Journal of Cellular Biochemistry 31:259–269 (1986)

Solubilization and Assay of a Colony-Stimulating Factor Receptor From Murine Macrophages

Yee-Guide Yeung, Paul T. Jubinsky, and E. Richard Stanley

Departments of Microbiology & Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

The colony-stimulating factor, CSF-1, selectively stimulates the survival, proliferation, and differentiation of mononuclear phagocytes. The solubilization, assay, and characteristics of the CSF-1 receptor from the J774.2 murine macrophage cell line are described. The recovery of cell-surface receptor in the postnuclear supernatant membrane fraction of hypotonically disrupted cells was 76%. Recovery of the ligand binding activity of the receptor after solubilization of this fraction with 1% Triton X-100 was ~150%. The binding of ^{125}I-CSF-1 to intact cells and membrane preparations was consistent with the existence of a single class of high-affinity receptor sites. In contrast, the equilibrium binding of ^{125}I-CSF-1 to the solubilized postnuclear fraction indicated the existence of two distinct classes of binding site (apparent K_ds 0.15 nM and 10 nM). A rapid assay was developed for the high-affinity sites, which were shown to be associated with the CSF-1 receptor. The function of the low-affinity sites, which have not been demonstrated on intact cells or cell membranes and which are 13 times more abundant than the high-affinity sites, is unknown. The solubilized high-affinity receptor-CSF-1 complex was stable on storage at 0°C and −70°C but dissociated at 37°C. Dissociation also occurred at 0°C in buffers of low pH (4.0) or high ionic strength (0.7 M NaCl).

Key words: CSF-1, CSF-1 receptor, mononuclear phagocytes, membrane proteins, colony-stimulating factor, murine macrophages

Colony-stimulated factors (CSFs) regulate the proliferation and differentiation of cells of the granulocytic and mononuclear phagocytic cell lineages [reviewed in 1]. CSF-1 is a lineage-specific growth factor eliciting a pleiotropic response by mononuclear phagocytes and their precursors [reviewed in 2]. It has been clearly discriminated from other CSFs by its detection in specific radioimmuno- and radioreceptor assays [3,4]. Murine and human CSF-1s are heavily glycosylated acidic glycoproteins

Y.-G. Yeung's present address is the University of Hong Kong, Hong Kong.

Received February 26, 1986; revised and accepted April 18, 1986.

© 1986 Alan R. Liss, Inc.

of M_r 45,000–76,000 consisting of two disulfide-bonded subunits of similar size and charge [5,6].

The pleiotropic response to CSF-1 includes regulation of survival, proliferation, cell morphology, and protein turnover [7,8]. The nature of the response depends on the differentiated state of the target mononuclear phagocyte [reviewed in 2]. Regulation of all components of the pleiotropic response is mediated via a high-affinity cell-surface receptor, which is selectively expressed on mononuclear phagocytic cells and their precursors [9–12] and is immunologically and functionally related to the c-fms proto-oncogene product [13]. Kinetic analyses of the binding reaction at 4°C or 37°C are consistent with the existence of a single class of high-affinity receptor sites on bone-marrow-derived and peritoneal exudate macrophages [14,15], and chemical cross-linking experiments with cell-surace-bound CSF-1 are consistent with the receptor being a polypeptide of ~ 165,000 M_r [16].

We selected the J774.2 clone [17] of the J774 cell line [18] as the starting cell population for the isolation of the CSF-1 receptor because of the ease with which it can be cultured in suspension. In this paper, we describe the solubilization, assay, and preliminary characterization of the CSF-1 receptor from J774.2 cells.

MATERIALS AND METHODS

Cell Culture and Harvesting

CSF-1 receptor-bearing cells of the J774.2 macrophage cell line [9,17] were maintained in spinner cultures of 10% (v/v) horse serum (Flow Laboratories) in alpha medium (Kansas City Biological). Cells were harvested in log-phase growth by centrifugation (400g, 30 min, 4°C), resuspended in 5 pellet volumes of 4 mM iodoacetic acid in phosphate-buffered saline, pH 7.4 (PBS-IAA) at 4°C, and counted by hemocytometer in the presence of trypan blue. The cells were discarded if fewer than 95% excluded dye.

