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Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor

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Two cDNA clones encoding a receptor for human granulocyte-macrophage colony-stimulating factor (hGM-CSF-R) were isolated by expression screening of a library made from human placental mRNA. Pools of recombinant plasmid DNA were electroporated into COS cells which were then screened for their capacity to bind radioiodinated hGM-CSF using a sensitive microscopic autoradiographic approach. The cloned GM-CSF-R precursor is a 400 amino acid polypeptide (Mr 45 000) with a single transmembrane domain, a glycosylated extracellular domain and a short (54 amino acids) intracytoplasmic tail. It does not contain a tyrosine kinase domain nor show homology with members of the immunoglobulin super gene family, but does show some significant sequence homologies with receptors for several other haemopoietic growth factors, including those for interleukin-6, erythropoietin and interleukin-2 (β-chain) and also to the prolactin receptor. When transfected into COS cells the cloned cDNA directed the expression of a GM-CSF-R showing a single class of affinity (K_D = 2–8 nM) and specificity for human GM-CSF but not interleukin-3. Messenger RNA coding for this receptor was detected in a variety of haemopoietic cells known to display hGM-CSF binding, and cross-linking experiments revealed a similar size for the glycosylated receptors in transfected COS and haemopoietic cells.

Key words: cDNA cloning/human GM-CSF/ligand binding/placenta/receptor

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein growth and differentiation factor active on cells of the neutrophil, eosinophil and monocyte/macrophage series (reviewed in Gough and Nicola, 1989). Molecular clones encoding murine (Gough *et al.*, 1984) and human (Wong *et al.*, 1985) GM-CSF have been isolated and recombinant protein tested in animal model systems (Metcalf *et al.*, 1987; Donahue *et al.*, 1986) and in phase I/II clinical trials in patients with a variety of haemopoietic disorders (reviewed in Morstyn *et al.*, 1989). In both animal experiments and clinical trials, GM-CSF has been found to elevate circulating levels of monocytes, neutrophils and eosinophils, to enhance the functional capacities of the circulating cells, and to enhance the rate of haemopoietic recovery following chemotherapy and/or bone marrow transplantation (Gough and Nicola, 1989; Morstyn *et al.*, 1989).

In both murine and human systems, autoradiographic analyses have indicated that GM-CSF receptors are present in low numbers (a few hundred per cell) on cells within the monocyte, neutrophil and eosinophil lineages (Nicola, 1987; DiPersio *et al.*, 1988). However, functional GM-CSF receptors have also been detected on non-haemopoietic cells including endothelial cells (Bussolino *et al.*, 1989), small cell lung carcinoma cell lines and SV40-transformed simian COS cells (Cocita Baldwin *et al.*, 1989).

Studies of the binding characteristics of GM-CSF receptors have raised a number of unresolved issues. While Walker and Burgess (1985) detected both high-affinity ($K_D \sim 30$ pM) and low-affinity ($K_D \sim 1$ nM) receptors on murine haemopoeitic cells, Park *et al.* (1986a) detected only a single receptor class of K_D = 1–3 nM. By contrast, only high affinity receptors have been described on human haemopoietic and endothelial cells (K_D = 30 pM) (Gasson *et al.*, 1986; Bussolino *et al.*, 1989; Park *et al.*, 1986b) although a receptor of lower affinity has been described on monkey COS cells (Cocita Baldwin *et al.*, 1989). The basis for this variable expression of high and low affinity GM-CSF receptors is at present unknown.

The apparent specificity of the GM-CSF receptor raises further issues. In the human system, the binding of [^{125}I]human GM-CSF to some haemopoietic cells is competed for only by GM-CSF while in others, at least part of the binding is competed for equally by GM-CSF and interleukin-3 (IL-3) (Lopez *et al.*, 1989; Park *et al.*, 1989). However, on murine haemopoietic cells the binding of GM-CSF to its receptor is not directly competed for by IL-3 although it can be indirectly down-modulated at 37°C by IL-3 and other factors (Walker *et al.*, 1985; Nicola, 1987).

Finally, cross-linking experiments have suggested a mol. wt for the murine GM-CSF receptor of 51 000 (Walker and Burgess, 1985) or 130 000 (Park *et al.*, 1986a) while the human receptor mol. wt has been estimated at 84 000 (DiPersio *et al.*, 1988).

In order to begin to resolve some of these apparent complexities in GM-CSF receptors and to determine possible mechanisms of signal transduction, we have isolated a cDNA clone encoding a GM-CSF receptor by expression screening of a human placental cDNA library. The receptor encoded by this clone, when expressed on COS cells, displays similar binding characteristics to that of the native receptor on human placental membranes. Moreover, mRNA for this receptor subunit is also expressed in haemopoietic cells and the cloned receptor in COS cells has a similar mol. wt to that seen for the native receptor on haemopoietic cells by chemical cross-linking. This suggests that the cloned receptor is at least a part of the GM-CSF-responsive elements in a variety of different cells.

