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The c-*fms* Proto-oncogene Product Is Related to the Receptor for the Mononuclear Phagocyte Growth Factor, CSF-1

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Summary

The feline c-*fms* proto-oncogene product is a 170 kd glycoprotein with associated tyrosine kinase activity. This glycoprotein was expressed on mature cat macrophages from peritoneal inflammatory exudates and spleen. Similarly, the receptor for the murine colony-stimulating factor, CSF-1, is restricted to cells of the mononuclear phagocytic lineage and is a 165 kd glycoprotein with an associated tyrosine kinase. Rabbit antisera to a recombinant v-*fms*-coded polypeptide precipitated the feline c-*fms* product and specifically cross-reacted with a 165 kd glycoprotein from mouse macrophages. This putative product of the murine c-*fms* gene exhibited an associated tyrosine kinase activity in immune complexes, specifically bound murine CSF-1, and, in the presence of the growth factor, was phosphorylated on tyrosine in membrane preparations. The murine c-*fms* proto-oncogene product and the CSF-1 receptor are therefore related, and possibly identical, molecules.

Introduction

Multiple and diverse etiologic agents of cancer have been proposed to act on a restricted subset of proto-oncogenes (or c-*onc* genes) that exhibit a latent potential for transforming cells and contributing to their malignant phenotype (Bishop, 1983; Heldin and Westermark, 1984). The normal role of these genes is presumably to regulate the processes of cell proliferation and differentiation by coding for products that describe critical steps in growth regulation. The term proto-oncogene may therefore be a misnomer, underscoring the role of these genes as determinants of malignant transformation rather than as elements defining a "mitogenic pathway." Of approximately two dozen proto-oncogenes now identified, one (c-*sis*) codes for a polypeptide chain of the platelet-derived growth factor (PDGF) (Waterfield et al., 1983; Doolittle et al., 1983), and another (c-*erb* B) is a cognate of the gene encoding the receptor for epidermal growth factor (EGF) (Downward et al., 1984). The EGF receptor is a member of the family of proteins that exhibit tyrosine-specific protein kinase activity (Ushiro and Cohen, 1980; Hunter, 1984). These enzymes have been identified as components of receptors for other polypeptide hormones, including PDGF (Ek et al., 1982; Nishimura et al., 1982), insulin (Kasuga et al., 1982), and the insulin-like growth factor 1 (Jacobs et al., 1983), as well as in several other viral transforming proteins including those specified by v-*src*, v-*abl*, v-*fes/fps*, v-*ros*, v-*yes/fgr*, and v-*fms* (recently reviewed in Hunter, 1985). The relationship of the c-*erb* B product to the EGF receptor suggests that other retroviral oncogene products might similarly represent altered forms of cell surface receptors for regulatory growth factors.

The viral oncogene (v-*fms*) of the McDonough strain of feline sarcoma virus (SM-FeSV) encodes a 140 kilodalton (kd) integral transmembrane glycoprotein (gp140$^{v\text{-}fms}$) (Anderson et al., 1982, 1984; Manger et al., 1984; Rettenmier et al., 1985b) whose expression at the cell surface is required for transformation (Roussel et al., 1984). The v-*fms* gene product exhibits biochemical and topological properties of known cell surface receptors. The mature glycoprotein is oriented in the plasma membrane with its glycosylated amino-terminal domain (approx. 450 amino acids) outside the cell and its carboxy-terminal domain (approx. 400 amino acids) in the cytoplasm (Hampe et al., 1984; Rettenmier et al., 1985b). Epitopes in the amino-terminal domain of gp140$^{v\text{-}fms}$ were detected on the surfaces of live transformed cells using fluorescent or peroxidase-labeled antibody conjugates (Anderson et al., 1984; Roussel et al., 1984; Manger et al., 1984; Rettenmier et al., 1985b), and the molecules were found to be associated with clathrin-coated pits and to gain access to endosomes (Manger et al., 1984). Nucleotide sequence analysis predicted that the cytoplasmic carboxy-terminal domain of the glycoprotein was closely related to sequences of prototypic tyrosine-specific protein kinases (Hampe et al., 1984); indeed, immune complexes prepared with antibodies to the v-*fms* product exhibit an associated tyrosine kinase activity that phosphorylates the glycoprotein in vitro (Barbacid and Lauver, 1981; Roussel et al., 1984).