Homogenization

Cells obtained as described above were centrifuged (400g, 10 min, 4°C) and the pellet was resuspended in 8 volumes of hypotonic buffer (75 mM sucrose, 1 mM iodoacetate, 0.5 mM ethyleneglycol-bis (β-aminoethyl ether) N,N^1-tetracetic acid (EGTA), 10 μg/ml leupeptin (Sigma), 0.5 unit/ml aprotinin (Sigma), 1,000 units/ml soybean trypsin inhibitor (type I-S, Sigma), 5 mM Tris-HCl, pH 8.0, at 0°C. Cells were allowed to swell (15 min) and were homogenized by 20–30 fast strokes in a Dounce homogenizer with a tight-fitting pestle. The percent cell breakage was determined by phase-contrast microscopy. When 90% of the cells were broken, 0.25 volume of compensating buffer (0.95 M sucrose, 26 mM Mg Cl_2, 0.15 M NaCl, 0.15 M KCl, 5 mM EGTA, 20 mM Tris-HCl, pH 7.4) was added to render the medium isotonic and stabilize the nuclei.

Fractionation of the Homogenate

The homogenate was centrifuged (1,000g, 45 sec, 4°C), the supernatant was saved, and the pellet was washed once with 0.33 homogenate volume of isotonic buffer (1 volume hypotonic buffer + 0.25 volume compensating buffer) by resuspension and centrifugation (1,000g, 20 sec, 4°C). Both supernatants were combined (postnuclear fraction) and layered on top of 0.3 volume of 15% sucrose, 5 μg/ml

leupeptin, 0.1 unit/ml aprotinin, and 100 units/ml soybean trypsin inhibitor, 100 mM Tris-HCl, pH 7.4, and centrifuged (Beckman SW27 rotor, 100,000g, 30 min, 4°C). The pellet was resuspended by Dounce homogenization with a loose-fitting pestle in 20 pellet volulmes of 10% sucrose, 50 mM NaCl, 10 μg/ml leupeptin, 1 unit/ml aprotinin, and 1,000 units/ml soybean trypsin inhibitor, 100 mM Tris-HCl, pH 7.4 (postnuclear particulate fraction).

Solubilization of the Receptor

The post nuclear particulate fraction was adjusted to 1% with respect to Triton X-100 by addition of 20% Triton X-100 and mixed by gentle homogenization in a Dounce homogenizer with a loose-fitting pestle for 5 min at 4°C. The resulting mixture was diluted by addition of an equal volume of 10% sucrose, 50 mM NaCl, 100m mM Tris-HCl, pH 7.4, centrifuged (100,000g, 4°C, 45 min) and the supernatant was saved (solubilized receptor preparation).

Assay of the Receptor in Intact Cells, Homogenates, and Suspension of the Cell Particulate Fraction

L cell CSF-1 was purified as previously described [5,10]. Purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing and non-reducing conditions and by complexing with rabbit anti-CSF-1 antibody [5,10]. CSF-1 concentration in units was determined by radioimmunoassay [3,10]. One unit equals ~0.44 fmol. CSF-1 was iodinated with carrier-free ^{125}I (Amersham) with full retention of biological activity [3,10]. The immunological activity of the ^{125}I-CSF-1, which reflects its biological activity [10], was assessed either by radioimmunoassay with ^{131}I-CSF-1 and double label counting or by immunoprecipitation with excess rabbit anti-CSF-1 antiserum [3,10]. The mean specific radioactivity [10] was approximately 400,000 cpm/ng of biologically active CSF-1 protein. To reduce retention of free ^{125}I-CSF-1 by the filters used in the assay of solubilized receptor (see below), the ^{125}I-CSF-1 was brought to 10% (v/v) with respect to normal rabbit serum, to 10% (w/v) with respect to polyethylene glycol (PEG 8000; 8,000 M_r, Sigma), centrifuged (2,000g, 10 min, 4°C), filtered (0.45 μm cellulose acetate membrane, EHWP, Millipore Corp.), dialyzed (assay buffer, see below, 16 hr, 4°C), and refiltered (0.22 μm cellulose acetate membrane) prior to storage at −20°C.

Incubation mixtures for receptor assays consisted of 10 μl of sample, 40 μl assay buffer (α medium containing 25 mM HEPES (Grand Island Biological Co.) in lieu of bicarbonate (α-HEPES), 0.2% bovine serum albumin (BSA) (A4378, Sigma) and 0.02% NaN_3, pH 7.35), 5 μl of either purified L cell CSF-1 (120,000 units per ml in PBS) or 5 μl PBS, and 5 μl of ^{125}I-CSF-1 (100,000 cpm; final concentration 1.7×10^6 cpm/ml). The competing unlabeled CSF-1 was added 30 min prior to the ^{125}I-CSF-1. Incubation with ^{125}I-CSF-1 was carried out at 0°C for 1 hr; then 50 μl of the mixture was filtered through a 0.22-μm membrane filter (GVWP, Millipore Corp.) which had been presoaked in 0.2% BSA in PBS at 0°C. The filter was washed three times with 0.4-ml aliquots of same BSA solution at 0°C prior to counting in a gamma counter. The filter was shown to retain 90% of the cpm of the postnuclear fraction of cells to which ^{125}I-CSF-1 had been prebound. In contrast, it retained 0.02% of the ^{125}I-CSF-1 in assay buffer in the absence of the postnuclear fraction.

