1 also resulted in a high affinity (K_d = 443 pM) and a low affinity (K_d = 40 nM) binding site (Table I). As cells transfected with the AIC2B cDNA alone did not show any specific IL-3 binding, as demonstrated before (Gorman *et al.*, 1990; Wang *et al.*, 1992), this low affinity site is probably due to binding to the SUT-1 protein. The dissociation constant of the high affinity mIL-3R generated by coexpression of SUT-1 with AIC2A was almost identical to that of SUT-1 with AIC2B and their K_d values were similar to those of hemopoietic cells (50−500 pM) (Schreurs *et al.*, 1990) (Table I). These results strongly demonstrate that pSUT-1 encodes a low affinity mIL-3 binding protein, which is distinct from AIC2A, and that the SUT-1 protein forms high affinity IL-3Rs with either AIC2A or AIC2B.

Although the SUT-1 protein has only weak sequence homology to hIL-3Rα, the results indicate that SUT-1 is a mouse IL-3R α subunit (mIL-3Rα). As AIC2B, a common β subunit between mouse GM-CSF and IL-5 receptors, is also involved in the formation of a high affinity mIL-3R, AIC2B is functionally identical to the human common β subunit, β_c. In contrast, AIC2A is not involved in the formation of high affinity mouse receptors for GM-CSF and IL-5 (Devos *et al.*, 1991; Kitamura *et al.*, 1991; Takakai *et al.*, 1991), but can function as a β subunit specific for

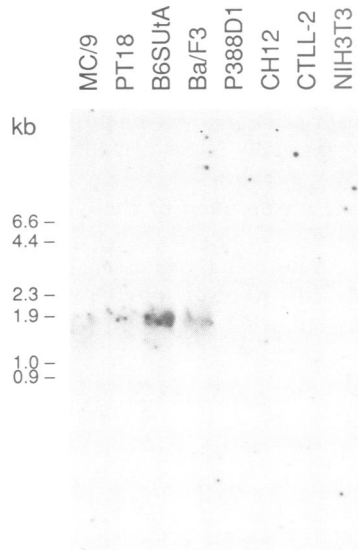

Fig. 3. Expression of the SUT-1 mRNA. Poly(A)$^+$ RNA samples (2 μg each) from various mouse cell lines were electrophoresed on a 1.3% agarose gel, transferred to a nitrocellulose membrane and hybridized with the ^{32}P-labeled SUT-1 cDNA. Positions of size standards are shown on the left side.

mIL-3R. Therefore, we designate AIC2A and AIC2B as β_{IL3} and β_c, respectively.

Binding characteristics of the high affinity mIL-3Rs reconstituted on CTLL-2 cells

To characterize the properties of the two high affinity mIL-3Rs further, the cDNAs were stably expressed in an IL-2 dependent mouse T cell line, CTLL-2. CTLL/AS and CTLL/BS are CTLL-2 transfectants expressing SUT-1 with either AIC2A or AIC2B, respectively, and both exhibited high affinity binding sites for mIL-3 (Figure 4A and B, Table I). CTLL/A, a transfectant expressing AIC2A alone, showed only a low affinity binding site (Figure 4C and Table I) and a CTLL transfectant expressing SUT-1 alone (CTLL/S) had a lower binding affinity for mIL-3 (Figure 4D and Table I). These binding properties were similar to those observed in COS7 cells as described above (Table I). The low affinity binding sites of CTLL/AS and CTLL/BS were barely detectable (Figure 4), probably because the expression level of SUT-1 is only slightly higher than that of AIC2A or AIC2B. As IL-3-responsive hemopoietic cell lines express both AIC2A and AIC2B simultaneously (Gorman *et al.*, 1990), we established a transfectant (CTLL/ABS-31 and 32) expressing AIC2A, AIC2B and SUT-1 and examined the binding properties. High affinity binding sites reconstituted on CTLL/ABS-31 (K_d = 352 pM) and CTLL/ABS-32 (K_d = 484 pM) were almost identical to those of PT18 cells (K_d = 381 pM) and B6SUtA cells (K_d = 418 pM) (Figure 4 and Table I). Although the apparent K_d values of CTLL/ABS cells seemed to be slightly higher than those of CTLL/AS or CTLL/BS (Table I), all these values are within the variation of the K_d values of high affinity mIL-3R previously reported (Schreurs *et al.*, 1990).

