Affinity Purification of Human Granulocyte Macrophage Colony-stimulating Factor Receptor α-Chain

DEMONSTRATION OF BINDING BY PHOTOAFFINITY LABELING*

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The human granulocyte macrophage colony-stimulating factor (GM-CSF) receptor α-chain, a low affinity component of the receptor, was solubilized and affinity-purified from human placenta using biotinylated GM-CSF. Scatchard analysis of 125I-GM-CSF binding to the placental membrane extract disclosed that the GM-CSF receptor had a dissociation constant (Kd) of 0.5–0.8 nM, corresponding to the KD value of the GM-CSF receptor α-chain on the intact placental membrane. Affinity labeling of the solubilized protein using a photoreactive cross-linking agent, N-hydroxysuccinimidyld-4-azidobenzoate (HSAB), demonstrated a single specific band at 70–95 kDa representing a ligand-receptor complex. Approximately 2 g of the placental membrane extract was subjected to a biotinylated GM-CSF-fixed streptavidin-agarose column, resulting in a single major band at 70 kDa on a silver-stained sodium dodecyl sulfate gel. The radioiodination for the purified material disclosed that the purified protein had an approximate molecular mass of 70 kDa and a pl of 6.6. Binding activity of the purified material was demonstrated by photoaffinity labeling using HSAB-125I-GM-CSF, producing a similar specific band at 70–95 kDa as was demonstrated for the crude protein.

The human granulocyte macrophage colony-stimulating factor (GM-CSF)* is a glycoprotein active on a variety of hematopoietic cells (Wong et al., 1985; Nicola, 1989) and on nonhematopoietic cells (Bussolino et al., 1989; Baldwin et al., 1989). The characterization of the cellular receptor for GM-CSF has been developed using intact cells or intact cellular membranes (DiPietro et al., 1988; Park et al., 1989; Chiba et al., 1990a, 1990b). A complementary DNA (cDNA) for GM-CSF receptor has recently been cloned screening a placental cDNA library (Gearing et al., 1989). However, biochemical analysis of the receptor proteins has yet been inadequate.

We have recently reported that the human GM-CSF receptor consists of at least two distinct proteins (Chiba et al., 1990b). A protein of around 80 kDa (α-chain) shows a lower affinity (Kd = 0.7–5 nM), faster dissociation, and broader expression not only on hematopoietic cells but also on various nonhematopoietic cells and tissues including placenta. In contrast, another protein of 135 kDa (β-chain) is related to higher affinity binding (Kd = 10–50 pM) and has slower dissociation and expression limited to hematopoietic cells. Thus, the placenta contains only the α-chain (Chiba et al., 1990b). The distinction between the two proteins was clearly demonstrated by the different proteolytic cleavage with the use of V8 protease. The cloned cDNA probably corresponds to the α-chain.

We report here (i) an assay of solubilized GM-CSF receptor α-chain utilizing polyethylene glycol precipitation, (ii) one-step affinity purification of the α-chain from placental tissues utilizing biotinylated GM-CSF, and (iii) identification of solubilized and purified receptor by photoaffinity labeling.

EXPERIMENTAL PROCEDURES

Recombinant Human GM-CSF and Its Radiolabeling—Recombinant human GM-CSF was expressed by Escherichia coli and was purified (Chiba et al., 1990b). It was iodinated by Bolton-Hunter reagent (ICN Chemicals, Radioisotope Division) (Bolton and Hunter, 1973) as described elsewhere (Chiba et al., 1990a) and yielded specific radioactivities of 5–10×10^6 cpm/ng of protein.

Preparation of photoactive N-hydroxysuccinimidyld-4-azidobenzoate (HSAB)-125I-GM-CSF was performed as described previously (Oka et al., 1985) with minor modifications. Briefly, 5 µg (0.33 nmol) of recombinant human GM-CSF was incubated with 37 MBq of Bolton-Hunter reagent in 10 µl of phosphate buffer (pH 8.0) for 8 h at 4 °C. Ten mg of HSAB (Pierce Chemical Co.) was freshly dissolved in 1 ml of dimethyl sulfoxide and diluted with distilled water to 1:100, and a 5-µl aliquot of the diluent (1.5 nmol) was added to the reaction mixture followed by a 30-min incubation at 24 °C in the dark. The reaction was quenched by adding 50 µmol of Tris-HCl, pH 7.4, and the reaction mixture was applied onto a Sephadex G-25 column equilibrated with phosphate-buffered saline without calcium and magnesium containing 0.25% gelatin. The separated HSAB-125I-GM-CSF was stored with the addition of bovine serum albumin at a final concentration of 0.1% in the dark at 4 °C for 4–6 weeks with a stable binding capacity. The entire procedure before irradiation using this preparation was carried out in the dark.