PEG 8000 Precipitation of the Solubilized Receptor-CSF-1 Complex

A concentrated membrane preparation from 7×10^6 cells was incubated with 2.7×10^7 cpm ^{125}I-CSF-1 per ml in assay buffer for 3.5 hr at 0°C (final volume =

0.3 ml). The preparation was brought to 1% with respect to Triton X-100 and centrifuged (188,000g, 19 hr, 0°C) through a 5–20% (w/v) sucrose gradient in 20 mM $MgCl_2$, 20 mM HEPES, 0.1% Triton X-100, pH 7.4, to separate the receptor-^{125}I-CSF-1 complex from free ^{125}I-CSF-1. The peak fraction of receptor-^{125}I-CSF-1 complex was used for the PEG 8000 precipitation study. This fraction was identified by its absence in a simultaneous centrifugation at pH 4.0 (a dissociating condition). The complex or ^{125}I-CSF-1 alone in 1/16 rabbit serum in 0.09 M HEPES, pH 7.4 (final volume 0.5 ml) was mixed with an equal volume of PEG 8000 of the appropriate concentration and filtered through a 0.45-μm cellulose acetate membrane. The precipitate was washed with 14 ml of the corresponding final concentration of PEG 8000 and then counted for radioactivity.

Assay of the Solubilized Receptor

Incubation buffer and conditions were as described above for the assay of the particulate receptor except that the assay buffer contained 0.1% Triton X-100. At the end of the incubation, 10 μl of 50% (v/v) normal rabbit serum in PBS at 0°C was added as carrier protein, followed by 70 μl of 20% (w/v) polyethylene glycol (PEG 8000; 8,000 M_r, Sigma) in 10 mM HEPES pH 7.4 at 0°C. The mixture was allowed to stand for 5 min. A 100-μl aliquot was filtered through a 0.45-μm cellulose acetate membrane (EHWP, Millipore Corp.) that had been soaked in 10% PEG 8000 in 10 mM HEPES pH 7.4 at 0°C. The precipitate retained by the filter was washed twice with 0.4-ml aliquots of 10% PEG 8000 in 10 mM HEPES pH 7.4 prior to counting in a gamma counter. All data were corrected for the binding of ^{125}I-CSF-1 to the membrane filters by subtracting the cpm retained from incubation mixtures that were incubated with the competing unlabeled CSF-1 for 30 min, followed by the addition of the rabbit serum carrier, PEG 8000, the ^{125}I-CSF-1, and immediate filtration. The retention of ^{125}I-CSF-1 by the filters was not prevented by presoaking them in 0.2% BSA or 1,000 units/ml CSF-1 and could amount to as much as 6% of the total cpm added to the incubation mixture. As it varied with the batch number of the filters, batches exhibiting the lowest retention of ^{125}I-CSF-1 were selected for use in the assay.

Protein Determinations

Protein determinations on the postnuclear particulate and solubilized postnuclear particulate fractions were carried out according to the method of Bradford [19]. One milliliter of 5% trichloroacetic acid (TCA) was added to 50 or 100 μl of the sample; the precipitate was collected by centrifugation and extracted twice with acetone, and the final pellet was dried in a gentle stream of N_2. The dried pellet was dissolved in 150 μl of 0.2 M NaOH; 50 μl of 0.5 M HCl was added and 10–50 μl was used with 2.5 ml of the Coomassie blue reagent.

RESULTS

Subcellular Fractionation of Cells Prebound With ^{125}I-CSF-1

Initial experiments on the localization of the receptor among subcellular fractions utilized the fact that at 2°C CSF-1 irreversibly binds its receptor on intact cells [10]. J774.2 cells were incubated with saturating concentrations of ^{125}I-CSF-1 for 1 hr at 2°C, separated from the free ^{125}I-CSF-1, and fractionated into nuclear and

postnuclear particulate (membrane) fractions, and the fractions were counted for ^{125}I. It can be seen (Table I) that 76% of the cell-surface receptor binding activity is recovered in the post nuclear particulate fraction. This postnuclear fraction was analyzed further by centrifugation in a six-step 10–55% (w/v) discontinuous sucrose gradient (69,000g, Beckman SW-41 rotor, 15 min, 4°C); ^{125}I was associated with subcellular particles that were broadly distributed over fractions in the 35–50% sucrose range. Because of the broad distribution of the CSF-1 receptor among these fractions, the postnuclear fraction was used without further purification as starting material for receptor solubilization.