To determine whether the high affinity IL-3R composed of SUT-1 and AIC2A is different from that composed of SUT-1 and AIC2B, the binding kinetics of [^{125}I]mIL-3 to each reconstituted high affinity receptor were analyzed using

Table I. Binding profiles of mIL-3 on transfected COS7 cells, CTLL-2 transfectants and IL-3-responsive hemopietic cell lines

Cell	Expression of mIL-3 receptor[a]	
	High affinity K_d (sites)	Low affinity K_d (sites)
COS7/AIC2A	–[b]	7 nM
COS7/AIC2B	–	–
COS7/SUT-1	–	45 nM
COS7/AIC2A + SUT-1	270 pM	32 nM
COS7/AIC2B + SUT-1	443 pM	40 nM
CTLL-2	–	–
CTLL/A	–	14 nM (5000)
CTLL/B	–	–
CTLL/S	–	61 nM (22 000)
CTLL/AS	154 nM (7700)	>20 nM (<8000)[c]
CTLL/BS	86 nM (5400)	>20 nM (<8000)[c]
CTLL/ABS-31	352 nM (19 000)	–
CTLL/ABS-32	484 pM (26 000)	–
PT18	381 pM (11 000)	>10 nM (<15 000)[c]
B6SUtA	418 pM (14 000)	–

[a]Dissociation constants and binding sites of each cell line were determined using the LIGAND program.
[b]Not detected.
[c]The LIGAND program did not give definitive affinity.

CTLL/AS and CTLL/BS cells (Figure 5). IL-3 rapidly associated with the receptor and reached a saturation level within 2 h in CTLL/AS, CTLL/BS and PT18 cells at 4°C, all in a similar manner. The bound [^{125}I]mIL-3 was released with similar slow kinetics from CTLL/AS, CTLL/BS and PT18 cells after addition of unlabeled mIL-3. Thus, the binding properties of the two reconstituted high affinity receptors were indistinguishable and seemed to be identical to that on the hemopoietic cells.

Chemical cross-linking of [^{125}I]mIL-3

Chemical cross-linking experiments were performed to characterize IL-3 binding proteins by using the CTLL-2 transfectants, PT18 and B6SUtA cells. In agreement with previous observations that cross-linking of [^{125}I]mIL-3 to hemopoietic cells generates 160, 140 and 80–90 kDa proteins cross-linked with mIL-3 (20 kDa) (May and Ihle, 1986; Nicola and Peterson, 1986; Park *et al.*, 1986; Sorensen *et al.*, 1986; Schreurs *et al.*, 1990), the bands with the same molecular weight were detected in PT18 and B6SUtA cells (Figure 6, lanes 6–9). Cross-linked proteins of 160, 140 and 80–90 kDa were also detected in CTLL/AS cells, while CTLL/BS cells showed bands of 150 and 90–90 kDa (Figure 6, lanes 2 and 3). All of these bands were diminished when an excess amount of unlabeled mIL-3 was added (data not shown) and were not detected in CTLL-2 parental cells (Figure 6, lane 1), indicating that they represent IL-3 binding proteins. As described previously, AIC2A generates two cross-linked proteins of 160 and 140 kDa (Itoh *et al.*, 1990). The reason for generating the two bands by AIC2A is unknown. CTLL/AS showed a broad band at 80–90 kDa in addition to these two bands. This broad band was also detected when the SUT-1 protein alone was highly expressed in COS7 cells (Figure 6, lanes 10 and 11). Subtraction of the M_r of mIL-3 resulted in an estimated M_r of 60–70 kDa which is consistent with the M_r of the hIL-3Rα. These results indicate that the 80–90 kDa band represents the SUT-1 protein crossed-linked with IL-3. This

is also consistent with the cross-linking pattern of CTLL/BS which showed the 150 kDa band and the 80–90 kDa band. The 150 kDa band seems to represent the AIC2B protein (130 kDa) cross-linked with IL-3, which is consistent with the fact that the M_r of AIC2B is slightly higher than that of AIC2A (120 kDa). Interestingly, cross-linking of mIL-3 to CTLL/ABS-31 and CTLL/ABS-32 showed a band at 160 kDa and a broad band of 140–150 kDa (Figure 6, lanes 4 and 5). The broad band seemed to be a mixture of 140 and 150 kDa bands representing AIC2A and AIC2B. A similar broad band of 140–150 kDa was also detected in PT18 cells and B6SUtA cells (Figure 6, lanes 6–9). These observations suggest that IL-3 binds to both types of high affinity receptors when they are coexpressed on the same cell. However, because it is difficult to estimate the exact expression levels of AIC2A and AIC2B in these transfectants or in hemopoietic cells, it is still not possible to tell whether one type of high affinity receptor is formed preferentially.

IL-3-dependent proliferation of the CTLL-2 transfectants

As the CTLL-2 transfectants expressing the α and β subunits of the hGM-CSF receptor proliferate in response to hGM-CSF (Kitamura *et al.*, 1991a), we examined whether