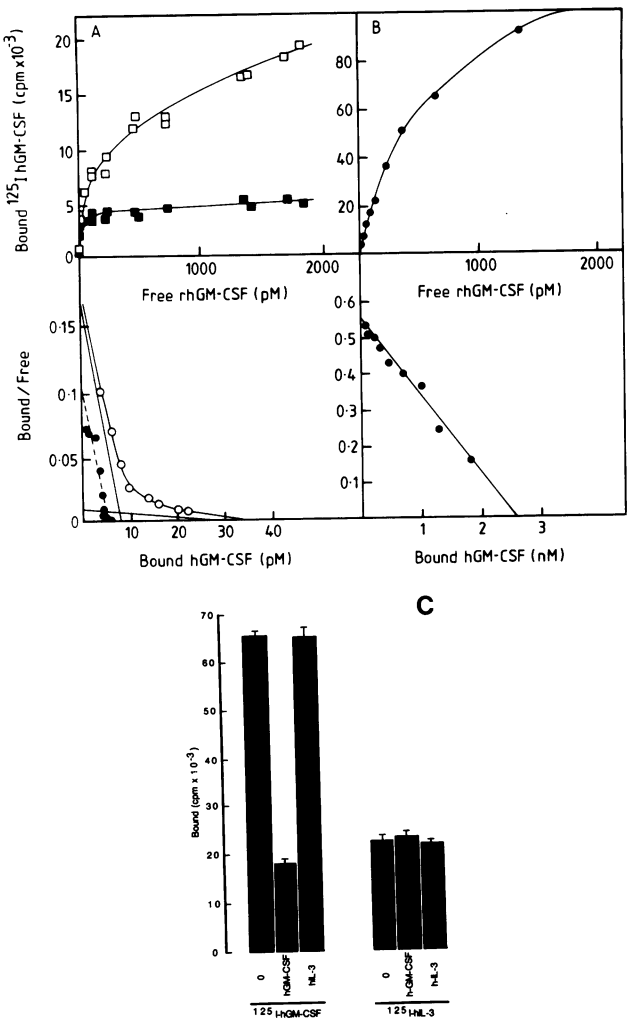

Fig. 1. Saturation binding isotherms and Scatchard analysis of [^{125}I]hGM-CSF binding to (**A**) HL60 cells after incubation in 1.25% dimethyl sulphoxide (DMSO) for 5 days and (**B**) purified human placental membranes., In (**A**), 5 × 10^6 DMSO-treated HL60 cells per point were incubated with increasing concentrations of [^{125}I]hGM-CSF at 4°C for 3 h and specific cell-associated radioactivity measured immediately after centrifugation (-□-) or after 10 min incubation in 1 ml PBS at 4°C (-■-). Scatchard analyses of these binding data are shown underneath before (-○-) or after (-●-) dissociation. The computer-derived high and low affinity binding components from undissociated cells are shown as solid lines. In (**B**), 40 μl of placental membrane suspension (see Materials and methods) were incubated with increasing concentrations of [^{125}I]hGM-CSF at 20°C for 1 h and specific binding subjected to Scatchard transformation under **panel B.** (**C**) Binding specificity of hGM-CSF and hIL-3 to receptors on human placental membranes. Membranes (40 μl) were incubated in duplicate with [^{125}I]hGM-CSF (200 000 c.p.m. in 110 μl HRF) with or without unlabelled hGM-CSF (500 ng) or hIL-3 (100 ng) for 1 h at 20°C. Similarly, 40 μl membranes were incubated with [^{125}I]hIL-3 (220 000 c.p.m. in 110 μl HRF) with or without the same amounts of unlabelled hGM-CSF or IL-3 as above. Total binding to each membrane preparation (mean ± range) is shown.

Results

Binding characteristics of human GM-CSF receptors

Published reports on the binding characteristics of human GM-CSF receptors have described a single affinity class of receptor on haemopoietic cells with an apparent equilibrum dissociation constant (K_D) of 20−600 pM (Gasson *et al.*, 1986; Park *et al.*, 1986b; Kelleher *et al.*, 1988). However, we have consistently detected both high and low affinity GM-CSF receptors on human bone marrow cells, primary human myeloid leukaemic cells and the human promyelocytic leukaemic cell line, HL-60 (Figure 1 and N.A.Nicola and L.Peterson, unpublished). Figure 1A shows the saturation binding isotherm at 4°C for [^{125}I]hGM-CSF binding to differentiated HL-60 cells. Transformation of this data by the method of Scatchard (1949) after curve fitting using the LIGAND program of Munson and Rodbard (1980) indicated that two rather than one, binding sites were required to fit the data ($P < 0.05$), with the high affinity receptors (40/cell) having a K_D of 46 pM and the low affinity receptors (130/cell) having a K_D of 2.9 nM. After allowing the same cells to dissociate bound GM-CSF for a brief time and then determining the remaining [^{125}I]hGM-CSF bound on the cells, only high affinity binding sites could be detected (Figure 1A) indicating that the low affinity receptors display a more rapid rate of ligand dissociation than do high affinity receptors.

We also detected specific binding of [^{125}I]hGM-CSF to purified human placental cell membranes (Figure 1B) but this binding was to a single class of low affinity receptor (3 × 10^9 receptors/mg placenta; K_D = 4.6 nM). The human placental GM-CSF receptor appeared to recognize only hGM-CSF and not h-interleukin-3 (hIL-3) (Figure 1C). Moreover, no hIL-3 receptors were detected on human placental membranes (Figure 1C).

Expression cloning of the hGM-CSF receptor

In order to clone the GM-CSF receptor, we have employed an expression screening strategy in simian COS cells. The low abundance of the GM-CSF receptor in all cell sources so far examined necessitated the screening of large cDNA libraries and consequent sensitive detection of positive transfected cells. The detection procedure we have adopted involves binding [^{125}I]hGM-CSF to transfected COS cells grown on glass microscope slides followed by direct microscopic autoradiography with photographic emulsion. This screening approach has two major advantages over previous receptor cloning procedures which used radioiodinated ligand as their detection tool (Sims *et al.*, 1988; D'Andrea *et al.*, 1989). Firstly, this procedure is extremely sensitive, since we could detect single clones from pools of 2 × 10^4 clones (cf 1 in 10^3, D'Andrea *et al.*, 1989; 1 in 350, Sims *et al.*, 1988). Secondly, our procedure allows easy identification of artefacts due to non-specific binding which would otherwise have made identification of a single GM-CSF-receptor positive cell, on a slide containing ~10^6 negative COS cells, impossible.

A human placental cDNA library of ~5 × 10^6 independent recombinants in a COS cell expression vector (obtained from Dr Brian Seed) was sub-fractionated into 500 pools of ~2 × 10^4 clones each and DNA from each pool was separately transfected into 1.5 × 10^6 COS cells by electroporation. The cells were cultured for 48 h on glass slides, incubated with a relatively high concentration of [^{125}I]hGM-CSF (~2 nM), fixed and then dipped in liquid photographic emulsion. After developing the slides they were individually examined microscopically and positive cells identified by the presence of autoradiographic grains. Of the first 250 pools screened, two gave rise to one or two positive cells (pools 29 and 138) (Figure 2). One of these pools (138) was partitioned into smaller pools (20 pools of 1500 recombinants then 60 pools of 80 recombinants and finally