As in the case of intact cells [9–11] the binding of ^{125}I-CSF-1 by postnuclear supernatant fraction at 2°C was saturable at 1 nM ^{125}I-CSF-1 and competed for by a preincubation with 100 nM CSF-1 (data not shown). The postnuclear supernatant fraction could be pelleted by centrifugation and resuspended by Dounce homogenization, as described in Materials and Methods, with a recovery of 60% of the ^{125}I-CSF-1 binding activity. Storage of the pellet for a week at − 70°C did not alter this recovery.

Assay of the Solubilized Receptor

Conventional assay for solubilized receptors require the efficient separation of the receptor-ligand complex from the free ligand. Initial experiments with Triton X-100-solubilized membrane fractions from cells pre-bound with ^{125}I-CSF-1 indicated that separation of the receptor-^{125}I-CSF-1 complex from free ^{125}I-CSF-1 could be effected by slight modification of the polyethylene glycol precipitation procedure of Cuatrecasas [20]. It can be seen (Fig. 1) that maximum precipitation of the complex (60% of the total) occurred between 9 and 12% (w/v) PEG 8000. While no significant precipitation of ^{125}I-CSF-1 was observed at concentrations of PEG 8000 as high as 18%, concentrations of PEG 8000 higher than 12% resulted in a drop in the amount of complex precipitated that was compatible with a PEG-induced dissociation of ^{125}I-CSF-1 from the complex. A concentation of 10% PEG 8000, which precipitated 60% of the total complex was therefore chosen for separation of receptor-^{125}I-CSF-1 complex from free ^{125}I-CSF-1 in the assay of the solubilized receptor.

TABLE I. Subcellular Fractionation Of Cells Prebound With ^{125}I-CSF-1*

Fraction	Total ^{125}I-CSF-1 bound (cpm × 10^{-6})	Recovery (%)
Intact cells	2.84	100.0
Nuclei	0.12	4.3
Postnuclear particulate fraction	2.17	76.0

*Cells (5×10^7) were incubated with 3.2×10^6 cpm of ^{125}I-CSF-1 (CSF = colony-stimulating factor) in 2.0 ml of assay buffer for 1 hr at 0°C. The cell suspension was carefully overlayered on 8 ml of horse serum and centrifuged (800g, 10 min, 4°C); the pellet was resuspended in 2.0 ml of assay buffer, and the centrifugation through horse serum was repeated prior to resuspending the cells in homogenization medium. Homogenization was carried out as described in the Methods.

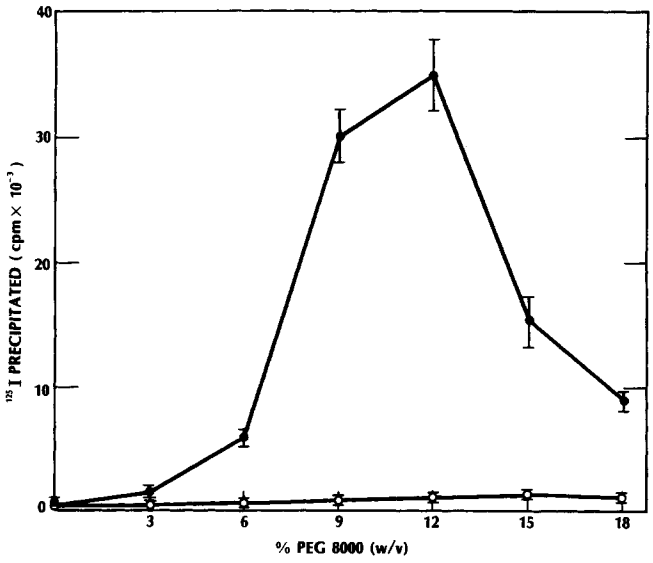

Fig. 1. Polyethylene glycol (PEG) 8000 precipitation of the solubilized receptor-^{125}I-CSF-1 (CSF = colony-stimulating factor) complex. Precipitation of the receptor-^{125}I-CSF-1 complex prepared by sucrose density gradient zone sedimentation (●) and of ^{125}I-CSF-1 (○). Incubation mixtures contained 54,000 cpm of receptor-^{125}I-CSF-1 complex or 44,000 cpm of ^{125}I-CSF-1.