The c-*fms* proto-oncogene is expressed at relatively high levels in cat spleen and at considerably lower levels in other cat tissues including bone marrow, liver, and brain. A 170 kd product of the c-*fms* gene identified in normal cat spleen was found to be a glycoprotein that functioned as a substrate in vitro for an associated tyrosine kinase (Rettenmier et al., 1985a). Because adult cat splenocytes consist predominantly of erythrocytes, lymphoid cells, mature granulocytes, and tissue macrophages, we reasoned that the c-*fms* proto-oncogene could encode a receptor for one of the known interleukins, erythroid growth factors, or granulocyte/macrophage-colony-stimulating factors (CSFs).

The macrophage growth factor, CSF-1, stimulates hematopoietic precursor cells to form colonies containing mononuclear phagocytes (Stanley and Guilbert, 1980). Unlike the granulocyte/macrophage-colony-stimulating factor and interleukin-3, which also directly induce mononuclear phagocyte proliferation (Burgess et al., 1977;

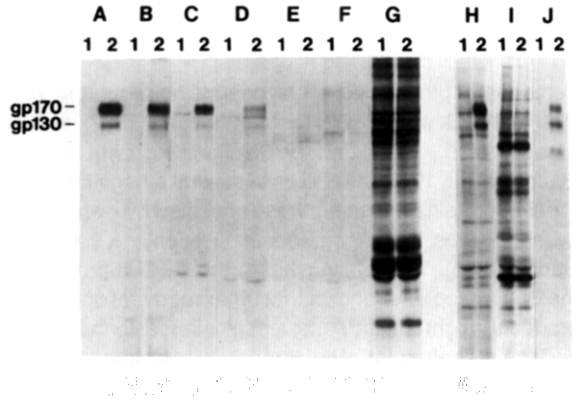

Figure 1. Expression of c-*fms* Gene Products in Cat Tissues

(Left) Acute inflammatory exudates were elicited by intraperitoneal inoculation of oyster glycogen into animals, and cells were recovered by peritoneal lavage (lanes A). Organs obtained at necropsy included spleen (lanes B), lymph nodes (lanes C), liver (lanes D), lung (lanes E), kidney (lanes F), and brain (lanes G). Tissue extracts were incubated either with control myeloma protein (lanes 1) or with a mixture of specific monoclonal antibodies (SM 2.6.3 and SM 5.15.4) to v-*fms*-coded epitopes (lanes 2). Washed immunoprecipitates were assayed for associated kinase activity, denatured, and run on polyacrylamide gels containing SDS.

(Right) Homogenates of total spleen cell suspensions (lanes H) were centrifuged to yield soluble cytosolic proteins (lanes I) and a membrane fraction (lanes J). After addition of detergents, immunoprecipitates were prepared as described above and assayed for associated kinase activity.

Bartelmez et al., 1985), CSF-1 is lineage-specific and alone does not stimulate the proliferation of granulocytic or erythroid precursor cells (reviewed in Stanley et al., 1983). CSF-1 is an acidic glycoprotein composed of two disulfide bonded, approximately 14 kd polypeptide chains that are variably glycosylated (Stanley and Heard, 1977; Das and Stanley, 1982). It binds specifically to mononuclear phagocytes and their precursors, irrespective of their tissue of origin or state of maturation (Guilbert and Stanley, 1980; Byrne et al., 1981), and selectively binds to macrophage and myelomonocytic cell lines in culture (Guilbert and Stanley, 1980; Stanley et al., 1984). The binding is of high affinity and consistent with the existence of a single class of binding sites at both 2°C and 37°C (Stanley and Guilbert, 1981; L. J. Guilbert, P. W. Tynan, and E. R. Stanley, unpublished data). The murine CSF-1 receptor has been purified and shown to be a glycosylated single polypeptide chain of approximately 165 kd exhibiting an associated tyrosine kinase activity that is stimulated by binding of the purified growth factor (Y. G. Yeung, P. T. Jubinsky, and E. R. Stanley, unpublished data). The similarity in tissue distribution and biochemical properties of the c-*fms* proto-oncogene product and the CSF-1 receptor raised the possibility that they were related molecules. We show that the c-*fms* gene product is expressed at high levels in mature macrophages, and that rabbit antibodies prepared against a recombinant v-*fms*-coded polypeptide specifically react with the murine CSF-1 receptor. The results indicate that the product of the c-*fms* proto-oncogene and the CSF-1 receptor are closely related, and possibly identical, molecules.