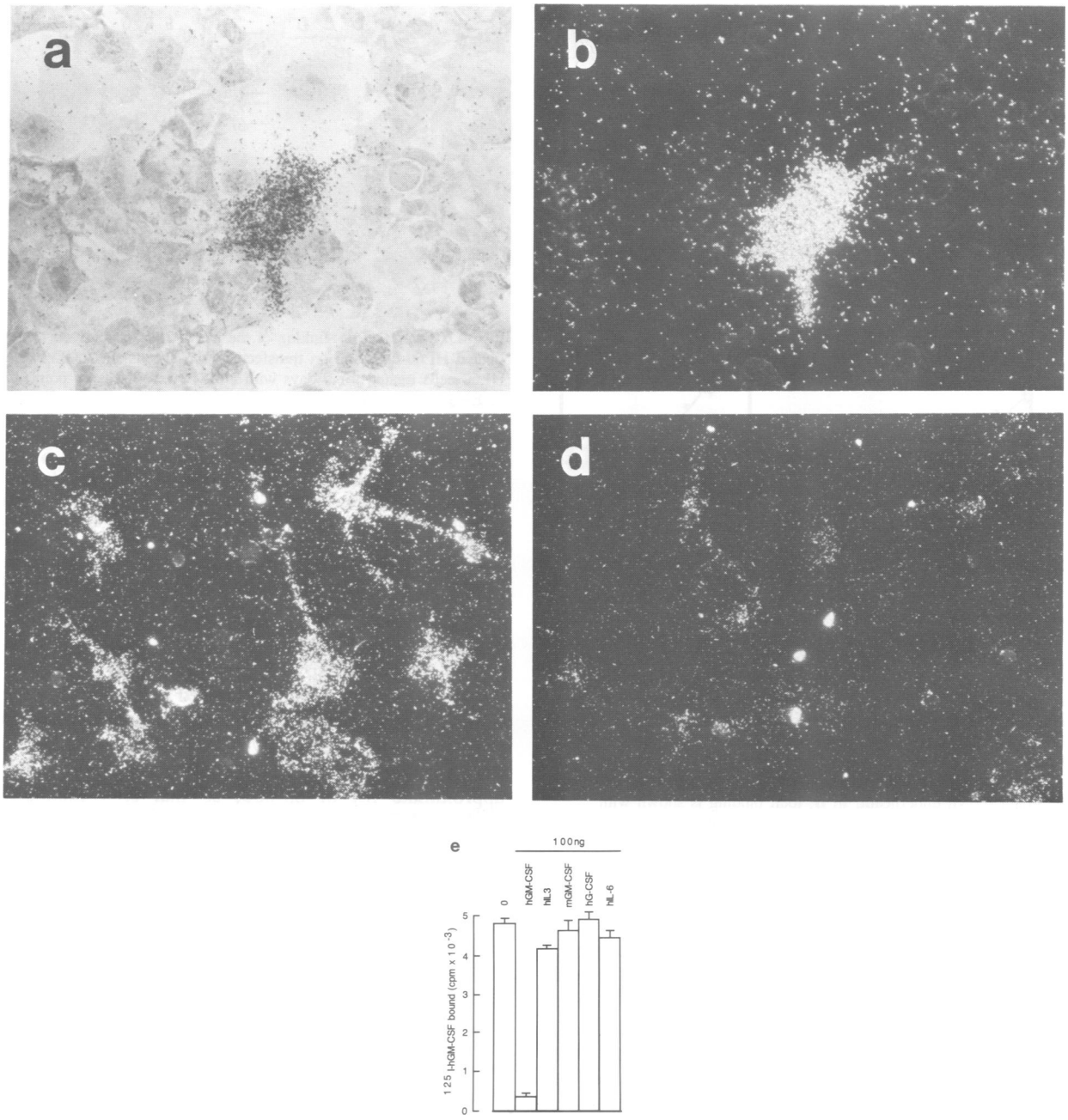

Fig. 2. Detection and specfciity of hGM-CSF receptors displayed on the surface of transfected COS-7 cells. (**a**) Photomicrograph of the cell autoradiograph showing the single clearly positive cell detected in 1.5×10^6 COS-7 cells transfected with DNA from pool 138 of the cDNA library (the COS cell covered by autoradiographic grains). Magnification ×15. (**b**) Same as (**a**) but photographed under dark field illumination. (**c**) Dark field illumination of a cell autoradiograph of COS-7 cells transfected with a pure hGM-CSF receptor clone (clone pGMR138) and incubated with 2 nM [^{125}I]hGM-CSF. Magnification ×7.5. (**d**) As for (**c**) except cells were also incubated with 20 nM unlabelled hGM-CSF. Note dramatic reduction in autoradiographic grains. (**e**) Specificity of the placental hGM-CSF receptor transfected into COs-7 cells. COS-7 cells transfected 48 h earlier with the pure clone (pGMR138) were assayed in duplicate for their ability to bind [^{125}I]hGM-CSF in the absence or presence of 100 ng unlabelled hGM-CSF, hIL-3, murine GM-CSF, human G-CSF or human IL-6 (mean ± range). Transfected cells (30 000 per point) were incubated for 1 h at 20°C in HRF containing 20 mM EDTA and 100 μg/ml chondroitin sulphate and [^{125}I]hGM-CSF (70 000 c.p.m. in 100 μl).

200 single clones) with autoradiographic screening at each stage until a single cDNA clone was obtained which could transfer high capacity [^{125}I]hGM-CSF-binding to COS cells. Following three rounds of selection in COS cells, the individual cDNA clone in a second positive pool of DNA (pool 29) was ultimately identified as coding for the GM-CSF receptor by colony hybridization with the 1.8 kbp insert of clone pGMR138.

Binding characteristics of the cloned receptor

The binding of [^{125}I]hGM-CSF to the transfected receptor on COS cells was specific since it could be competed for by unlabelled hGM-CSF but not by murine GM-CSF (which has no activity on human cells) or human IL-3,G-CSF or IL-6 (Figure 2e). The binding characteristics of the cloned GM-CSF receptor when transfected into COS cells are shown in Figure 3. The saturation binding isotherm for

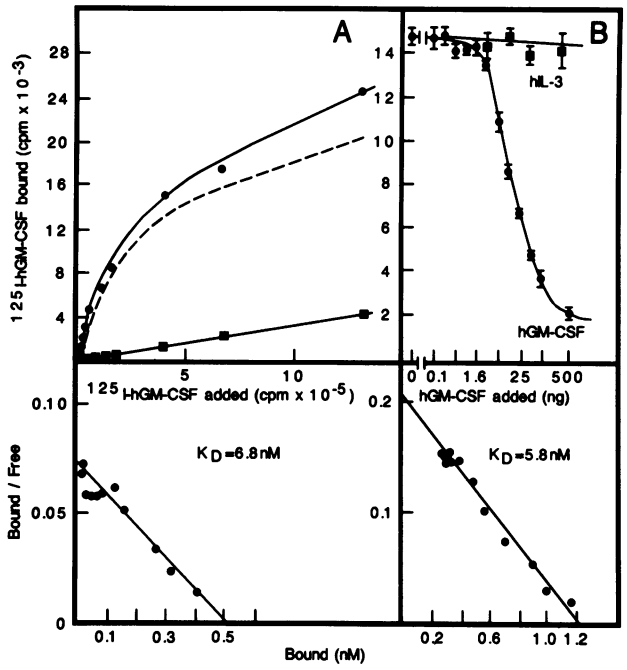

Fig. 3. Saturation and competition binding analysis of the placental hGM-CSF receptor transfected to COS-7 cells. COS-7 cells (33 000 per point) transfected 48 h earlier with the pure clone (pGMR138) were incubated with increasing concentrations of [^{1256}I]hGM-CSF with or without excess unlabelled hGM-CSF (**A**) or with a constant amount of [^{125}I]hGM-CSF (200 000 c.p.m.) and increasing concentrations of unlabelled hGM-CSF or hIL-3 (**B**) in a constant volume of 85 μl HRF/20 mM EDTA/100 μg/ml chondroitin sulphate at 20°C for 1.5 h. In **A**, total binding (-●-), non-specific binding (-■-) and specific binding (---) are shown with Scatchard transformation of the specific binding data shown underneath. In **B**, total binding is shown with Scatchard transformation of the specific binding data shown underneath.

[^{125}I]hGM-CSF binding to transfected COS cells at 20°C showed a single class of binding site with an equilibrium dissociation constant of 6.8 nM and 600 000 receptors per cell. In contrast, untransfected COS cells or COS cells transfected with vector alone showed no significant binding at the cell concentrations used for binding and chemical cross-linking analyses of the transfected receptor ($3-7 \times 10^4$ cells per point). Autoradiographic analyses indicated that only ~20% of the transfected cells were receptor positive (reflecting the transfection efficiency) so that positively transfected cells probably displayed $\sim 3 \times 10^6$ receptors per cell compared with a reported value of 1700 receptors per cell for untransfected COS cells (Cocita Baldwin *et al.*, 1989). Displacement of [^{125I}]hGM-CSF by unlabelled hGM-CSF also demonstrated only a single class of receptor with K_D = 5.8 nM and this binding was not displaced by unlabelled hIL-3 (Figure 3B). The similar binding affinities observed when the concentration of either labelled or unlabelled hGM-CSF was varied indicates that iodination did not significantly alter the binding affinity of hGM-CSF for this receptor. In several different experiments, binding of [^{125}I]hGM-CSF to transfected COS cells was determined in suspension (in binding medium with or without 20 mM EDTA and 100 μg/ml chondroitin sulphate to prevent cell aggregation), or on aherent cells and the apparent K_D varied from 4 to 8 nM indicating that neither calcium nor adherence significantly altered the binding characteristics of the transfected receptor.

