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Cloning of the low-affinity murine granulocyte–macrophage colony-stimulating factor receptor and reconstitution of a high-affinity receptor complex

(cytokine receptor/hematopoietic growth factor)

LINDA S. PARK*, UNJA MARTIN, RICK SORENSEN, SEAN LUHR, PHILIP J. MORRISSEY, DAVID COSMAN, AND ALF LARSEN

Immunex Research and Development Corporation, 51 University Street, Seattle, WA 98101

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ABSTRACT **A cDNA clone (clone 71) that encodes a low-affinity receptor for murine granulocyte–macrophage colony-stimulating factor (GM-CSF) has been isolated by direct expression. This molecule is the homologue of the human GM-CSF receptor α subunit, although homology between these molecules is surprisingly low (less than 35% amino acid identity). The cDNA encodes a polypeptide of 387 amino acids, which contains the conserved features of the hematopoietin receptor superfamily. When expressed in COS-7 cells, this clone encodes a protein that binds radiolabeled murine GM-CSF with low affinity. Coexpression of clone 71 with a cDNA corresponding to a low-affinity interleukin 3 (IL-3) receptor (AIC2A) did not alter the affinity of binding of either GM-CSF or IL-3. However, coexpression of clone 71 with the IL-3 receptor-related cDNA AIC2B generated high-affinity binding sites for murine GM-CSF but not murine IL-3. These studies show that clone 71 and AIC2B are capable of forming an $\alpha\beta$ complex capable of binding murine GM-CSF with high affinity, while AIC2A appears not to be a component of the murine GM-CSF receptor.**

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a cytokine that has been characterized as a molecule capable of stimulating proliferation and differentiation of granulocyte/macrophage progenitor cells as well as modulating the functional activity of mature granulocytes and macrophages (1). Recent studies have documented a broader range of biological activities for GM-CSF, which overlap substantially with the effects of interleukin 3 (IL-3) (2). Insights into the molecular basis of the overlapping biological functions of these two molecules have come from studies of the cell surface receptors to which they bind. We (3, 4) and others (5–9) have described the existence of a heterogeneous population of receptors for GM-CSF and IL-3 on a variety of human primary cells and cell lines. GM-CSF and IL-3 are capable of directly competing for binding to at least a subset of these cell-surface receptors.

The recent cloning of component subunits of the human GM-CSF and IL-3 receptors has contributed substantially to our understanding of the molecular nature of this complex receptor system. The first cDNA isolated encoded a human GM-CSF-binding subunit (termed the α subunit) which was shown to manifest only low-affinity GM-CSF binding (10, 11). Subsequently, a second subunit of the human GM-CSF receptor (GM-CSFR) was cloned that lacked direct binding activity but was capable of generating high-affinity GM-CSF binding when coexpressed with the GM-CSFR α subunit (12). It was proposed that this second subunit (termed the β subunit) might be a shared component of a separate IL-3 receptor (IL-3R) complex, thereby explaining both the cross-competition of binding and overlapping biological activities of GM-CSF and IL-3 (12). The very recent cloning of a low-affinity human IL-3R subunit has now confirmed this hypothesis (13). Gaining an understanding of the GM-CSF/IL-3 receptor system has been complicated by the fact that significant differences in this system appear to exist between mice and humans. It has been reported (14) that on some types of murine cells, IL-3 is capable of down-modulating the GM-CSFR at 37°C. This is quite different from the direct cross-competition for receptor binding seen with GM-CSF and IL-3 on human cells, which occurs at 4°C. No direct cross-competition of this type has been reported on murine cells.