Preparation and Solubilization of Placental Cellular Membranes—The placental cellular membrane fraction containing microsomal and other membrane proteins was prepared from placentae as described by the method of DiPietro et al. (1988) with some modifications. The placental cellular membrane fraction was obtained from the homogenate at a concentration of 0.1% in the dark at 4 °C for 4–6 weeks with a stable binding capacity. The entire procedure before irradiation using this preparation was carried out in the dark.

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plasma membranes was prepared from three normal human fresh full-term placentae as described elsewhere (Posner, 1971). The homogenizing buffer contained 0.3 M sucrose, 25 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, 200 trypsin inhibitor units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The membrane fraction obtained as a 100,000 × g pellet was solubilized with 1.5% Triton X-100 containing 0.1% Nonidet P-40 and 0.5 M NaCl in 5 ml of 0.5 M HEPES, pH 7.4, 0.1% NaCl, 0.1% Triton X-100 was added at a final concentration of 0.1-0.125%, followed by the addition of 25% polyethylene glycol (M, 6,000) (PEG; Genzyme, Boston, MA) at a final concentration of 12.5%. The samples were mixed vigorously and allowed to stand on ice for 15 min, followed by centrifugation at 12,000 × g for 5 min at 4 °C. Phthalate oil was then layered on and centrifuged at 12,000 × g for a further 2 min at 4 °C to wash the pellets and the walls of the tubes. The radioactivity in the pellets was counted with a γ-counter (Aloka ARC-300). Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled GM-CSF. The binding data were transformed by the method of Scatchard, and the affinity to the affinity-purified material was also carried out by the same protocol.

Affinity Purification through Biotinylated GM-CSF Fixed to Streptavidin-Agarose—Five mg of GM-CSF was incubated with 300 μg of N-succinimidyl biotin (NSB, Pierce Chemical Co.) in 7.5 ml of phosphate buffer (10 mM, pH 8.3) for 30 min on ice. The biotinylated GM-CSF was then separated from the unincorporated NSB through Sephadex G-25 (equilibrated with 10 mM phosphate buffer containing 0.1% gelatin, pH 7.4) packed in a 2-cm diameter, 30-cm length column. Protein recovery was approximately 80% according to the Bio-Rad protein assay kit. Incorporation of NSB into GM-CSF was confirmed by enzyme-linked immunosorbent assay using affinity-purified rabbit anti-human GM-CSF polyclonal antibody and horseradish peroxidase avidin (Vector Laboratories, Inc., Burlingame, CA). Subsequently, biotinylated GM-CSF was mixed with a prewashed 5-ml bed volume of streptavidin-agarose (Pierce Chemical Co.) for 30 min at room temperature. After centrifugation, the agars were stored at 4 °C. Phosphate-buffered saline without calcium and magnesium containing 0.1% Nonidet P-40 (detergent buffer) and stored at 4 °C.

All of the following procedural steps were performed at 4 °C. The stored placental membrane extract (20 mg/ml, 100 ml) was thawed and diluted with 25 mM HEPES (pH 7.4) containing the protease inhibitors described above in order to reduce the concentration of Triton X-100. The final concentration of Triton X-100 was 0.75%, and we confirmed that GM-CSF fully bound the solubilized receptor in the presence of less than 1% Triton X-100 (data not shown). This sample was applied directly onto NSB-GM-CSF coupled gel with three cycles of absorption. The column was then washed with 3 volumes of the column buffer. Elution of GM-CSF receptor was carried out with 15 ml of citrate buffer (20 mM, pH 3.0) containing 0.1% Nonidet P-40 and 0.5 mM NaCl in 5 ml of 0.5 mM HEPES, pH 7.4, in order to compensate for the pH. The gradient for the pH or NaCl concentration was not advantageous for elution, probably due to the extremely rapid dissociation of the ligand from the α-chain as was shown previously (Chiba et al., 1990a). The eluted column was concen-