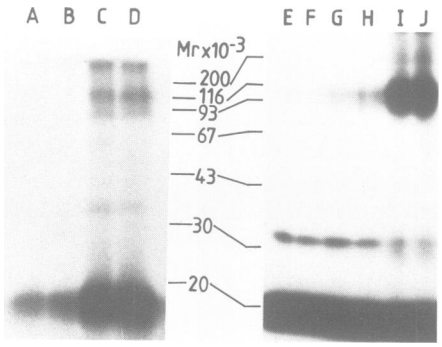

Fig. 4. Chemical cross-linking of the hGM-CSF receptor on DMSO-treated HL60 cells and on transfected COS-7 cells. In A–D 5×10^6 HL60 cells treated for 9 days with DMSO were used per point, while in **E–J**, 7×10^4 transfected COS-7 cells were used per point. In each case binding was for 3 h at 4°C with [^{125}I]hGM-CSF at 2 nM. Tracks **C** and **D** were with or without 10 min dissociation in 1 ml PBS to remove low affinity binding and **A** and **B** are as for **C** and **D** except that 20 nM unlabelled hGM-CSF was included during the binding reaction. In each case 1 mM DSS was use for cross-linking (15 min on ice). Tracks **E–J** represent transfected COS cells allowed to bind [^{125}I]hGM-CSF and then cross-linked with **E**, 0 mM; **F**, 0.01 mM; **G**, 0.05 mM; **H**, 01 mM; **I**, 0.5 ml and **J**, 1 mM DSS for 15 min on ice. Gel electrophoresis was on (**A–D**) 10% SDS gels or (**E–J**) 8% SDS gels and autoradiographs were exposed for (**A–D**) 4 weeks or (**E–J**) 2 days. Mol. wt markers (Pharmacia and BioRad) are shown.

The molecular size of the hGM-CSF receptor on transfected COS cels was compared to that on HL-60 cells by chemical cross-linking with disuccinimidyl suberate (DSS) (Figure 4). As observed by others (DiPersio *et al.*, 1988) the glycosylated GM-CSF receptor on HL-60 cells had an approximate M_r of 85 000, so that cross-linking with [^{125}I]hGM-CSF (Mr 15 000) gave an M_r of 100 000. Both before and after rapid ligand dissociation (with or without low affinity binding, respectively—see Figure 1) a major cross-linked band of M_r 100 000 and a minor cross-linked band of M_r 90 000 (representing receptors of M_r 85 000 and 75 000, respectively) were seen possibly representing different glycosylation variants of a single binding subunit. Cross-linking of [^{125}I]hGM-CSF to the transfected receptor on COS cells gave a major band of similar mol. wt (90 000–110 000) although it was somewhat broader than that seen on HL-60 cells, possibly reflecting more variable glycosylation. Similar cross-linking gels run under reducing conditions gave the same cross-linked receptor mol. wt indicating that the mature receptor was not comprised of disulphide-linked subunits (not shown).

Sequence analysis of the hGM-CSF receptor clones

The inserts of clones 29 and 138 were subcloned and sequenced by standard techniques (Figure 5). In the composite sequence shown in Figure 5, clone 29 is represented by nucleotides 1–1709 and clone 138 by nucleotides 7–1807. The two sequences are identical except for a single silent base difference (G → A) at position 1148. Each sequence contains a large open reading frame (ORF) encoding a polypeptide of 400 amino acids preceded by a short ORF of 22 codons. The methionine codon beginning the larger reading frame is in a context which corresponds well to the consensus sequence (RCCATGG) for translation initiation sites (Kozak, 1987) while the shorter ORF begins with a methionine codon in a poor context. The large ORF begins with a presumed signal peptide sequence of 22 amino

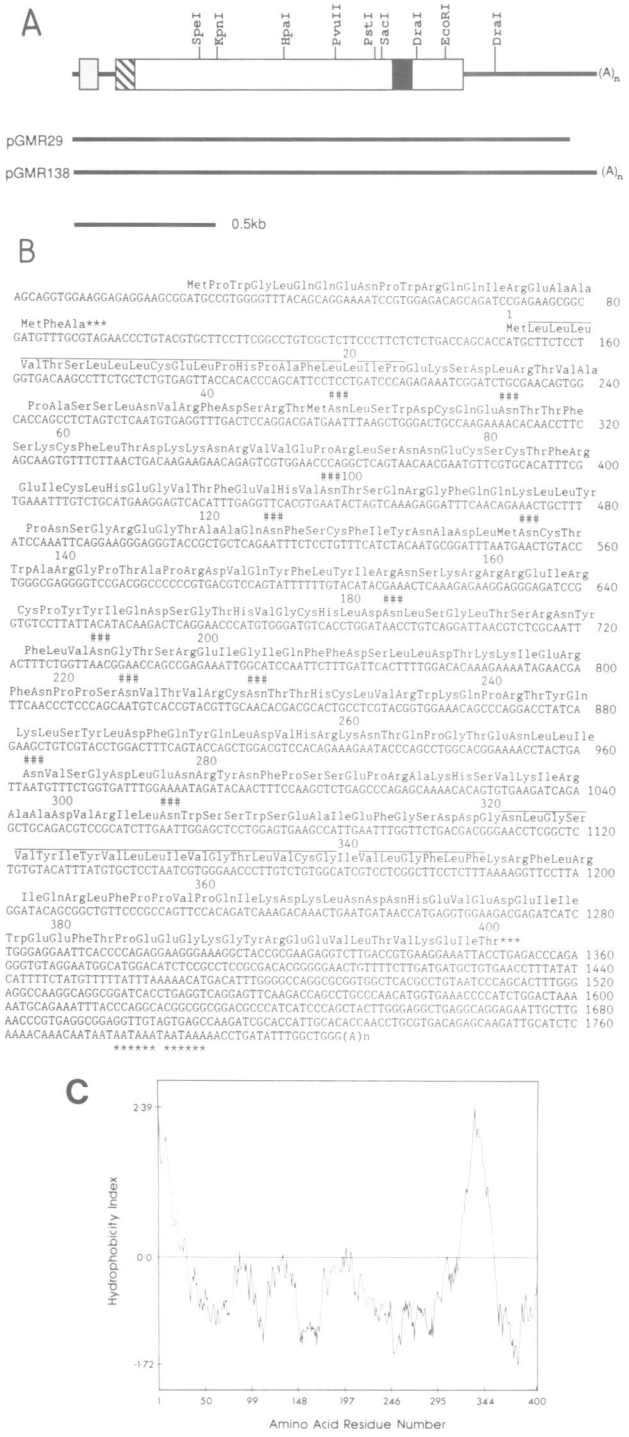

Fig. 5. (**A**) Restriction endonuclease cleavage map of the insert of cDNAs of pGMR138 and pGMR29. Boxes represent open reading frames. The hatched and filled regions present the signal sequence and transmembrane region of the GM-CSF-R coding region, respectively. The stippled box represents the upstream open reading frame. (**B**) Combined nucleotide sequence and deduced amino acid sequence of the insert cDNAs of pGMR138 and pGMR29. Numbers at the right margin indicate positions of nucleotides and numbers above the sequence refer to the amino acid sequence. The hatch marks indicate potential *N*-glycosylation sites (Asn-X-Ser/Thr). The overlined regions indicate the presumed signal peptide and transmembrane region respectively. The sets of six asterisks below the nucleotide sequence identify possible poly(A) addition signals. The poly(A) tail in clone 138 was 61 nucleotides long. (**C**) Hydrophobicity plot of the hGM-CSF receptor sequence according to Hopp and Woods (1983) (span length = 15). Note the two hydrophobic regions corresponding to the signal sequence and transmembrane domain respectively.

acids and residue Glu23 can be assigned as the first amino acid of the mature protein by comparison with typical signal peptide cleavage sites (von Heijne, 1986).