Two apparent components of the murine GM-CSF/IL-3 receptor system have been cloned to date, termed AIC2A (15) and AIC2B (16). These molecules are greater than 90% identical; however, AIC2A has been shown to bind murine IL-3, while AIC2B does not. Neither molecule binds GM-CSF. AIC2A and AIC2B are homologous to the human GM-CSFR β subunit, which does not bind GM-CSF or IL-3; however, a human homologue of AIC2A, which binds IL-3 with low affinity, has not been found (12). The specific roles AIC2A and AIC2B may play in the murine GM-CSF/IL-3 receptor system, and how these relate to the corresponding human system, are not yet clear. Recently, reconstitution studies in which the human GM-CSFR α subunit was coexpressed in CTLL cells with either AIC2A or AIC2B have suggested that AIC2B is likely to be the β subunit of the mouse GM-CSFR (17). In this paper we describe the molecular cloning of a murine (mu)GM-CSFR subunit, with distant but significant homology to the previously described human GM-CSFR α subunit.† When expressed alone, this protein binds muGM-CSF with low affinity and does not bind IL-3. We demonstrate that coexpression of this muGM-CSFR α subunit with AIC2B, but not AIC2A, results in generation of a high-affinity GM-CSFR complex.

MATERIALS AND METHODS

Isolation of NS1.1 Cell Line. Spleen cells from 4-day-old C57BL/6J mice were plated at 2×10^6 cells per well in 24-well plates (Costar) and stimulated with recombinant muGM-CSF (20 ng/ml) in a final volume of 1 ml. After 2 days of incubation at 37°C, 10^6 irradiated (100 gray) $\psi 2$ cells producing ΔRM retrovirus (18) were added directly to the cultures and

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Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; GM-CSFR, GM-CSF receptor; IL-3, interleukin 3; IL-3R, IL-3 receptor; mu-, murine.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M85078).

incubation was continued for 3 additional days. Cells were then harvested, washed, and recultured in soft agar at a density of 10^6 cells per dish in the absence of GM-CSF. After 7 days, macroscopic colonies were plucked and cultured individually. Cells in wells exhibiting active growth were expanded in liquid culture. From this procedure, two lines of cells, termed NS1.1 and NS1.2, were established that had similar if not identical characteristics. Cell surface antigen expression by these lines was assessed by immunofluorescent staining and flow cytometric analysis.

Cell Lines and Tissue Preparations. NS1.1 and LSTRA cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and antibiotics. FDCP2-ID cells were maintained as above with the addition of muGM-CSF, and CTLL-2 and FDCP2 cells were maintained as previously described (19). Unfractionated bone marrow was isolated from femurs of C57BL/6J mice, and peritoneal macrophages were prepared by incubating whole peritoneal cell suspensions for 1 hr at 37°C in plastic tissue culture dishes and harvesting adherent cells.

Binding Assays and Affinity Crosslinking. Radiolabeling of recombinant muGM-CSF and binding assays were done as previously described (19). For binding assays with COS-7 cells, subconfluent monolayers of cells were transfected with isolated cDNA clones or empty vector controls, and 8 hr later cells were treated with trypsin and reseeded in Costar 6-well plates. Cell monolayers were assayed for binding 2 days later as previously described (20). Affinity crosslinking was also performed with adherent monolayers of transfected COS-7 cells (in 10-cm dishes) essentially as previously described for human interleukin 4 (20).

cDNA Library Construction, Screening, and Analysis. Total cellular RNA was isolated from NS1.1 cells as described below and polyadenylylated RNA was prepared as previously described (21). Double-stranded, oligo(dT)-primed cDNA was prepared with a commercial kit (Amersham) and size fractionated by velocity sedimentation through a sucrose gradient containing 0.5 M sodium acetate. cDNAs larger than ≈2 kilobases (kb) were ligated into the mammalian expression vector pDC302 (22) and used to transform *Escherichia coli* strain DH5α as previously described (23). Screening of cDNA pools by direct expression in COS-7 cells was performed by an *in situ* autoradiographic plate binding assay (24) as previously described (25). Plates were incubated with 2 nM ^{125}I-labeled muGM-CSF for 1 hr at 25°C. Sequences of isolated cDNA clones were analyzed by using computer programs of the Wisconsin sequence analysis package (GCG; Madison, WI) (26).