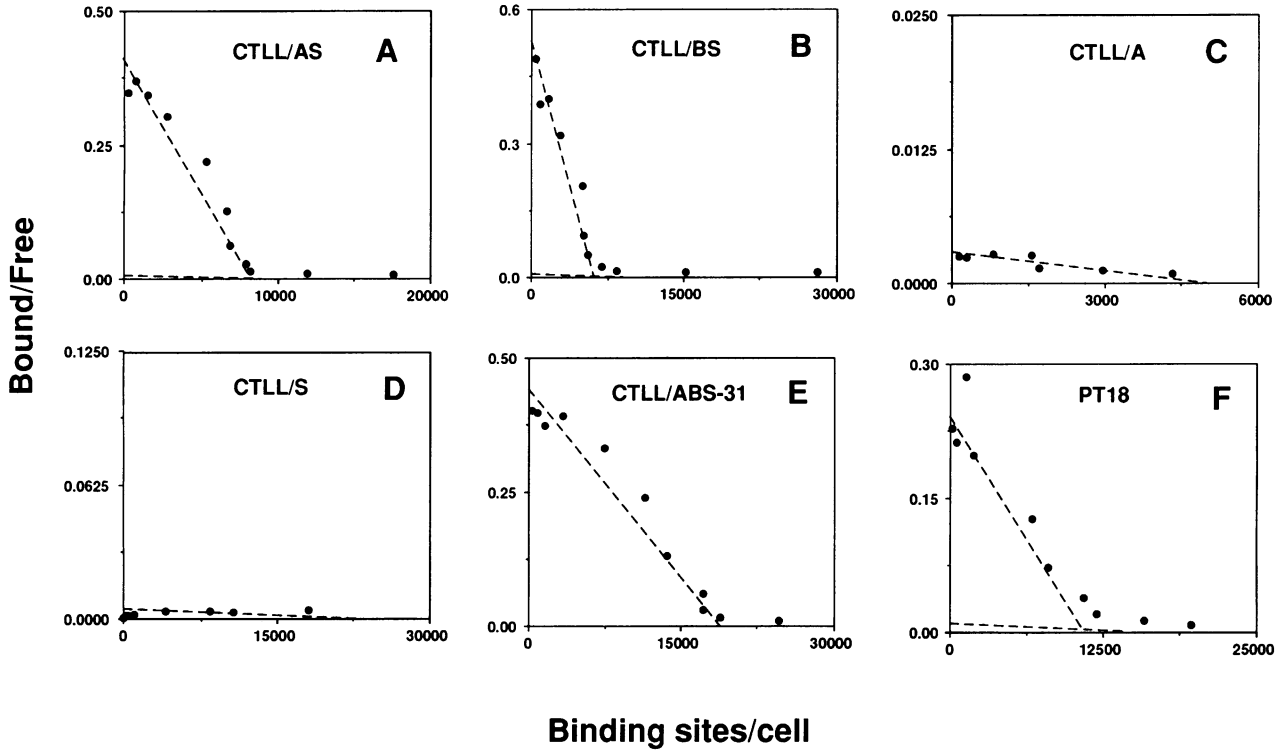

Fig. 4. Binding of [^{125}I]mIL-3 to the CTLL-2 transfectants and PT18 cells. Binding assay was carried out as described in Materials and methods. Scatchard analyses of binding data using CTLL-2 transfectant expressing AIC2A and SUT-1 (CTLL/AS) (**A**), AIC2B and SUT-1 (CTLL/BS) (**B**), AIC2A alone (CTLL/A) (**C**), SUT-1 alone (CTLL/S) (**D**), AIC2A, AIC2B and SUT-1 (CTLL/ABS-1) (**E**) and the PT18 cells (**F**) are shown.

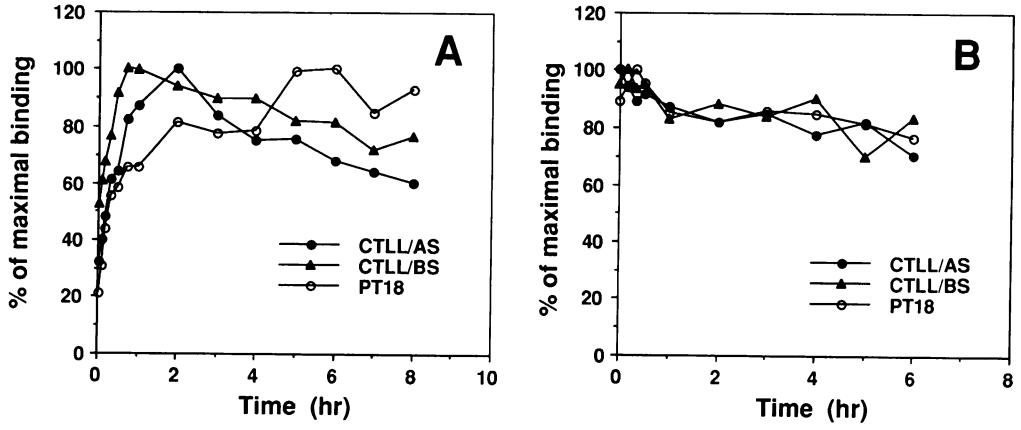

Fig. 5. Binding kinetics of the mIL-3 receptor expressed on the CTLL-2 transfectants and PT18 cells. (**A**) Association rate. Cells (5×10^5) were incubated with 200 pM [^{125}I]mIL-3 at 4°C for various times and the cell-bound radioactivity was counted. (**B**) Dissociation rate. Cells (5×10^5) were pre-incubated with 200 pM [^{125}I]mIL-3 at 4°C for 3 h and then an excess of unlabeled mIL-3 (100 nM) was added. After incubation at 4°C for various times, residual cell-bound radioactivity was measured. Closed circles: CTLL-2 transfectant expressing AIC2A and SUT-1; closed triangles: CTLL-2 transfectant expressing AIC2B and SUT-1; open circles: PT18 cells.