The predicted 378 amino acid mature GM-CSF receptor is calculated to have a mol. wt of 43 728, which is approximately half the size of the receptor observed by cross-linking of [^{125}I]GM-CSF to HL-60 cells and to COS-7 cells transfected with clone pGMR138 (Figure 4). This difference between the predicted size of the core receptor polypeptide and the mature receptor on cells is probably due to the attachment of carbohydrate to the numerous (11) potential N-linked glycosylation sites in the putative 297 amino acid extracellular domain since we have shown that the mature receptor does not contain disulphide-linked subunits. A hydrophobicity plot (Figure 5C) suggests that a stretch of 27 uncharged amino acids extending from Gly320 to Phe346 (Figure 5B) represents a transmembrane domain. This putative transmembrane domain is followed by a 54 amino acid intracellular domain which begins with a short stretch of basic amino acids, a feature common to the cytosolic face, next to the membrane-spanning segments, of many transmembrane proteins.

In the 3′-untranslated region of the GM-CSF-receptor mRNA there is a sequence element homologous to the 'Alu' family of repetitive elements (residues 1493−1760) which make up ~3% of the human genome (Jelinek and Schmid, 1982). Further downstream there are two poly(A) addition signals immediately prior to the poly(A) tail (Figure 5B).

The GM-CSF receptor transcript

Since the cDNA clone pGMR138 was isolated from a human placental cDNA library, it was of interest to determine whether mRNA corresponding to this transcript was also present in haemopoietic cells known to express the GM-CSF receptor. Northern blot analysis (e.g. Figure 6A) revealed that HL-60 cells, which express high affinity GM-CSF receptors, contain a 2.1 kb transcript hybridizing at high stringency to the pGMR138 probe (tracks 3−5), whereas receptor-negative CEM T-lymphoblastoid cells and HepG2 hepatic carcinoma cells contain no detectable transcript hybridizing with this probe (tracks 1 and 2).

Because of the low abundance of this RNA species, a more sensitive survey of RNA from various haemopoietic and non-haemopoietic cells was undertaken using PCR-based amplification of cDNA. Such analyses (e.g. Figure 6B) revealed the presence of GM-CSF receptor transcripts in a variety of human myeloid cell lines known to express functional GM-CSF receptors, including HL-60, U937 and AML 193, but not in CEM T-lymphoblastoid, Raji Burkitt lymphoma nor HepG2 hepatic cells. Interestingly HeLa cells, which also display GM-CSF receptors (M.Ward and N.A.Nicola, unpublished), also have transcripts corresponding to this cDNA clone (Figure 6B).

Discussion

GM-CSF was originally defined by its ability to stimulate the proliferation and differentiaton of granulocyte/macrophage progenitor cells, but more recently, it has become apparent that it can also stimulate the proliferation of progenitor cells of other haemopoietic lineages (Metcalf *et al.*, 1980) and cells of non-haemopoietic origin. The latter include human bone marrow fibroblasts, osteogenic sarcoma cell lines and

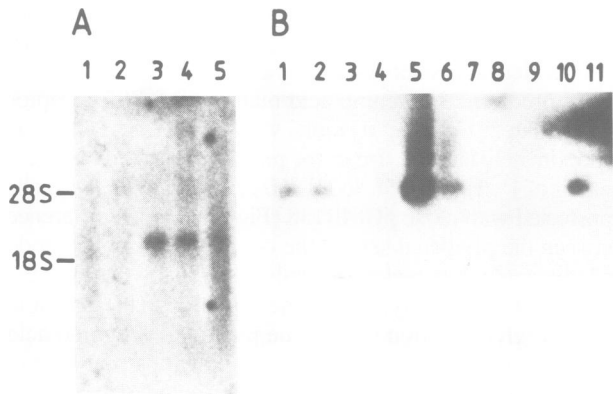

Fig. 6. Detection of the human GM-CSF receptor transcript. (**A**) RNA from the following sources was probed for LIF transcripts by Northern blot analysis as described in Materials and methods: CEM cells (**track 1**); HepG2 cells (**track 2**); HL60 cells (**tracks 3** and **4**); HL60 cells cultured at 5×10^5 cells/ml with 100 ng/ml TPA for 3 days (**track 5**). The positions of the 28S and 18S rRNA molecules are indicated. On other gels, RNA size standards (BRL) were also included. Exposure time of the autoradiograph: 5 days; (**B**) RNA from the following sources was probed for LIF transcripts by PCR-based amplification of cDNA as described in Materials and methods: U937 cells (**track 1**); AML193 cells (**track 2**); HL60 cells (**tracks 5** and **6**); CEM cells (**track 7**); Raji cells (**track 8**); HepG2 cells (**track 9**); HeLa cells (**track 10**). **Tracks 3, 4** and **11** contained the following negative controls: 'RNA blank' from no cells, carried through cDNA synthesis and PCR reactions; HL60 RNA not subjected to cDNA synthesis; RNA from murine L60T cells, respectively.

a breast carcinoma cell line (Dedhar *et al.*, 1988), human small cell carcinoma cell lines (Cocita Baldwin *et al.*, 1989), human endothelial cells (Bussolino *et al.*, 1989) human osteoblast-like cells (Evans *et al.*, 1989) and human placental cells (Wegman *et al.*, 1989).

In view of these biological results it is apparent that non-haemopoietic GM-CSF receptors can be functional, despite the fact that only high affinity receptors were detected on endothelial cells and only low affinity receptors were detected on fibroblasts and placental membranes.

Apparent diversities of human GM-CSF receptors

On haemopoietic cells, low affinity hGM-CSF receptors were distinguished by a rapid rate of ligand dissociation and poor internalization at 37°C whereas high affinity receptors displayed a much slower rate of ligand dissociation and were efficiently internalized (N.A.Nicola and L.Peterson, unpublished). In addition to this complexity, two types of high affinity hGM-CSF receptor have been described on some, but not all, normal haemopoietic cells and cell lines. One type recognizes only hGM-CSF and is the only type of GM-CSF receptor on human neutrophils, while the other type apparently recognizes hGM-CSF and hIL-3 with nearly equal affinity and represents 80% of GM-CSF receptors on eosinophils (Lopez *et al.*, 1989). Reciprocally, hIL-3-specific or cross-reactive receptors have also been described (Park *et al.*, 1989).

We have now cloned the functional, low-afffinity hGM-CSF receptor from placenta and shown that it recognizes only GM-CSF and not IL-3. This receptor, when transfected into COS cells, shows nearly identical low affinity binding and the same specificity as the receptor on placental membranes. Moreover, like the low affinity binding hGM-CSF receptor on haemopoietic cells the transfected receptor is characterized by a rapid ligand dissociation rate ($t_{½}$ = 5 min) and poor internalization (~10% after 2 h at 37°C) (N.A.Nicola and M.Ward, unpublished).