RESULTS

Biochemical and Biological Properties of HSAB, 125 I-GM-CSF—The GM-CSF with double modification by 125 I-Bolton-Hunter reagent and HSAB was examined for physiological integrity and biological activity. HSAB-125 I-GM-CSF as well as 125 I-GM-CSF migrated on a SDS gel as a single band at approximately 15 kDa (Fig. 1). We compared the biological activities of unlabeled and HSAB-125 I-GM-CSF. The growth stimulatory effect of HSAB-125 I-GM-CSF on TF-1 cells (Kimagura et al., 1989) was very close to that of unlabeled GM-CSF (data not shown) when estimated by a colorimetric assay. The binding property of HSAB-125 I-GM-CSF was also tested by Scatchard analysis of its binding to U-937 cells, which has two affinity classes of receptor, yielding biphasic plots (data not shown) similar to those observed using 125 I-GM-CSF without HSAB (Chiba et al., 1990a).

Assay of Solidolized GM-CSF Receptor—When we initially mixed γ-globulin (final concentration, 0.05%) and PEG (final concentration, 12.5%) with 125 I-GM-CSF, and centrifuged the mixture at 12,000 × g, approximately 95% radioactivity was detected in the supernatant. This predicted that the receptor-bound form of GM-CSF could be separated from free GM-CSF by PEG. 125 I-GM-CSF bound to solubilized GM-CSF receptor in a specific and saturable manner (Fig. 2B). The recovery of the protein from the membranes through solubilization was 70-80%, but the recovery of GM-CSF binding capacity was more than 100%, indicating that the GM-CSF binding activity was not decreased but rather increased through the solubilizing procedure. Triton X-100 did not affect the amount of specific binding at the concentration of 0.1-1% (data not shown). Scatchard analysis for the solubilized placental membranes was similar to that for the intact membranes (Fig. 2, A and B), indicating the existence of a single class of receptor with a Kd of 0.5-0.8 nM. The Scatchard

FIG. 1. Migration of GM-CSFs modified by Bolton-Hunter reagent alone (lanes a–c) or Bolton-Hunter reagent plus HSAB (lanes d–f) on sodium dodecyl sulfate-15% polyacrylamide gel. Approximately 15,000 cpm (lanes a and d), 30,000 cpm (lanes b and e), and 60,000 cpm (lanes c and f) were loaded.
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Fig. 2. Scatchard analysis of $^{125}$I-GM-CSF (A and B) or HSAB-$^{125}$I-GM-CSF (C) binding to intact placental membranes (A) and the solubilized samples (B and C). The samples were incubated at 4°C with increasing concentrations of $^{125}$I-GM-CSF or HSAB-$^{125}$I-GM-CSF in the binding buffer. Separation of receptor-bound radioactivities from free radioactivities was performed using 12.5% PEG for the solubilized samples. See details under “Experimental Procedures.” In the inset, the symbols represent specific binding (○), total binding (●), and nonspecific binding (□). Each experiment was performed in duplicate. Each panel shows mean values and standard deviations obtained from a representative experiment among five (A), six (B), or two (C) independent experiments which yielded similar results.

Fig. 3. Affinity labeling of intact placental membranes with HSAB-$^{125}$I-GM-CSF. A, following the binding reaction in the dark, the cross-linking reaction was carried out by increasing the time of irradiation. The cell extract using 1% Triton X-100 was subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. The binding was performed in the presence (lane h) or the absence (lanes a–g and i) of a 100-fold excess of unlabeled GM-CSF. The binding mixtures were irradiated for the following times: 0 s (lane a), 10 s (lane b), 20 s (lane c), 30 s (lane d), 1 min (lane e), 3 min (lane f), 5 min (lanes g and h), and 10 min (lane i). B, comparison between the irradiation protocol (lane a) and the protocol using DSS without irradiation (lane b) to cross-link HSAB-$^{125}$I-GM-CSF and α-chain receptor. A faint radioactivity spread around 50–60 kDa and a sharp band running at 48 kDa on lane a probably represent the degradation product by ultraviolet rays, since these bands were variably observed in the cross-linking experiments using the photoaffinity protocol.