RNA Analysis. Total cellular RNAs were isolated and prepared for Northern analysis as previously described (23). Filters were probed with a ^{32}P-labeled antisense RNA prepared by T7 RNA polymerase transcription of a fragment of the clone 71 sequence between nucleotides 932 and 1604 generated by PCR. High-stringency blot hybridization and wash conditions were as previously described (27) with the exception of a final 20-min wash at 68°C in 0.025× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

Isolation of cDNA Clones Corresponding to AIC2A and AIC2B. The AIC2A and AIC2B type receptor cDNA clones, 3-2 and 42-324, respectively, were prepared by PCR of NS1.1 cDNA using the oligonucleotide primers 5'-CCTTGCCCATAGCTTGTGCAGCCACC-3' and 5'-ACCTTCCCAGACTGGCTATTGTCCCAAGG-3', which flank the 2.8-kb coding region of the AIC2A sequence of Itoh *et al.* (15).

RESULTS

Isolation and Characterization of NS1.1 Cell Line. To isolate a cDNA encoding a muGM-CSFR, we utilized the NS1.1 cell line, which was established from neonatal mouse spleen cells stimulated with GM-CSF and transformed with a retrovirus expressing the *ras* and *myc* oncogenes. These cells grew as an adherent population independent of exogenous growth factors and phenotypically resembled immature macrophages. They were highly phagocytic, expressed Mac1, Fc receptors, and major histocompatibility complex (MHC) class I antigen, and were negative for expression of MHC class II, Mac2, Mac3, surface immunoglobulin, CD4, CD8, Thy1, and B220 antigens. The expression of MHC class II antigens could be induced by interferon γ, while culture in GM-CSF resulted in increased expression of Fc receptors (data not shown).

Equilibrium binding studies with ^{125}I-labeled muGM-CSF showed that NS1.1 cells produced a curvilinear Scatchard plot (Fig. 1*A*) as opposed to the linear Scatchard plot observed with a representative control (Fig. 1*B*). The curvilinear nature of the NS1.1 binding curves was due to the expression of a large number of low-affinity binding sites, in addition to a small number of high-affinity sites with a binding affinity similar to that seen on other cell lines (19). From the average of 13 binding experiments, GM-CSF binding to NS1.1 cells displayed a high-affinity component with an apparent K_a of $3.1 \pm 2.5 \times 10^9$ M^{-1} and 1090 ± 580 specific sites per cell and a low-affinity component with an apparent K_a of $2.0 \pm 1.7 \times 10^7$ M^{-1} and $1.6 \pm 0.9 \times 10^5$ specific binding sites per cell. The large number of total binding sites suggested that these cells would be an excellent source of mRNA for cDNA library constructions.

Isolation and Sequence Analysis of GM-CSFR cDNA. A sized NS1.1 cDNA library was prepared in the mammalian expression vector pDC302. Plasmid DNA from pools of ≈900 individual transformants was transfected into COS cells and screened for ^{125}I-GM-CSF binding by contact autoradiography. Four positive clones were identified, and the restriction map of each conformed to that of clone 71 shown in Fig. 2*A*. Sequencing of this insert revealed a 1782-bp cDNA with a single open reading frame predicted to encode a 387-amino acid polypeptide with a signal sequence of 29 residues and a membrane-spanning region of 25 residues (Fig. 2*B*). The mature receptor polypeptide is predicted to contain an extracellular region of 295 amino acid residues and a short