Solubilization of the CSF-1 receptor was achieved by treatment of the postnuclear fraction with 1% Triton X-100, and subsequent removal of residual insoluble material was achieved by centrifugation. For rapidity and convenience, the initial incubation period chosen for the assay of the solubilized receptor was 1 hr. The other conditions (^{125}I-CSF-1 concentration, temperature, and pH) have been shown to result in saturation of the receptor sites in 1-hr incubations of ^{125}I-CSF-1 with intact cells [9–11]. Subsequent experiments demonstrated that at the ^{125}I-CSF-1 concentration used (0.15 nM, 1.7 × 10^6 cpm/ml) only 45% of the high-affinity receptor sites were occupied at equilibrium (see Fig. 4) and that only 62.5% of the maximum binding at equilibrium was achieved in the 1-hr-incubation, 10% of which was due to binding by a lower-affinity site (see Fig. 3). Thus, the overall efficiency of the assay was 0.6 (fraction of complex precipitated by PEG 8000) × 0.45 × 0.625 × 1.1 = 0.19 (19%). Assay results were reproducible under the defined conditions and the amount of binding by the solubilized receptor was directly proportional to the amount of solubilized membrane protein in the assay (Fig. 2).

Recovery and Stability of the Solubilized Receptor

The recovery of the ^{125}I-CSF-1 binding activity of the receptor following its solubilization with Triton X-100 from the postnuclear particulate fraction is presented in Table II. Addition of Triton X-100 to the postnuclear fraction resulted in an apparent reduction of the recovered receptor-associated ^{125}I-CSF-1. This may be explained by the lower efficiency of the assay for solubilized receptor compared with that of the membrane-bound receptor. Most of the ^{125}I-CSF-1 binding activity was recovered in the solubilized fraction; less than 4% of the recovered activity was particulate. Correction for the efficiency of the solubilized receptor assay indicated

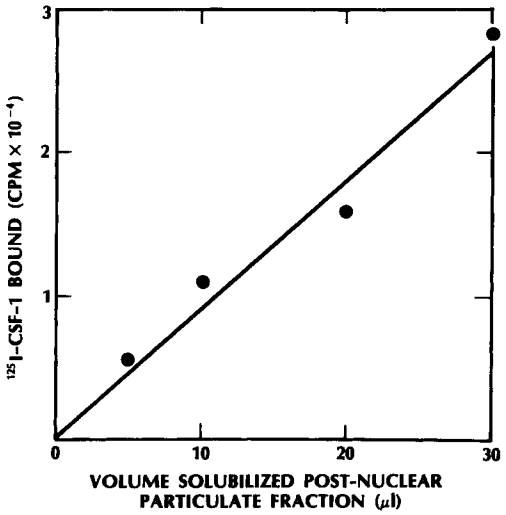

Fig. 2. Linearity of the relationship between sample volume and ^{125}I-CSF-1 bound in the assay of the solubilized CSF-1 receptor. Means of duplicate incubations. Protein concentration of solubilized postnuclear particulate fraction, 1.7 mg/ml.

TABLE II. Solubilization of the CSF-1 Receptor*

Fraction	Total ^{125}I-CSF-1 bound (cpm × 10^{-6})	Recovery of ^{125}I-CSF-1 binding activity (%)	Calculated recovery of receptor sites[a]
Postnuclear particulate fraction	103.4	100.0	100.0
Postnuclear particulate fraction in 1% Triton X-100	34.0	32.9	171.4
Solubilized postnuclear particulate fraction	29.3	28.3	147.4
Triton X-100 insoluble post-nuclear particulate fraction	1.3	1.3	6.8

*Cells (6.5×10^8) were fractionated and the fractions were assayed for receptor protein as described in Materials and Methods. Means of duplicate determinations.

[a]Recovery of receptor sites corrected for the fractions of receptor-CSF-1 complexes precipated by 10% polyethylene glycol (PEG) 8000 (0.6) and the fraction of the total number of high-affinity sites binding ^{125}I-CSF-1 in the 1-hr incubation (from Fig. 3) with 100,000 cpm ^{125}I-CSF-1 (from Fig. 4) ($0.45 \times 0.625 = 0.32$, see text).

that the recovery of receptor sites in the solubilized postnuclear fraction was ~150%. Thus solubilization of the postnuclear particulate fraction actually increases the number of receptor sites available for binding.

The ^{125}I-CSF-1 binding activity of the solubilized postnuclear fraction was stable on storage overnight at 4°C and for prolonged periods of time at −70°C in the presence or absence of dimethyl sulfoxide. Activity was not lost following a tenfold concentration by pressure dialysis at 4°C.