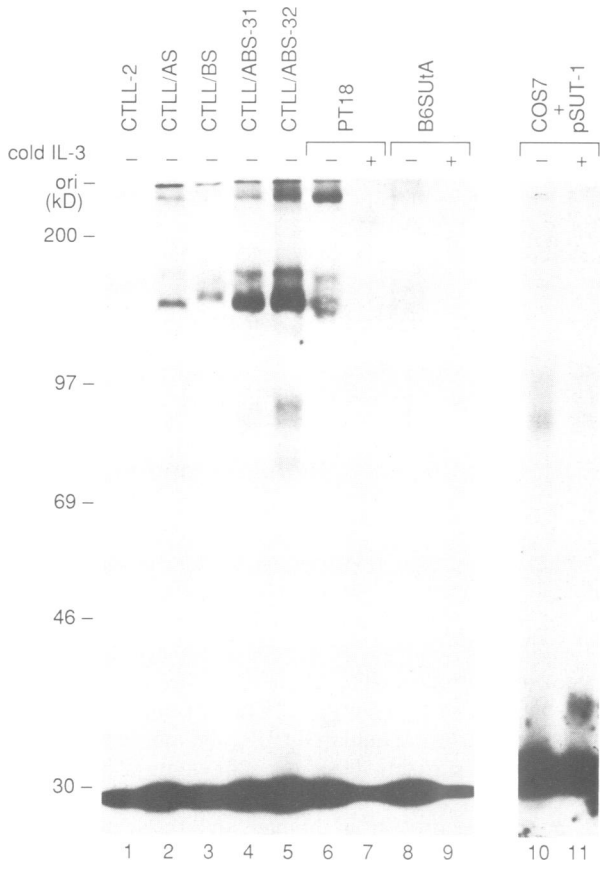

Fig. 6. Chemical cross-linking of [^{125}I]mIL-3. Cells (2×10^6) were incubated with 3 nM (lanes 1–9) or 20 nM (lanes 10 and 11) of [^{125}I]mIL-3 in the absence (lanes 1–6, 8 and 10) or presence (lanes 7, 9 and 11) of unlabeled mIL-3 (2 μM) at 4°C for 2 h and cross-linked with disuccinimidyl suberate. The proteins were analyzed by SDS–7.5% PAGE. The X-ray film was developed after 5 days exposure. Cell lines analyzed were parental CTLL-2 (lane 1), CTLL-2 transfectants expressing AIC2A and SUT-1 (lane 2); AIC2B and SUT-1 (lane 3); AIC2A, AIC2B and SUT-1 (lanes 4 and 5, independent clones C31 and C32 respectively), PT18 (lanes 6 and 7), B6SUtA (lanes 8 and 9) and COS7 cells transfected with the SUT-1 cDNA clone (lanes 10 and 11). Positions of molecular weight standards are shown on the left.

the reconstituted high affinity mIL-3Rs on CTLL/AS and CTLL/BS cells transduce growth signals in response to mIL-3, CTLL/AS and CTLL/BS cells proliferated equally in response to mIL-3 whereas no stimulation of growth by mIL-3 was observed in CTLL/A, CTLL/B and CTLL/S cells (Figure 7). The dose–response curves of CTLL/AS and CTLL/BS cells were similar to that of IL-3-dependent MC/9 cells (data not shown). In addition, CTLL/AS and CTLL/BS cells proliferated continuously in the presence of mIL-3 (Figure 7B). These observations indicate that the high affinity receptor composed of AIC2A and SUT-1 as well as that of AIC2B and SUT-1 are equally functional in their ability to respond to IL-3. In CTLL/S cells which express SUT-1 alone, a small population of cells survived in the presence of mIL-3 and started to grow slowly after 2 weeks of culture. The growth pattern of the CTLL/S cells was clearly distinct from those of CTLL/AS and CTLL/BS cells in the mIL-3-containing culture (Figure 7B). A subpopulation of the CTLL/S cells might have acquired a growth pathway by induction of an endogenous gene product, since a small amount of AIC2B mRNA was detected in the mIL-3-responsive CTLL/S cells (data not shown).