The placental receptor appears to form at least part of a functional GM-CSF receptor since GM-CSF has been shown to stimulate the growth of placental cells *in vitro* and to increase placental and foetal weight as well as decrease the rate of foetal resorption in a murine model of spontaneous abortion (Wegman *et al.*, 1989). This has revealed a somewhat unexpected role of at least two haemopoietic growth factors, GM-CSF and M-CSF (Rettenmier *et al.*, 1986) in foetal trophoblast growth and action.

Possible relationships of the cloned hGM-CSF-R to other forms of hGM-CSF-R

The relationship of the low affinity hGM-CSF binding subunit we have cloned to other forms of the GM-CSF receptor, is at present, unclear. The evidence that it forms at least a subunit of the GM-CSF receptor on haemopoietic cells includes the observations that GM-CSF receptor-positive haemopoietic cells express mRNA coding for the cloned receptor (Figure 6) and that cross-linking experiments on haemopoietic cells, performed under low-affinity or high-affinity conditions, have identified a binding subunit of the same molecular size (M_r ~85 000) as that seen on transfected COS cells (Figure 4). This suggests the possibility that the low affinity receptor (rapidly dissociating) may be converted to a high affinity receptor (slowly dissociating) by association with another protein(s) ('adaptor subunit') in the cell membrane, as has been shown for the interleukin-2 receptor (Tcshigawara *et al.*, 1987; Hatekeyama *et al.*, 1989). Since pre-existing GM-CSF receptors on placental or COS cell membanes are exclusively of the low affinity type (DiPersio *et al.*, 1988) these cells may lack the postulated second subnit of the high affinity receptor, which would explain the absence of high affinity receptors on transfected COS cells. Moreover, such a hypothesis would be consistent with the variable expression of high and low affinity receptors on different haemopoietic cells as well as our observation, in the murine system, that GM-CSF receptors, which display predominantly high affinity binding characteristics on FDCP-1 cells, are all converted to a low affinity form (K_D ~4 nM) when solubilized from cells in detergent solution (N.A.Nicola and D.Carey, unpublished). This hypothesis might be tested by transfecting the present cloned receptor into murine FDCP-1 cells.

The second type of high affinity human GM-CSF receptor on haemopoietic cells appears to be unique for the human system since it apparently cross-reacts with GM-CSF and IL-3 with nearly equal affinity (Park *et al.*, 1989; Lopez *et al.*, 1989). This may represent a novel receptor not related to the receptors recognizing only GM-CSF or only IL-3. However, a receptor recognizing both hGM-CSF and hIL-3 seems unlikely given the lack of sequence homology between these factors. A second possibility is that the binding of GM-CSF to its receptor communicates information to unoccupied IL-3 receptors (for example by physicial association or enzymic modification) to render them unable to bind IL-3 and vice versa. Alternatively, an extension of the 'adaptor subunit' hypothesis could also lead to apparent cross-reactivity of GM-CSF and IL-3 receptors. If the 'adaptor subunit' required for affinity conversion is common to GM-CSF and IL-3 receptors, then binding of IL-3 to its high

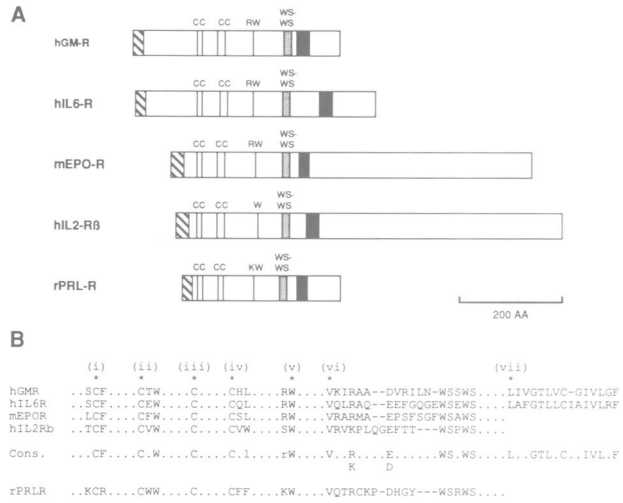

Fig. 7. Alignment of the hGM-CSF-receptor with other growth factor receptors. (**A**) Schematic of alignment. Boxes represent coding regions. The hatched and solid boxes represent signal sequences and transmembrane regions respectively. The positions of four conserved cysteine residues (C) and a conserved tryptophan residue (W) are indicated by vertical lines. The stippled box represents the 'WS-WS' box (see text). Sequences are aligned via the first conserved cysteine residues. (**B**) Details of sequence alignment. Reference positions (*) are marked bove the sequence (i−vii) and corresponds to the following amino acid numbers of each sequence (numbers in brackets refer to positions (i)−(vii) respectively; NH, no homology). hGMR (126, 136, 165, 178, 236, 294, 331), hIL-6R (121, 132, 165, 176, 233, 290, 374), mEPOR (52, 62, 90, 106, 165, 219, NJ), hIL2-Rβ (36, 46, 60, 74, 126, 182, NH) and rPRLR (31, 41, 70, 81, 146, 199, NH). Cons; consensus sequence based on haemopoietic receptor sequences. Except in the consensus, dots indicate variable spacing and dashes indicate gaps introduced to align the sequences.

affinity complex might prevent the 'adaptor subunit' from dissociating from that complex and forming a high affinity GM-CSF receptor complex (and vice-versa). This model of receptor competition for a limiting 'adaptor subunit' predicts that apparent cross-reactivity would only be seen at the level of high affinity receptors and is now testable since it predicts that transfection of the low affinity GM-CSF receptor to cells displaying high affinity IL-3 receptors would generate GM-CSF receptors that are both high affinity and 'cross-reactive'.

GM-CSF receptor primary structure: homology with other haemopoietic growth factor receptors

The deduced amino acid sequence of the GM-CSF receptor (Figure 5B) has many characteristics of growth factor receptors. It has a large glycosylated extracellular segment, a hydrophobic transmembrane segment and a short intracellular domain. The ligand binding domain (amino acids 23−319) contains 11 cysteine residues, but these do not appear to be organized in a manner characteristic of receptors of the immunoglobulin superfamily (Williams, 1987) nor does the intracellular domain have any apparent sequence homology with the catalytic domain of the tyrosine kinase family of growth factor receptors and oncogenes (Hanks *et al.*, 1988).

However, comparison of the GM-CSF receptor sequence with those of other haemopoietic growth factor receptors has revealed some significant regions of homology with those for human interleukin-6 (Yamasaki *et al.*, 1988), murine erythropoietin (D'Andrea *et al.*, 1989) and the human interleukin-2 receptor β-chain (Hatekeyama *et al.*,1989) but not to receptors for murine interleukin-1 (Sims *et al.*, 1988) or the α subunit of the human interleukin-2 receptor (Leonard *et al.*, 1984; Nikaido *et al.*, 1984). A similar haemopoietic growth factor receptor family has been described, including the murine interleukin-4 receptor, by R.Iderda, P.Beckman, K.March and D.Cosman (D.Urdal, personal communication).

As shown in Figure 7, the positions of four cysteine residues are approximately conserved in all four receptors and the context in which three of these residues are found is identical between the four receptor types (Figure 7). These four cysteine residues, which might form pairs of intra-chain disulphide bonds, do not coincide with either of the two cysteine residues of the IL-6 receptor associated with the putative immunoglobulin domain structure (Yamasaki *et al.*, 1988).