Binding of ^{125}I-CSF-1 by the Solubilized Receptor Preparation at 0°C

The solubilized postnuclear particulate fraction was used to examine the binding kinetics and equilibrium binding of ^{125}I-CSF-1 to components of the solubilized receptor preparation. Maximal binding in the presence of 0.15 nM ^{125}I-CSF-1 was attained by approximately 4 hr of incubation at 0°C. Almost all of the binding was competed for by a 30-min preincubation with 30-fold excess CSF-1 that was not removed during the subsequent incubation with ^{125}I-CSF-1. In order to examine the reversibility of binding, solubilized membrane preparations that had been incubated with the ^{125}I-CSF-1 for 2 hr were brought to 9 nM with respect to CSF-1 and the incubation was continued. A small proportion ($\sim$ 10%) of the ^{125}I-CSF-1 bound at 2 hr dissociated rapidly. The remainder failed to dissociate over the following 4-hr period. The amount of ^{125}I-CSF-1 dissociated in the experiment described in Figure 3 equals the amount bound to the low-affinity binding sites at this ^{125}I-CSF-1 concentration (calculated from Fig. 4, see below). It is therefore likely that the measured dissociation of ^{125}I-CSF-1 is from the low-affinity sites.

Stable maximal binding of ^{125}I-CSF-1 to the solubilized receptor preparation was also observed within 4 hr of incubation at one-hundredth the concentration of ^{125}I-CSF-1 used in Figure 3. In contrast to incubations with peritoneal exudate macrophages [10], unoccupied receptor sites were stable under these conditions so that equilibrium binding studies could be carried out. During a 4-hr incubation, it can be seen (Fig. 4) that the binding of ^{125}I-CSF-1 to components of the solubilized postnuclear fraction did not saturate even at very high concentrations of ^{125}I-CSF-1. Scatchard analysis [21] of the binding that was competed by 50-fold excess CSF-1 was consistent with the existence of two classes of binding sites. The high-affinity sites (apparent $K_d \sim 0.15$ nM; the irreversibility of binding to the high-affinity site invalidates determination of the true K_d by the method of Scatchard) were present at a concentration of .0415 nM. The low-affinity sites ($K_d \sim 9.9$ nM) were present at a concentration of 0.534 nM. Several observations (summarized in the Discussion) indicate that the high-affinity binding is to the CSF-1 receptor.

Properties of the Solubilized CSF-1 Receptor

All binding activity of the solubilized receptor was lost following incubation of the solubilized postnuclear fraction at 90°C for 1 min or within 1 min of its exposure to 0.1% SDS at 4°C.

The stability of the ^{125}I-CSF-1-receptor complex was initially investigated by using sucrose density gradient centrifugation to separate free ^{125}I-CSF-1 from the complex. The ligand-receptor complex was found to remain irreversibly bound after 5 days at −71°C or after 3 days at 0°C. As with CSF-1 bound at the cell surface, dissociation occurs on warming. One third of the complex was dissociated following a 1-hr incubation at 37°C. At 0°C the complex was stable in the presence of 0.13 M NaCl but completely dissociated in 0.67 M NaCl or in 0.1 M acetate buffer, pH 4.0.

DISCUSSION

Previous work on the interaction of CSF-1 with its receptor has been restricted to studies with intact cells. The assay and characterization of the solubilized receptor were carried out in order to obtain an active soluble receptor preparation which could be used to further characterize the receptor and its interactions with ligand in a cell-