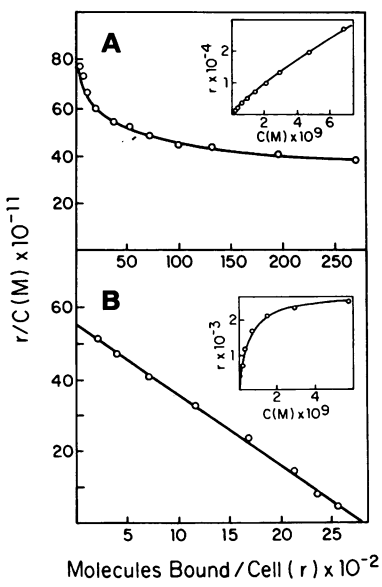

FIG. 1. Binding characteristics of muGM-CSFR. NS1.1 (*A*) and LSTRA (*B*) cells were incubated with various concentrations of ^{125}I-muGM-CSF for 60 min at 37°C and assayed for binding. Data are corrected for nonspecific binding and plotted in the Scatchard (28) coordinate system from the direct binding data shown in the *Insets*. C(M), molar concentration.

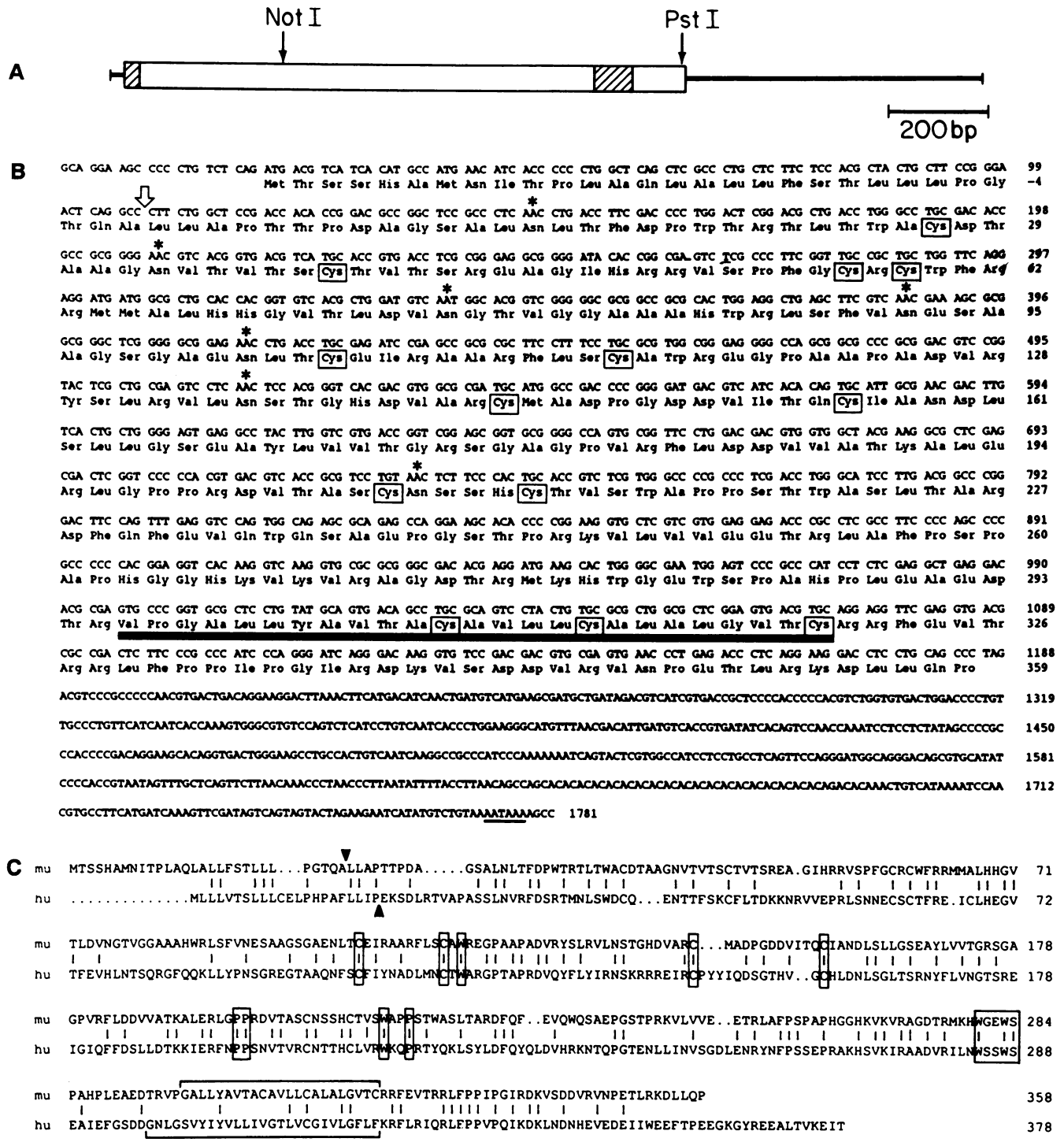

FIG. 2. muGM-CSFR cDNA clone 71. (*A*) Schematic representation and restriction map. Restriction sites are indicated for *Not* I and *Pst* I. Coding sequences are shown as wide bars, noncoding sequences as narrow bars. The predicted signal and transmembrane sequences are shown as hatched bars. bp, Base pairs. (*B*) Nucleotide and deduced amino acid sequence. The predicted signal peptide cleavage site is shown by an open arrow, and the predicted N terminus of the mature protein is designated residue 1. The predicted membrane-spanning sequence is indicated by heavy underline. Cysteine residues are boxed and potential N-linked glycosylation sites are indicated by asterisks. A potential poly(A) addition signal is shown by a light underline. (*C*) Homology of the murine and human GM-CSFR amino acid sequences. Amino acid identities are indicated by vertical bars. The deduced signal peptide cleavage sites are shown by the arrows, and the transmembrane regions by horizontal lines. Sequence features conserved in hematopoietin receptor family members are boxed.

cytoplasmic domain of 38 residues. The amino acid sequence of the murine receptor precursor showed a maximum of 35% amino acid identity to the 400-residue human GM-CSFR precursor sequence (10) and contained a cytoplasmic region 16 residues shorter than the human (Fig. 2*C*).