Trp236 is conserved and in three of the receptors is adjacent to an Arg residue. A further homology between all four receptor sequences is found just N-terminal of the transmembrane domain (Figure 7). The consensus sequence starting at position 294 of the GM-CSF receptor (the 'WS-WS' box) is found in all four receptors. The position of this sequence is close to the transmembrane domain in three of the receptors (GM-CSF-R, EPO-R and Il2-Rβ chain) but is further away in the IL-6 receptor.

The structure of the GM-CSF receptor most closely resembles that of the IL-6 receptor (Figure 7). Indeed, a striking homology between these two receptors can also be seen in their transmembrane regions. The transmembrane sequence Leu331−Leu342 of the GM-CSF receptor is 50% identical to the sequence Leu374−Leu386 of the IL-6 receptor transmembrane domain, including in each case the only Cys residue in the transmembrane domain. However, the position of the intramembrane cysteine residue relative to the putative inner face of the membrane [represented by Arg388 (IL-6-R) and Lys347 (GM-CSF-R)] differs between the two receptors. This particular conservation of residues in the transmembrane region may suggest a common ability to associate with other transmembrane proteins or lipids in their respective membranes. A similarly placed residue in the transmembrane region of the Semliki Forest virus E1 spike protein, corresponding to the middle of the inner leaflet of the membrane, has been shown to be a site of palmitoylation (Schmidt *et al.*, 1988). Neither the EPO nor IL2-Rβ receptors have transmembrane Cys residues, whilst there are three in this region of the IL-1 receptor which might therefore play a similar role.

A string based on the 'WS-WS' box [$VXXRX_{(6-11)}$ WSXWS] was used to search our most up-to-date database (Protein Research Foundation, Japan; April 1989) and a region homologous to this string was found in the rat prolactin receptor (rPRL receptor; Boutin *et al.*, 1988) just N-terminal to its transmembrane domain (Figure 7). Strikingly, all four of the extracellular cysteine residues in the rPRL receptor as well as a Lys−Trp doublet, are found in approximately the same relative positions as those of the four haemopoietic receptors (Figure 7A). The rPRL receptor also has a transmembrane cysteine residue (Boutin *et al.*, 1988) but the transmembrane sequence is not homologous to the hGM-CSF receptor. By contrast, the 'WS-WS' box is not found in a close relative of the prolactin receptor, the growth hormone receptor (Leung *et al.*, 1988), which in other regions shares 75−100% sequence similarity with the prolactin receptor (Boutin *et al.*, 1988). The hGM-CSF

receptor therefore appears to be a member of a new subset of growth and differentiaton factor receptors, defined by the set of five receptors described above.

In the GM-CSF receptor mRNA, prior to the long ORF encoding the GM-CSF receptor, is a short 22 codon ORF. Interestingly, such short ORFs are also found 5′ of the main receptor coding region in the DNA sequences for the human IL-6 receptor (Yamasaki *et al.*, 1988), the murine IL-1 receptor (Sims *et al.*, 1988) and the human IL-2 receptor α and β chains (Nikaido *et al.*, 1984; Hatekeyama *et al.*, 1989) and might act, if translated, to depress the translation of the main receptor coding regions. Such a mechanism might partly explain the low levels of expression of these receptors in normal cell types.

Concluding remarks

The availability of a cloned human GM-CSF receptor subunit should allow resolution of some of the complexities of GM-CSF and IL-3 binding. It should help in the analysis of some aspects of signal transduction by determining which cell types acquire GM-CSF responsiveness after receptor cDNA transfection and it should also allow more efficient screening of potential agonists or antagonists of GM-CSF action that may be relevant clinically. Moreover, soluble forms of this receptor may prove useful as therapeutic agents to abrogate the toxic effects of over-production of GM-CSF noted in animal model systems (Lang *et al.*, 1987; Johnson *et al.*, 1989) and in clinical trials (Morstyn *et al.*, 1989) and may possibly be used to inhibit the growth of some types of GM-CSF-dependent myeloid leukemias (Lang *et al.*, 1985; Young and Griffin, 1986).

Materials and methods

Screening of the cDNA library

A cDNA library derived from poly(A)$^+$ human placental RNA, constructed in the COS cell expression vector πH3M (Aruffo and Seed, 1987) and consisting of $\sim 5 \times 10^6$ independent clones was obtained from Dr B.Seed (Massachusetts General Hospital, Boston, USA). 10^7 clones, produced by transforming *Escherichia coli* MC1061/p3 cells, were sorted into 500 pools of $\sim 2 \times 10^4$ clones and glycerol stocks prepared. Miniprep DNA from each pool was transfected into COS-7 cells by electroporation. Briefly, 1.5×10^6 COS-7 cells in 180 μl phosphate-buffered saline (PBS) pH 7.3 were mixed with 20 μl DNA (~ 3 μg) and chilled on ice for 5 min. Cells were electroporated in 0.4 cm gap cuvettes at 300 V and 125 μFD (τ = 8.2–10.5 ms), returned to ice for 5 min and finally cultured in 2 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) in glass slide-based flaskettes (Lab-Tek, Nunc Inc., Naperville, USA). These conditions led to a 15–20% transfection frequency in surviving COS-7 cells as assessed by control transfections of ICAM/CDM8 (Simmons *et al.*, 1988) labelled with a radioiodinated α-ICAM monoclonal antibody, W-CAM-1 (Boyd *et al.*, 1988). Preliminary mixing experiments using dilutions of ICAM/CDM8 with 'blank' CDM8 vector (Seed, 1987) demonstrated a linear relationship between the dilution of ICAM/CDM8 DNA and the number of strongly ICAM-positive transfectants, down to a dilution of 1 in 10^4 ICAM/CDM8 to CDM8 molecules (D.Gearing and N.Nicola, unpublished observations). Using the conditions described above we expected to see up to 5–10 positive cells on a slide derived from transfecting a single primary pool of 10^4 recombinants. After 48 h, the medium was removed and the transfected monolayers were assessed for binding of radioiodinated human GM-CSF ($4-8 \times 10^5$ c.p.m. (1–2 nM) in 1 ml of 10 mM HEPES-buffered RPMI medium, pH 7.2/10% FCS) for 60 min at 20°C. The monolayers were washed twice in medium, fixed in 2.5% glutaraldehyde/PBS and dipped in 1% gelatine as described (Nicola and Metcalf, 1985). The slides were dipped in Kodak NTB2 photographic emulsion at 42°C and exposed in the dark for 48 h at 4°C in light proof boxes containing drierite. Slides were developed for 3 min in Kodak D19 developer (40 g/500 ml water), rinsed in water and fixed in Agfa G433C fixer before staining in 10% filtered Giemsa in water. Slides were screened at 10–20× magnification. Glycerol stocks of *E.coli* transformants corresponding to positive pools were partitioned into smaller pools as above until single positive cDNA clones were obtained.

Nucleotide sequencing

The inserts and various internal fragments of clones 29 and 138 were subcloned into M13 vectors and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the modified T7 polymerase (Tabor and Richardson, 1987; Sequenase, USB) and primers internal to subcloned segments. All subcloning boundaries were resequenced on full length clones. Compressions were resolved using dITP. Primers corresponding to some subclones were used to extend sequence to adjacent segments. Both strands of clone 138 were sequenced in their entirety (average gel characters per contig character = 4.87). The mRNA-synonymous strand of clone 29 was completely sequenced and ambiguities resolved using oligonucleotides on the opposite strand.