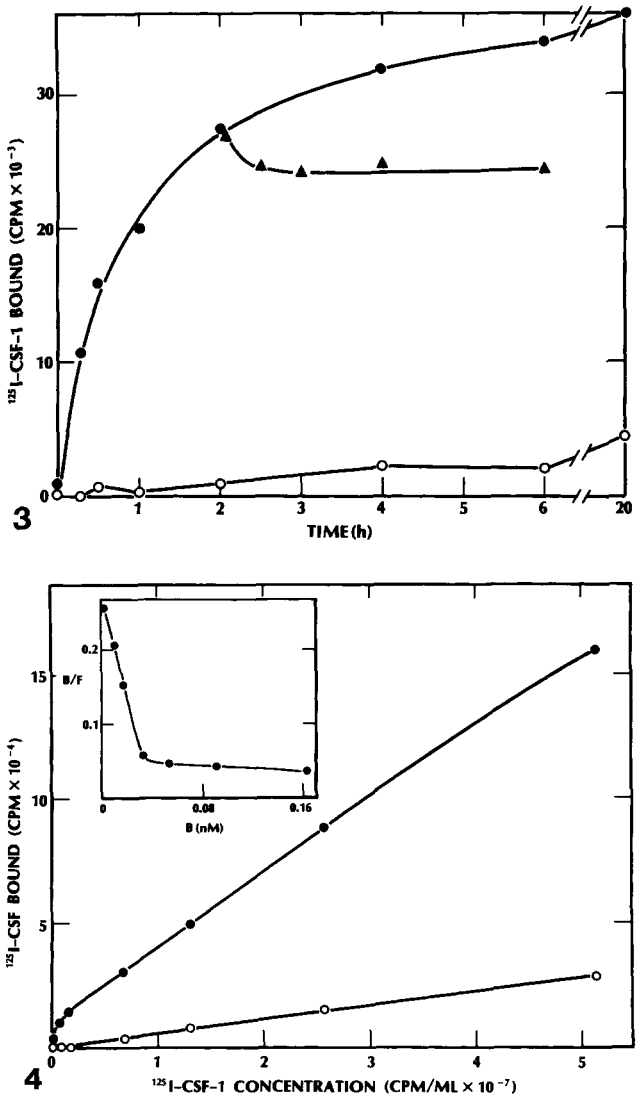

Fig. 3. Time course of binding of ^{125}I-CSF-1 to the solubilized postnuclear particulate fraction at 0°C. (●), Binding in the presence of 0.154 nM ^{125}I-CSF-1 (1.7 × 10^6 cpm/ml). (○), Binding in the presence of 0.15 nM ^{125}I-CSF-1 following a 30-min preincubation in 4.5 nM CSF-1. (▲), Incubations containing 0.15 nM ^{125}I-CSF-1 that were made 9 nM with respect to CSF-1 by addition of 2 μl of concentrated CSF-1 after 2 hr of incubation. Means of duplicate 60-μl incubations each containing 55 μg of postnuclear fraction protein.

Fig. 4. Equilibrium binding of ^{125}I-CSF-1 to the solubilized postnuclear particulate fraction at 0°C. (●), Incubation for 4 hr in ^{125}I-CSF-1 alone. (○), Incubation for 4 hr in ^{125}I-CSF-1 following a 30-min preincubation in a 50-fold-higher concentration of CSF-1. Incubation mixtures (70 μl) contained 12.5 μg of solubilized postnuclear fraction protein. Inset: Plot of data according to the method of Scatchard [21]. Abscissa, bound ^{125}I-CSF-1; ordinate, ratio of bound to free ^{125}I-CSF-1 (unitless).

free system. Results of subcellular fractionation of cells prebound with ^{125}I-CSF-1 indicated that there was a 76% recovery of cell-surface binding sites in the postnuclear supernatant fraction. The essentially irreversible, saturable, and high-affinity binding exhibited by whole cells was mimicked by this membrane preparation. The recovery of receptor sites in the postnuclear supernatant fraction after solubilization, however, was greater than 140%. This is probably due to the existence of " inside-out" vesicles containing inaccessible receptors which become available after solubilization [22]. Even without the contribution from the receptors within the vesicles, the overall recovery of cellular binding sites in solubilized membrane fraction is extremely good (~28%, Table II). These procedures have been used in the initial part of the purification of the CSF-1 receptor-kinase (unpublished observations). Although the extent to which intracellular receptor sites contributed to the overall recovery was not determined, it has been estimated that there are only twice as many intracellular sites as cell-surface sites on bone-marrow-derived macrophages [14].

The procedure used to assay the solubilized receptor is relatively rapid and highly reproducible. Under the defined conditions, a low-affinity binding component, also present in the solubilized postnuclear fraction, contributes to the total binding to a small degree (~10%). This low-affinity binding is readily reversible compared to the high-affinity binding. Thus its contribution may be eliminated, if necessary, by a 30-min incubation with CSF-1 (final concentration, 10 nM), after the 1-hr incubation with ^{125}I-CSF-1 (Fig. 3). Evidence that the assay is detecting the solubilized CSF-1 receptor comes from several sources. The measured recovery of solubilized binding sites from cells is consistent with a good recovery of estimated total cellular receptor pool (see above). Furthermore, both the kinetic analysis of the binding to intact cells and the equilibrium analysis of the binding of CSF-1 to the high-affinity component of the solubilized postnuclear supernatant fraction are consistent with the existence of a single class of binding sites [14,15, this paper]. Finally, the purification of the solubilized CSF-1 receptor by using this assay yields a molecule of M_r 165,000, which corresponds to the M_r of receptor derived from chemical cross-linking studies (unpublished observations). The purified receptor also possesses functional characteristics (specific CSF-1 binding, CSF-1-stimulated autophosphorylation and kinase activity) of the receptor in membrane preparations.