Disuccinimidyl suberate (DSS) instead of irradiation was also used to label intact cellular membranes with HSAB-$^{125}$I-GM-CSF. A very similar band running at 70–95 kDa was visualized (Fig. 3B).

Purification of GM-CSF Receptor α-Chain by NSB-GM-CSF Affinity Chromatography—The solubilized GM-CSF receptor in the crude sample, the flow-throughs, and the eluate from the NSB-GM-CSF-streptavidin-agarose column were identified in a specific manner using HSAB-$^{125}$I-GM-CSF followed by application of the irradiation protocol (Fig. 4). A marked reduction in the nonspecific bands was noted in lanes g and h (the eluate) suggesting the purification of the receptors. A band of 70–95 kDa was detected in lane g and not detected in the presence of excess unlabeled GM-CSF as shown in lane h. The purification efficiency is summarized in Table I. When the eluate from the NSB-GM-CSF-streptavidin-agarose column was analyzed by SDS-gel electrophoresis and silver staining under reducing conditions, a single major band was detected approximately at 70 kDa (Fig. 5, lane a). After radioiodination of the purified material by the Bolton-Hunter method, the 70-kDa band as well as other minor lower molecular weight bands could be detected (Fig. 5, lane b). In two-dimensional electrophoresis, a single major radioactive spot was detected around 70 kDa with a pI of 6.6 (Fig. 6).
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Fig. 4. Photoaffinity labeling of the solubilized and affinity-purified GM-CSF receptor α-chain. The crude lysate, the first and the second flow-throughs passing the affinity column, or the eluate were mixed with 3 nM HSAB-125I-GM-CSF in the presence (lanes b, d, f, and h) or the absence (lanes a, c, e, and g) of excess unlabeled GM-CSF in the binding buffer at a final volume of 100 µl in a 96-well plate. After 90 min of incubation at 4 °C, 3 min of irradiation was performed with ultraviolet rays. The mixtures were directly added by 1/4 volumes of 5 × Laemmli sample buffer, boiled for 3 min, and subjected to SDS-polyacrylamide gel electrophoresis. The position of the band which specifically disappeared in the presence of excess unlabeled GM-CSF is shown by an arrow. The samples were loaded on a gel without separating receptor-bound radioactivity from free radioactivity, which thus caused fairly high background.

Table I

| Step                     | Protein (µg) | Maximal binding (nmol/mg) | Purification fold | Yield (%) |
|--------------------------|-------------|---------------------------|------------------|-----------|
| Placental membrane extract | 2,000,000   | 0.001                     | 1                | 100       |
| NSB-GM-CSF affinity column | 7          | 30                        | 20,000           | 11        |

Protein amount was estimated by the relative intensities of bands in silver-stained SDS gels.

The maximal binding was variable probably due to fine alteration of the condition of the precipitation. The value represents a mean of four individual experiments.

Discussion

Previous works on interaction of human GM-CSF and its receptor have been restricted to studies with intact cells (DiPersio et al., 1988; Gearing et al., 1988; Chiba et al., 1990a, 1990b; Miyagawa et al., 1990) or intact cellular membranes (Chiba et al., 1990b). In this paper, we first described the binding assay for the solubilized GM-CSF receptor by separating the bound form of 125I-GM-CSF from the free form by using PEG. The solubilized placental cellular membranes were successfully assayed for the GM-CSF receptor, demonstrating a single affinity class of receptor with a Kd of approximately 0.5–0.8 nM. The assay of the solubilized receptor could be used for further studies of the interaction of the ligand and receptor in a cell-free system.