A computer search of several data bases found homologies only between the muGM-CSFR and other members of the hematopoietin receptor family (29). In each case, the homology was confined to the membrane-proximal ≈200 residues of the murine receptor extracellular region, which contains the sequence features that define the hematopoietin receptor family (boxed in Fig. 2*C*). This region of the muGM-CSFR is preceded by an N-terminal "cap" of ≈90 residues, which is unrelated to the immunoglobulin-like sequence identified at

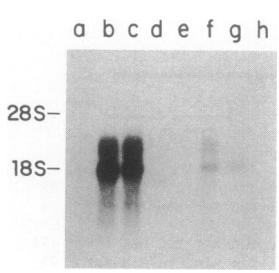

FIG. 3. Northern blot analysis of GM-CSFR mRNA. All lanes contain 2.0 μg of either polyadenylylated RNA (lanes a–e) or total cellular RNA (lanes f–h) from the following sources: LDA 11 (a), NS1.1 (b), LSTRA (c), peritoneal macrophage (d), CTLL-2 (e), unfractionated bone marrow (f), FDCP2-1D (g), and FDCP2 (h). The positions of the 18S and 28S ribosomal RNAs are indicated.

the N terminus of the interleukin 6 and granulocyte colony-stimulating factor receptors (23, 30) but appears homologous to the corresponding region of the human GM-CSFR sequence.

Expression of GM-CSFR mRNA. A 672-bp antisense RNA probe derived from the clone 71 sequence was used to probe Northern blots of RNAs isolated from a variety of sources (Fig. 3). Hybridizing bands of approximately 1.8 and 2.6 kb were detected in RNA isolated from cells known to bind GM-CSF, including the NS1.1 and LSTRA cell lines and unfractionated bone marrow and peritoneal macrophages from normal mice. Interestingly, although these transcripts were evident in RNA from the GM-CSF-responsive FDCP2-1D cell line, they were not detected in the unresponsive line FDCP2. Under the stringent conditions used, no specific hybridization was seen with RNAs isolated from the T-cell line CTLL or the bone marrow stromal cell line LDA11.