Discussion

Our strategy for cloning the mIL-3R α subunit cDNA was based on the affinity conversion of a low affinity mIL-3 binding protein, AIC2A, because several lines of evidence suggested that AIC2A is a part of a high affinity IL-3R (Schreurs *et al.*, 1991; Ogorochi *et al.*, 1992). Although a number of receptor cDNAs have been isolated using ligand binding (D'Andrea *et al.*, 1989; Fukunaga *et al.*, 1990), there were inherent difficulties in our cloning strategy due to coexpression of cDNAs with the AIC2A protein which itself binds IL-3. A very sensitive method utilizing film emulsion autoradiography to detect receptor expressing cells, which we employed for cloning of the human IL-3Rα (Kitamura *et al.*, 1991b), gave a high background due to IL-3 binding to the AIC2 protein. We overcame this problem by selecting stringent binding conditions to distinguish high and low affinity binding sites: i.e. [^{125}I]IL-3

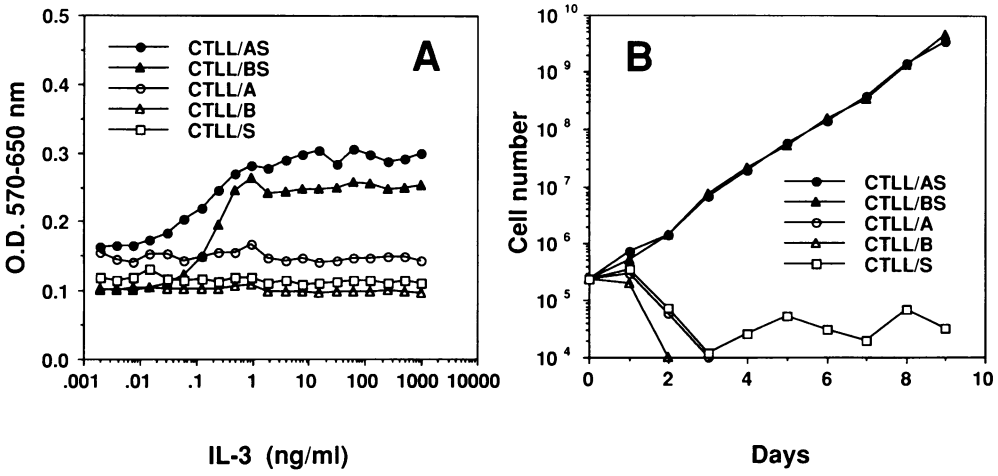

Fig. 7. IL-3 dependent proliferation of CTLL-2 transfectants expressing high affinity mIL-3R. (**A**) Cells were incubated for 24 h in the presence of increasing concentrations of mIL-3 and cell growth was examined by MTT assay. (**B**) Cells were independently cultured in the presence of mIL-3 (2 ng/ml) and viable cells were counted daily. CTLL-2 transfectants expressing AIC2A and SUT-1 (closed circles), AIC2B and SUT-1 (closed triangles), AIC2A alone (open circles), AIC2B alone (open triangles) and SUT-1 alone (open squares) were used.

bound to the low affinity site was removed by incubating with unlabeled IL-3.

Characterization of mIL-3Rα and the reconstituted high affinity mIL-3Rs as well as previous characterization of the human IL-3, GM-CSF and IL-5 receptors (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991b; Tavernier *et al.*, 1991) may provide a molecular explanation for the differences in IL-3 and its receptor between mouse and human. First, the sequence homology of IL-3 between mouse and human is unusually low (29% identity) (Yang *et al.*, 1986) compared with other cytokines (Arai *et al.*, 1990). Secondly, there are some differences in biological activities. The most significant difference is that mIL-3 has a very potent ability to stimulate mast cell growth (Yokota *et al.*, 1984), whereas hIL-3 does not (Saito *et al.*, 1988). Thirdly, the binding properties of IL-3 to the receptors are different between the two species: mIL-3 binds to high and low affinity sites (Schreurs *et al.*, 1990), but only a high affinity binding site has been found for hIL-3 (Gesner *et al.*, 1988; Park *et al.*, 1989; Kuwaki *et al.*, 1989; Uckun *et al.*, 1989; Budel *et al.*, 1990; Kitamura *et al.*, 1991c). Moreover, cross-competition of binding between IL-3 and GM-CSF has been described in human (Gesner *et al.*, 1988; Eliott *et al.*, 1989; Park *et al.*, 1989; Budel *et al.*, 1990; Taketazu *et al.*, 1991), but not in mouse receptors (Walker *et al.*, 1985; unpublished observations).