Equilibrium binding of ^{125}I-CSF-1 to intact cells at 4°C could not be carried out due to the long time period required to attain equilibrium at low concentration of ligand and the relative instability of unoccupied binding sites compared with occupied binding sites over this period [10]. However, kinetic analyses of the binding to the receptor on intact peritoneal exudate or bone-marrow-derived macrophages are consistent with the interaction of CSF-1 with a single class of receptor sites with a K_d of $\leqslant 10^{-13}$ M [14,15]. The stability of the receptor in the solubilized postnuclear supernatant fraction and the relatively rapid attainment of stable maximum binding at low concentrations of ligand has allowed equilibrium binding to be analyzed in this system. In contrast to binding to the receptor on intact cells, two distinct classes of binding site were observed with apparent K_ds of 0.15 nM and ~10 nM. The reason for the difference in apparent K_d between the membrane-associated and the solubilized (apparent K_d, 0.15 nM) receptor has not been established, although similar differences have been observed for other receptors [23–25]. The low-affinity site (K_d ~ 10 nM) was ~ 13 times more abundant than the high-affinity site. As such, it seems too abundant to represent the "cryptic" internal pool of receptors shown to be readily

available to the macrophages on rapid internalization of cell-surface receptors [14]. The possible role of the lower-affinity sites and their functional relationship (if any) to CSF-1 remain to be explained.

ACKNOWLEDGMENTS

This work was supported by NIH grant CA 26504 and by the AECOM Cancer Center Core Grant. P.T.J. is supported by NIH training grant GM 7288 from the NIGMS. E.R.S. is the recipient of a Irma T. Hirschl Career Scientist Award. We thank Dr. D. Yeung for reviewing the manuscript, Michelle Hankins, Suzanne Saltzman, and Fran Pellerito for technical assistance, and Lenore Grollman for help in preparing the manuscript.

REFERENCES

1. Stanley E.R.: In Stewart WE, Hadden JW (eds): "The Lymphokines." Humana Press, N.J., 1981, pp 102–132.
2. Stanley ER, Guilbert LJ, Tushinski RJ, Bartelmez SH: J Cell Biochem 21:151–159, 1983.
3. Stanley ER: Proc Natl Acad Sci USA 76:2969–2973, 1979 (erratum ibid 76:5411).
4. Das SK, Stanley ER, Guilbert LJ, Forman LW: Blood 58:630–641, 1981.
5. Stanley ER, Heard PM: J Biol Chem 252:4305–4312, 1977.
6. Das SK, Stanley ER: J Biol Chem 257:13679–13684, 1982.
7. Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER: Cell 28:71–81, 1982.
8. Tushinski RJ, Stanley ER: J Cell Physiol 116:67–75, 1983.
9. Guilbert LJ, Stanley ER: J Cell Biol 85:153–159, 1980.
10. Stanley ER, Guilbert LJ: J Immunol Methods 42:253–284, 1981.
11. Byrne PV, Guilbert LJ, Stanley ER: J Cell Biol 91:848–853, 1981.
12. Chen D-M, Lin H-S: Exp Cell Res 140:323–329, 1982.
13. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER: Cell 41:665–676, 1985.
14. Guilbert LJ, Stanley ER: J Biol Chem, 261:4024–4032, 1986.
15. Guilbert LJ, Tynan PW, Stanley ER: J Cell Biochem, 31:203–216, 1986.
16. Morgan CJ, Stanley ER: BBRC 119:35–41, 1984.
17. Diamond B, Bloom BR, Scharff MD: J Immunol 121:1329–1333, 1978.
18. Ralph P, Prichard J, Cohn M: J Immunol 114:898–905, 1975.
19. Bradford M: Anal Biochem 72:248–254, 1976.
20. Cuatrecasas P: Proc Natl Acad Sci USA 69:318–322, 1972.
21. Scatchard G: Ann NY Acad Sci 51:660–672, 1949.
22. Deutsch PJ, Rosen OM, Rubin CS: J Biol Chem 257:5350–5358, 1982.
23. Shiu RPC, Friesen HG: J Biol Chem 249:7902–7911, 1974.
24. Yarden Y, Harari I, Schlessinger J: J Biol Chem 260:315–319, 1985.
25. Rosengurt E, Collins M, Brown KD: J Biol Chem 257:3680–3686, 1982.