Characteristics of Recombinant muGM-CSFR. The binding characteristics of the muGM-CSFR clone 71 were assessed after expression in COS-7 cells alone, or when expressed with clone 3-2 (AIC2A) or 42-324 (AIC2B). Clone 71 expressed either alone or in combination with clone 3-2 exhibited only low-affinity binding (Fig. 4*A*) with a K_a between 10^7 and 10^8 M^{-1}. However, when clone 71 was coexpressed with clone 42-324 (Fig. 4*B*), a subpopulation of high-affinity GM-CSF binding sites (5000 ± 100) was observed with a K_a of 2.7 ± 0.9 × 10^{10} M^{-1}. Clone 71 alone did not bind muIL-3, nor did muIL-3 compete for ^{125}I-muGM-CSF binding when clone 71 was coexpressed with clone 3-2 or 42-324. In addition, coexpression with clone 71 did not alter the low-affinity

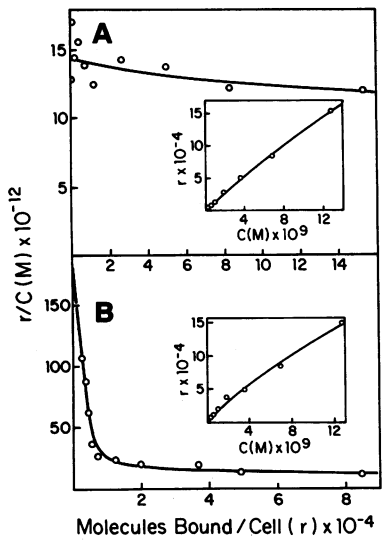

FIG. 4. Binding characteristics of muGM-CSFR coexpressed with AIC2A or AIC2B. Clone 71 (50 μg) was cotransfected into COS-7 cells with 50 μg of either clone 3-2 (*A*) or clone 42-324 (*B*), and binding of ^{125}I-GM-CSF to transfected cells was assayed as described in *Materials and Methods* and the legend to Fig. 1.

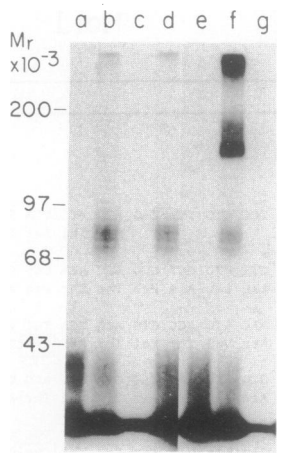

FIG. 5. Crosslinking of ^{125}I-muGM-CSF to muGM-CSFR clone 71. Monolayers of COS-7 cells transfected with clone 71 alone (lanes a–d) or cotransfected with clones 71 and 42-324 (lanes e–g) were crosslinked with ^{125}I-muGM-CSF (2 nM) in the absence of unlabeled competitors (lanes b and f), in the presence of unlabeled muGM-CSF (lanes c and g), or in the presence of unlabeled muIL-3 (lane d), and samples were analyzed by SDS/PAGE. Lanes a and e show control samples in which all procedures were conducted except for the addition of crosslinker.

binding of muIL-3 to expressed clone 3-2, nor did it induce clone 42-324 to bind muIL-3.

The molecular nature of the muGM-CSFR was examined by affinity crosslinking as depicted in Fig. 5. Clone 71 expressed either alone or in the presence of clone 3-2 generated a single, relatively broad crosslinked complex of M_r 70,000–80,000 (Fig. 5, lane b). This would correspond to a receptor protein of M_r 50,000–60,000, which is somewhat smaller than the expressed molecular weight of the human GM-CSFR α subunit (10). Crosslinking to this species was inhibited by unlabeled muGM-CSF (Fig. 5, lane c) but not by unlabeled muIL-3 (Fig. 5, lane d). When clone 71 was coexpressed with clone 42-324, an intense new crosslinked species was observed with a M_r of approximately 150,000. This would correspond to a receptor species of 130,000 which is similar to the expressed molecular weight reported for the AIC2A protein (15).