We have previously shown that the human high affinity IL-3 and GM-CSF receptors are composed of a ligand-specific α subunit and a common β subunit (β_c, or KH97) which is shared between IL-3 and GM-CSF receptors (Kitamura *et al.*, 1991b). The same β subunit is also involved in the high affinity IL-5R (Tavernier *et al.*, 1991). These findings explain cross-competitive binding of IL-3, GM-CSF and IL-5 on the human receptors. In contrast, the mouse has two distinct genes, AIC2A and AIC2B, both of which are homologous to human β_c (Hayashida *et al.*, 1990). Previous results indicate that AIC2B is a β subunit for both mouse GM-CSF and IL-5 receptors (Kitamura *et al.*, 1991a; Shanafelt *et al.*, 1991; Takaki *et al.*, 1991; Devos *et al.*, 1991). In this paper, we demonstrate that both AIC2A and AIC2B form high affinity mIL-3Rs with SUT-1 and both types of high affinity receptors are equally functional in CTLL-2 cells. Therefore the mouse clearly has two distinct β subunits for high affinity mIL-3Rs: an IL-3 specific one (AIC2A or β_{IL3}) and a common one (AIC2B or β_c) (Figure 8). This finding provides a molecular explanation for the absence of cross-competition in the mouse receptors. Cross-competition is dependent on the ratio of α and β subunits. If the expression level of the β subunit is higher than those of α subunits, cross-competition is not observed even in the human system (Kitamura *et al.*, 1991b). On the other hand, if the α subunit of mIL-3R is expressed more than α subunits of GM-CSF and IL-5 receptors and both β subunits (although this is not the case in most cells), either IL-5 or GM-CSF binding may be inhibited by IL-3 even in the mouse system. In addition, this model predicts that regardless of the expression levels of α subunits, neither GM-CSF nor IL-5 can inhibit IL-3 binding in mouse, because AIC2A and AIC2B are coexpressed in hemopoietic cells (Gorman *et al.*, 1990) and AIC2A lacks the ability to interact with α subunits of GM-CSF and IL-5 receptors (Kitamura *et al.*, 1991a; Takaki *et al.*, 1991; Devos *et al.*, 1991). Cross-competition between GM-CSF and IL-5 may

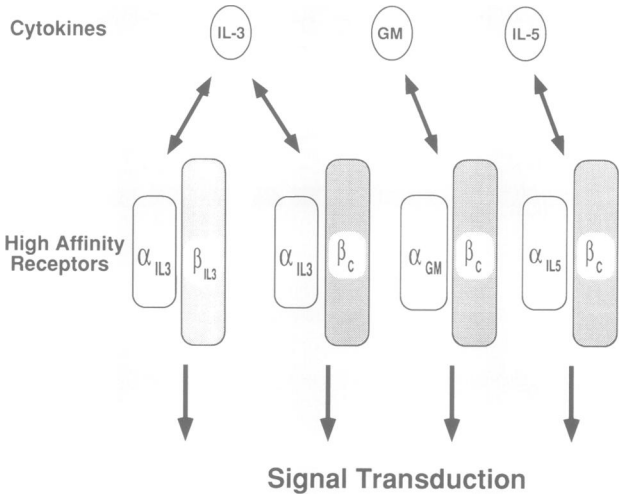

Fig. 8. Mouse high affinity receptors for IL-3, GM-CSF and IL-5. Based on the evidence shown by Devos *et al.* (1991), Kitamura *et al.* (1991a), Shanafelt *et al.* (1991), Takaki *et al.* (1991) and this article, multi-subunit structures of the high affinity receptors for mIL-3, mGM-CSF and mIL-5 are proposed. An α subunit of mGM-CSF has not been isolated yet.

occur in the mouse, although there has been no such report. This model also explains the difference of binding isotherms between mouse and human IL-3Rs: the mouse has high and low affinity sites but the human has only a high affinity site. This is probably due to the absence of an AIC2A-like IL-3 binding protein in human and the extremely low affinity of hIL-3Rα.

However, this model raises the question of why the mouse has two distinct β subunits while the human has only one. The AIC2A and AIC2B genes are extremely homologous (95% at the nucleotide level) and appear to be created by a gene duplication of a common ancestral gene (Gorman,D.M., Itoh,N., Jenkins,N.A., Gilbert,D.J. Copeland,N.G. and Miyajima,A., unpublished results). As AIC2B and human β_c are functionally identical, AIC2B (β_c) may be the prototype gene and the gene duplication may have occurred after divergence between mouse and human. If this is the case, AIC2A has gained the ability to bind IL-3 and has lost the ability to interact with the α subunits of GM-CSF and IL-5 receptors during the course of evolution. Then, is there any advantage for the mouse to have AIC2A? Although the high affinity mIL-3Rs consisting of either AIC2A or AIC2B are equally functional in stimulating CTLL-2 cells, it is still possible that AIC2A mediates some AIC2A-specific signal(s) because AIC2A and AIC2B are not identical. As mIL-3 can stimulate mast cell growth very potently (Yokota *et al.*, 1984), while hIL-3 does not show the same activity (Saito *et al.*, 1988), one AIC2A function may be related to mast cell growth. However, as mast cells express both AIC2A and AIC2B (Gorman *et al.*, 1990) and as our results suggest that both types of high affinity mIL-3R are formed in PT18 mast cells (Figure 6), it may be unlikely that only AIC2A stimulates mast cell growth. Based on the model presented in this paper, IL-3 binding to the high affinity receptor consisting of AIC2A is not inhibited by GM-CSF or IL-5, even when the expression levels of α subunits of GM-CSF and IL-5 receptors are high. In such cells, AIC2A should be capable of transmitting an IL-3 signal in the presence of either GM-CSF or IL-5. It is possible that the IL-3 signal is absolutely required during the development of mouse

hemopoietic cells: e.g. IL-3 elicits self-renewal while GM-CSF or IL-5 induce differentiation to each lineage. The specific functions, if any, associated with AIC2A remain to be explored.