Although a cross-linking technique using DSS worked well to identify the receptor in intact membranes, our initial trial using DSS in order to identify the solubilized GM-CSF receptor yielded very high backgrounds. Therefore, we developed labeling of 125I-GM-CSF with a heterobifunctional, photoreactive cross-linker, HSAB, and utilized it as a tool for affinity labeling of the receptor. The conjugation of 125I-GM-CSF with HSAB could be carried out without a detectable change of binding properties of 125I-GM-CSF judged from a Scatchard analysis of the binding to intact U-937 cells (data not shown) and to the solubilized placental membranes (Fig. 2, B and C). Very similar cross-linked bands were detected for intact placental membranes by utilizing either HSAB-125I-GM-CSF (either by the irradiation protocol or the protocol using DSS without irradiation; see Fig. 3) or 125I-GM-CSF (by the protocol using DSS (Chiba et al., 1990b)). Although HSAB is incorporated into the amino residues of the protein and

Fig. 5. Silver staining and radiolabeling of the purified material. A 5-µl aliquot of the purified and then concentrated material was applied to SDS-8% polyacrylamide gel electrophoresis and stained with silver reagent under reducing conditions (lane a). The same material was radiiodinated by the Bolton-Hunter method and directly electrophoresed with SDS-8% polyacrylamide gel (lane b). Some minor bands at approximately 35–40 kDa and smear radioactivities at a lower position were probably due to trace contaminants accumulated in the labeling.

Fig. 6. Two-dimensional electrophoresis of the purified and then radiiodinated material. Ten µl of the material was mixed with a 100-µl sample solution for isoelectric focusing containing 8.5 M urea, 2% Nonidet P-40, and 4% Ampholine (pH 4–8, Bio-Rad). The isoelectric focusing gel contained 0.4% acrylamide, 0.02% bis, 8.5 M urea, 2% Nonidet P-40, and 2% Ampholine with the same pH range. The radioactivity at the gel front probably represents again trace contaminants accumulated in the labeling or unincorporated Bolton-Hunter reagent. The arrow indicates the spot of interest.
GM CSF was iodinated with Bolton-Hunter reagent which is also incorporated into the amino residues, we could easily incorporate both molecules into GM-CSF retaining full binding capacity. This was so probably because human GM-CSF has seven free amino residues and some of them are not involved in the receptor binding site.

We previously suggested that human GM-CSF receptor consists of two proteins: a low affinity 80-kDa protein (α-chain) and a 135-kDa one (β-chain) which related to the high affinity binding. We also showed that human placental membranes and nonhematopoietic tumor cells contain the same α-chain (Chiba et al., 1990b; Miyagawa et al., 1990), although the molecular size of the placental α-chain was slightly smaller than that of the α-chain on the hematopoietic cells (Chiba et al., 1990b). Utilizing HSAB-Iz51-GM-CSF, the solubilized placental α-chain was successfully cross-linked for detection at 70–95 kDa (maximum radioactivity at 83 kDa), which was the same size as a band detected using the intact membranes, suggesting that the placental α-chain did not suffer from proteolysis or loss of binding activity during the solubilizing procedure. Although a high background which represents nonspecific cross-linking was observed when the membranes were solubilized, the specifically cross-linked band was apparent (Fig. 4).

The purified sample was applied to reversed-phase high performance liquid chromatography (HPLC) and a single major peak with an absorbance at 215 and 280 nm, and according to this the purity of the sample was more than 95% (data not shown). Therefore, some minor bands detected in the iodinated sample as well as dense radioactivity at the gel front (Fig. 5, lane b, and Fig. 6) were due to trace contaminants accumulated in the labeling. A direct sequencing of the N terminus on a gas-phase sequenator (470A, ABI) was carried out for the protein eluted at the peak (470A, ABI) was carried out for the protein eluted at the peak (i.e. subtracting 15 kDa of GM-CSF from 83 kDa). Its specific molecular weight of the purified protein corresponded more than 95% to that predicted for the α-chain by cross-linking studies (Chiba et al., 1990b; Miyagawa et al., 1990), although a high background which represents nonspecific cross-linking was observed when the membranes were solubilized, the specifically cross-linked band was apparent (Fig. 4).

In this paper, we describe the purification of the α-chain. We have to emphasize again, however, that the α-chain represents only the low affinity component of the GM-CSF receptor and that the GM-CSF receptor on hematopoietic cells consists of a multichain containing the β-chain which is concerned with the high affinity binding. Thus, it is necessary to study the β-chain to understand the nature of the GM-CSF receptor and the signal transduction mechanism operating through it.

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