DISCUSSION

This report describes the molecular cloning of a low-affinity binding subunit of the muGM-CSFR by using a direct expression approach. Although this molecule is clearly the homologue of the human GM-CSFR α chain (10), isolation of a cDNA by cross-species hybridization was precluded by the relatively low homology (less than 35% at the amino acid level) it exhibits relative to its human counterpart. In fact, we have been unable to demonstrate any detectable hybridization of either murine or human GM-CSFR α probes to mRNA samples from the opposite species. Not unexpectedly, the isolated muGM-CSFR cDNA was found to be a member of the hematopoietin receptor superfamily (29). It contained all four cysteine residues and the WSXWS motif conserved in this family, although this motif was imperfect (WGEWS). It also contained the approximately 90-amino acid N-terminal cap region characteristic of the human GM-CSFR α subunit. The significance of this region, which is not a general feature of the hematopoietin receptor family, is currently unknown. Several members of the hematopoietin receptor family have been shown to exist as both membrane-bound and soluble forms (29). Recently the cloning of a cDNA encoding a potentially soluble form of the human GM-CSFR α subunit was reported (31), as well as a cDNA encoding an isoform

with an alternative cytoplasmic domain (32). During this study, we did not isolate any muGM-CSFR cDNA clones that would be predicted to encode soluble forms or to contain alternative cytoplasmic domains, although this does not rule out the possibility that such cDNA forms may exist.

The low-affinity human GM-CSFR α subunit can be converted to a high-affinity GM-CSF binding complex in the presence of a β subunit (12), which has very recently been shown to also serve as a high-affinity converter protein for low-affinity human IL-3 receptor (IL-3R) (13) and interleukin 5 receptor (IL-5R) (33) α subunits. Competition between low-affinity binding subunits of human GM-CSF, IL-3, and IL-5 for this common β chain appears to be the mechanism by which these three ligands are capable of inhibiting each other's binding to cell-surface receptors (13, 33). The human β subunit, KH97, was originally cloned by cross-species hybridization using a probe specific for the AIC2A and AIC2B molecules (12). Interestingly, this β subunit was the only AIC2A/B homologue found in humans, raising the question of why there are two murine β-type molecules and what their roles are. We have examined the ability of AIC2A and AIC2B to serve as high-affinity converter proteins for the muGM-CSFR α subunit and found that only AIC2B is able to generate a high-affinity muGM-CSF binding complex. AIC2B has also been recently shown to be a high-affinity converter protein for the murine IL-5R α subunit, while AIC2A was ineffective (34, 35). Although cloning of a murine IL-3R α subunit homologous to that isolated from human cells has not been reported, it has been suggested that AIC2A may be a high-affinity converter exclusive to this putative muIL-3R α subunit (13). Whether AIC2B could also serve as a high-affinity converter for a muIL-3Rα subunit is unknown. It is interesting to note, however, that no cross-competition between IL-3, GM-CSF, and IL-5 binding to murine cells has been reported in the literature. The fact that the muGM-CSFR and muIL-5R α subunits share a common β chain suggests that cross-competition could occur, as has been observed in the human system (33, 36, 37). The reason it has not been observed in the mouse system may simply be that the AIC2A and AIC2B β subunits may be abundantly expressed relative to the α subunits.

Why these apparent differences exist between the mouse and human systems is not clear, but it would appear that divergence is present in both the receptor and ligand sides of the system. Just as the efforts to clone a muGM-CSFR α subunit by cross-species hybridization were precluded due to low homology, the efforts several years ago to clone a human IL-3 molecule from its murine counterpart by this method were also initially unsuccessful for the same reason (38). As the components of this receptor system are further explored, it will be of considerable interest to investigate how the divergence between the human and murine systems is carried through to the signal transduction machinery of these receptors and what mechanisms may exist to mediate ligand-specific signaling.

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