The sequence homology between mouse and human IL-3R α subunits is very low (30% identity), which coincides with the low homology between mouse and human IL-3 (29% identity) (Yang *et al.*, 1986). This observation suggests that the evolution of IL-3 and IL-3Rα genes was closely related: diversity of mouse and human IL-3 might be compensated for by the structural changes of IL-3Rα. This contrasts to the high sequence conservation between mouse and human β subunits (55% identity). As β_c interacts with IL-3, GM-CSF and IL-5 as well as their α subunits, evolutional diversity of β may be restricted. Generally cytokines are folded into four α-helical bundles and the N-terminal helices of GM-CSF and IL-5 appear to be responsible for the interaction with β_c (Shanafelt *et al.*, 1991). As the amino acid sequences of N-terminal α-helices are relatively conserved between mouse and human IL-3 (Shanafelt *et al.*, 1991), this region may be responsible for the interaction with both β subunits. Several regions conserved between mouse and human IL-3Rα (Figure 2) may be important for association with β subunits, IL-3, or other associated molecules.

The receptor system of IL-3, GM-CSF and IL-5 provides a unique model for studying the mechanism of hemopoietic cell regulation initiated by cytokines. IL-3, GM-CSF and IL-5 each have unique biological activities as well as sharing functions such as eosinophil colony stimulating activity. As IL-3, GM-CSF and IL-5 receptors share β_c, common signals may be delivered by β_c while specific signals associated with each ligand might be given by its α subunit. Based on the response of hemopoietic cells to these cytokines, it is conceivable that β_c is expressed in various lineages of cells and that the expression of the α subunit determines the specificity of a cell to a given cytokine. The AIC2A along with SUT-1 may provide another route to deliver IL-3 specific signals in mouse.

Materials and methods

Cells and reagents

A mouse myeloid progenitor cell line, B6SUtA (Greenberger *et al.*, 1983), and a mouse mast cell line, PT18 (Pluznik *et al.*, 1982), were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol (2-ME) and 50 U/ml mGM-CSF. An IL-2-dependent T cell line, CTLL-2 (Cerottini *et al.*, 1974), was maintained in RPMI medium containing 10% FCS, 50 μM 2-ME and 100 U/ml of mIL-2. COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. Anti-Aic2 monoclonal antibody (mAb) (rat IgM) (Yonehara *et al.*, 1990) and 9D3 mAb (rat IgG_{2a}) (Ogorochi *et al.*, 1992) have been described previously. Purified HB mAb (hamster IgG) (Yonehara,S., unpublished results) was generously provided by Dr S.Yonehara (Tokyo Metropolitan Institute of Medical Science).

Recombinant cytokines

Recombinant mouse IL-3 and GM-CSF were produced in silkworm and yeast respectively (Miyajima *et al.*, 1986, 1987). Recombinant mouse IL-2 produced in *Escherichia coli* was provided by G.Zurawski (DNAX, Palo Alto, CA). Radioiodination of mIL-3 was performed using Iodogen (Pierce) as described (Schreurs *et al.*, 1989). The specific radioactivity of [^{125}I]IL-3 was $5-20 \times 10^7$ c.p.m./μg protein ($1-4 \times 10^6$ c.p.m./pmol).

Construction of the cDNA library

Poly(A)$^+$ RNA was prepared from B6SUtA cells using a Fast-Track kit (Invitrogen, San Diego, CA). As described previously (Kitamura *et al.*, 1991b), double-stranded cDNA was synthesized, ligated with *Bst*XI linkers, size fractionated (>1.0 kb) and cloned into the *Bst*XI site of pME18S (K.Maruyama and A.Miyajima, unpublished), a derivative vector of pCEV4 (Itoh *et al.*, 1990). The total number of independent clones of the cDNA library was 1.6×10^7.