RNA blot analysis

Cytoplasmic polyadenylated RNA (~ 1.5 μg), prepared essentially as described (Gough, 1988) was fractionated on 1% agarose gels containing 20 mM morpholinopropane sulphonic acid, 5 mM sodium acetate, 1 mM EDTA (pH 7.0), and 6% (v/v) formaldehyde and transferred to nitrocellulose. Prior to hybridization, filters containing RNA were soaked in 2 × SSC containing 0.2% Ficol, 0.2% plolyvinylpyrollidone, 0.2% bovine serum albumin, 2 mM sodium pyrophosphate, 1 mM ATP, 50 μg/ml denatured salmon sperm DNA and 50 μg/ml *E.coli* tRNA at 67°C for several hours. Hybridization was in the same buffer containing 0.1% SDS at 67°C. The hybridization probe was the gel-purified 1300 bp *Xho* I–*Eco*RI fragment spanning the 5′ end of cDNA clone pGMR138, radiolabelled to a specific activity of $\sim 10^9$ c.p.m./μg by random priming (Feinberg and Vogelstein, 1983) and included in the hybridization at $\sim 5 \times 10^7$ c.p.m./ml. Filters were washed extensively in 2 × SSC, 0.1% SDS at 67°C and finally in 0.2 × SSC at 67°C prior to autoradiography.

RNA detection using the polymerase chain reaction

RNA (~ 1 μg) was subjected to first strand cDNA synthesis in a 20 μl reaction containing 50 mM Tris–HCl (pH 8.3 at 42°C), 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM of each dNTP, 20 μg/ml oligo(dT)$_{15}$ and 20 units AMV reverse transcriptase (Boehringer Mannheim) for 40 min at 42°C. Alter completion of first strand synthesis, the reaction was diluted to 100 μl with distilled water and 5 μl used for each PCR reaction. PCR reactions (in a volume of 50 μl) contained 200 μM of each dNTP, 1 μM of each specific primer, buffer as supplied in the GeneAmp kit (Cetus Corp., USA) and 1.25 units *Taq* polymerase. The primers used for PCR reactions were 5′-CTTCTCTCTGACCAGCA (position 131–147) and 5′-ACATGGGTTCCTGAGTC (Position 676–660) defining a 530 bp fragment. The PCR reaction conditions were: 2 min at 94°C; 2 min at 65°C; 3 min at 72°C for 25 cycles in a Perkin-Elmer-Cetus DNA thermal cycler. A portion of the PCR reaction was electrophoresed through a 1.2% agarose gel and transferred to nitrocellulose. Filters were pre-hybridized, hybridized and washed as described above. The hybridization probe was the gel-purified 1.9 kbp cDNA insert of pGMR138, radiolabelled to a specific activity of $\sim 10^9$ c.p.m./μg by random priming and included in the hybridization at $\sim 2 \times 10^7$ c.p.m./ml.

Radioligands

Purified, recombinant human GM-CSF produced in non-glycosylated form in *E.coli* was a kind gift from Dr J.Cebon (Ludwig Institute for Cancer Research, Melbourne Branch). It was radiodinated by incubating 2 μg of protein (2 μl) with 1 mCi Na^{125}I (New England Nuclear, Dreieich, FRG) in 40 μl of 0.2 M Na phosphate buffer pH 7.2 containing 0.02% Tween 20 and adding iodine monochloride (0.04 mM in 2 M NaCl) in two lots of 3 μl and 6 μl while vortex mixing the solution. The reaction mixture was passed through a column of Sephadex G-25M (Pharmacia, Uppsala, Sweden) to separate macromolecular radioactivity from free iodine (Hilton *et al.*, 1989). [^{125}I]hGM-CSF was 100% bindable (Calvo *et al.*, 1983) and displayed a specific radioactivity of 20 000–40 000 c.p.m./ng by the self-displacement analysis of Calvo *et al.* (1983). Unlabelled and labelled (specific radioactivity 40 000 c.pm./ng) human IL-3 was purchased from Amersham (Buckinghamshire, UK).

Binding experiments

HL60 cells grown for 5 days in medium containing 1.25% dimethyl sulphoxide were resuspended at 5×10^6 cells/50 μl in HEPES (10 mM, pH 7.2) buffered RPMI medium (HR) containing 10% foetal calf serum (HRF). Aliquots (50 μl) of cells were incubated with increasing concentrations of [^{125}I]hGM-CSF (0–2 nM) with or without unlabelled hGM-CSF (0.3 μM) at 4°C for 4 h. Cell suspensions were then layered over 180 μl

chilled foetal calf serum and centrifuged for 5 min at 700 *g* in small plastic centrifuge tubes and the cell pellet removed by cutting the tube with a scalpel blade. Cell-associated and free radioactivity were separately determined by counting duplicate tubes in a γ-counter. Transfected COS-7 cells were harvested 48–72 h after transfection by removing the supernatant and incubating the adherent cells in 40 mM EDTA in HR at 37°C for 30 min followed by adding an equal volume of HRF containing 200 μg/ml chondroitin sulphate and incubating at 37°C for a further 40 min (Padmanabhan *et al.*, 1988). The detached and disaggregated cells were centrifuged at 700 *g* for 5 min and resuspended in HRF with or without 20 mM EDTA and 100 μg/ml chondroitin sulphate. Saturation binding isotherms or competition experiments were performed as for HL60 cells. Human placental membranes were prepared from fresh term placentas essentially as described by Yeung *et al.* (1987) with 6 g of placenta yielding 4 ml of membrane suspension. For each binding point, 40 μl of membrane suspension was mixed with 40 μl HRF and increasing concentrations of [^{125}I]hGM-CSF with or without excess unlabelled hGM-CSF (0.3 μM). After 1 h incubation at 20°C the membranes were centrifuged at 30 000 *g* for 5 min, the supernatant removed with a fine Pasteur pipette and membrane pellets and supernatants counted separately in a γ-counter.

Cross-linking experiments

Binding of [^{125}I]hGM-CSF to cells in solution was performed at 4°C as described above and the cell pellets resuspended in 1 ml of ice-cold Na phosphate buffered (20 mM, pH 7.2) saline (0.15 M). Disuccinimidyl suberate (Sigma, Missouri, USA) in anhydrous acetonitrile (10 μl) was immediately added to give a final concentration of 0–1 mM and the cells incubated for 15 min on ice before centrifuging the cell pellet at 13 000 *g* for 1 min. The cell pellet was treated with DNase in the presence of protease inhibitors and prepared for SDS–PAGE as described (Nicola and Peterson, 1986).

Analysis of binding data

Specific binding was determined as the difference between binding in the absence or presence of excess unlabelled hGM-CSF. Specifically bound c.p.m. were converted to molar concentrations using the specific radioactivity of [^{125}I]GM-CSF determined by self-displacement analysis. Curve fitting of the binding data was performed using the LIGAND program of Munson and Rodbard (1980) before conversion to the Scatchard transformation. Two binding site fits were used only if the fit to the data was significantly improved ($P < 0.05$) over the one binding site fit.

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