Screening of the library by a coexpression system

A screening method based on the binding assay was basically the same as that of Fukunaga *et al.* (1990). One thousand pools of 50 clones each were prepared from an original stock of the cDNA library. Two of these pools were combined and plasmid DNAs were prepared by the alkaline lysis method (Maniatis *et al.*, 1982). Semi-confluent COS7 cells grown in 6-well plates were transfected with 1 μg of each plasmid DNA and 0.1 μg of the AIC2A plasmid DNA (AIC2-26) (Itoh *et al.*, 1990) by the DEAE–dextran method as described (Fukunaga *et al.*, 1990). After 3 days culture, COS7 cells were washed with DMEM containing 10% FCS and incubated in 0.6 ml of 200 pM [^{125}I]mIL-3 (5×10^5 c.p.m) at 4°C for 2 h. Medium containing unbound radioactive ligand was removed, and cells were incubated with an excess amount of unlabeled mIL-3 (100 nM) at 4°C for 1 h. Cells washed twice with DMEM and once with phosphate-buffered saline (PBS) were trypsinized, and the radioactivity retained on cells was measured by using AUTO-GAMMA (Packard). Background binding of [^{125}I]mIL-3 on COS7 cells transfected with the AIC2A cDNA alone was ~600 c.p.m. We found one candidate pool (78A2) which showed higher binding (1050 c.p.m.). Since this pool was a mixture of two pools of 50 clones (7A2 and 8A2), we assayed each of them and confirmed that significant binding occurred in one of them: 7A2 showed 650 c.p.m. while 8A2 was 1200 c.p.m. Subsequently, 400 single colonies from the 8A2 pool were grown in 96-well plates, and plasmid DNA mixtures of each row (12 clones) and each column (eight clones) were prepared and subjected to the binding assay. Finally a single positive clone was identified in one well (row G: 5060 c.p.m.; column 5: 5400 c.p.m.) of the plate.

Binding assay and chemical cross-linking

The binding assays using [^{125}I]mIL-3 were carried out as described previously (Schreurs *et al.*, 1990). Scatchard plot analyses were performed using the LIGAND program (Munson, 1983). Chemical cross-linking was carried out as described previously (Schreurs *et al.*, 1990). Briefly, cells ($1-6 \times 10^6$ per 100 μl) incubated with 100 μl [^{125}I]mIL-3 (6 or 40 nM) with or without cold mIL-3 (4 μM) at 4°C for 2 h were washed twice with PBS and incubated in 1 ml of PBS containing 0.2 mM disuccinimidyl suberate (DSS) at 4°C for 20 min. Cells were lysed with 15 μl of PBS containing 1% Triton X-100, 2 mM EDTA, 2 mM (4-amidinophenyl)-methanesulfonylfluoride, 10 μM leupeptin, 100 μM benzamidine and 5 μg/ml aprotinin. The clear cell lysates were then subjected to electrophoresis on a 7.5% polyacrylamide gel in the presence of SDS (Laemmli, 1970) followed by autoradiography.

Hybridization and nucleotide sequence analysis

Northern blot analysis was carried out according to the standard method (Maniatis *et al.*, 1982). As a probe, the 1.3 kb cDNA insert of pSUT-1 was labeled using the random primer labeling kit (Boehringer). Supercoiled plasmid DNA was denatured and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) by using the Sequenase sequencing kit (US Biochemicals).

Construction of stable transfectants

The drug selection marker gene, *Neo*^{r} or *Hyg*^{r}, was inserted into the pSUT-1 plasmid and introduced into CTLL-2 cells by the electroporation method as described by Potter *et al.* (1984). Briefly, cells (2×10^6) were resuspended in 0.5 ml of the buffer (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM $NaHPO_4$, 1.46 mM KH_2PO_4 and 5 mM $MgCl_2$) containing 30 μg of linearized DNA and subjected to electroporation at 960 μF and 200 V using a Gene Pulser (Bio-Rad). Transfectants were selected with either G418 (1 mg/ml) or hygromycin B (1 mg/ml). A CTLL-2 transfectant expressing AIC2A and SUT-1 (CTLL/AS) or AIC2B and SUT-1 (CTLL/BS) was obtained by transfection of a SUT-1 construct containing a drug-resistant gene into CTLL-2 transfectants expressing AIC2A (CTLL/A) or AIC2B (CTLL/B), provided by T.Kitamura (DNAX) respectively. Transfectants expressing AIC2A, AIC2B and SUT-1 (CTLL/ABS-31 and CTLL/ABS-32) were prepared by cotransfection of the AIC2A plasmid and the SUT-1 construct into the CTLL/B cells. Expression of the AIC2A and/or the AIC2B protein on the transfectants was confirmed by FACScan using the AIC2A specific antibody (9D3), an AIC2B specific antibody (HB) and the anti-Aic2 antibody which recognizes both AIC2A and AIC2B.

Flow cytometry and cell proliferation assay

Cells (10^5-10^6) were incubated with 50 μl of PBS containing 5% FCS and first antibody (10 μg/ml), stained with fluorescein isothiocyanate (FITC)-conjugated corresponding second antibody (10 μg/ml) and analyzed using a FACScan (Becton Dickinson, Mountain View, CA). To detect a short-term response to mIL-3, a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetreazolium bromide (MTT) (Sigma) was performed as described by Mosmann (1983). In brief, 10^4 cells (50 μl) were mixed with 50 μl of various concentrations of mIL-3 in 96-well plates. After 24 h culture, 10 μl of MTT (5 mg/ml in PBS) was added per well and further incubated at 37°C for 4 h prior to the colorimetry.

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