Results

Expression of the Feline c-*fms* Product in Macrophages

Although different cat tissues express a characteristic 4 kb c-*fms* messenger RNA species, by far the highest levels of c-*fms* transcription have previously been detected in cat spleen. Using monoclonal antibodies to v-*fms*-coded epitopes, a c-*fms*-coded glycoprotein of 170 kd ($gp170^{c\text{-}fms}$) was precipitated from spleen extracts, but was not detected in homogenates of other tissues that contained significantly lower levels of c-*fms* RNA (Rettenmier et al., 1985a). These results were attributed to the fact that, even in spleen, the c-*fms* product is not readily detected, necessitating the use of a sensitive immune complex kinase reaction to radiolabel the glycoprotein in vitro.

We undertook a more extensive survey of c-*fms* expression and used the immune complex kinase assay to screen for $gp170^{c\text{-}fms}$ in other adult cat tissues. Extracts prepared from freshly necropsied spleen, lymph nodes, liver, lung, kidney, and brain were immunoprecipitated with monoclonal antibodies to the v-*fms*-coded glycoprotein, and the washed precipitates were incubated for 10 min at 30°C with [γ^{32}P]ATP in the presence of buffer containing manganese ions. The phosphoprotein products were then denatured, separated by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS), and detected by autoradiography. Similar experiments performed using extracts of cells from peritoneal inflammatory exudates are described in greater detail below. As shown in Figure 1 (left), relatively high levels of c-*fms*-coded products were detected in spleen (lanes B) and lymph node homogenates (lanes C), whereas lower levels were seen in liver (lanes D). As previously reported, two c-*fms*-coded glycoproteins of 170 and 130 kd were detected in positive tissues and represent differentially glycosylated forms of the polypeptide; only $gp170^{c\text{-}fms}$ is sensitive to neuraminidase digestion and therefore is presumed to represent a mature cell surface glycoprotein (Rettenmier et al., 1985a). Although cat brain homogenates exhibited high levels of endogenous kinases (lanes G), no specifically precipitated c-*fms*-coded products were demonstrated in this tissue or in lung (lanes E), kidney (lanes F), skeletal muscle, or cultured feline fibroblasts.

To determine whether the c-*fms* products were expressed in parenchymal splenocytes and to assay the glycoproteins for association with membranes, single cell suspensions were prepared from the spleen and mechanically disrupted in hypotonic buffer. After centrifugation to remove nuclei, a crude microsomal fraction was sedimented from the homogenate, treated with detergents to disrupt the vesicles, and tested for c-*fms*-associated kinase activity in an immune complex reaction. Figure 1 (right) shows that the relevant kinase activity detected in splenocytes (lanes H) was exclusively associated with the membrane preparation (lanes J), whereas no c-*fms*-related kinase was detected in the cytosol (lanes I).

Like $gp140^{v\text{-}fms}$, $gp170^{c\text{-}fms}$ is phosphorylated on tyrosine in the immune complex kinase reaction (Rettenmier

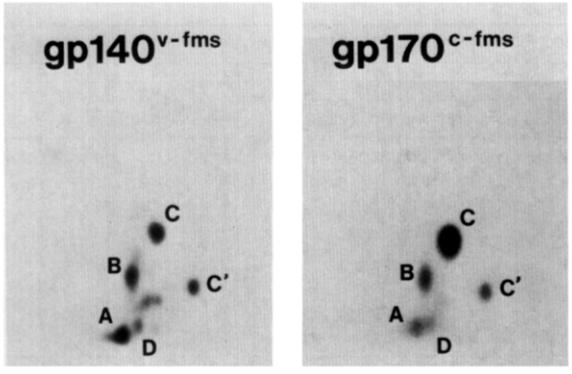

Figure 2. Two-Dimensional Analysis of Tryptic Phosphopeptides from Feline gp140$^{v\text{-}fms}$ and gp170$^{c\text{-}fms}$

The glycoproteins phosphorylated in immune complexes were separated electrophoretically. Gel slices were digested with trypsin. Eluted [^{32}P]labeled peptides were spotted on cellulose-coated thin layer plates at the lower left corner of each panel and subjected to electrophoresis (left to right) and chromatography (bottom to top). Radiolabeled peptides were detected by autoradiography. Five major spots labeled A-D designate phosphopeptides that map in a linear order from the amino terminus to the carboxy terminus of gp140$^{v\text{-}fms}$ labeled in vitro; spots C and C′ are probably overlapping tryptic peptides that share a common amino acid sequence.

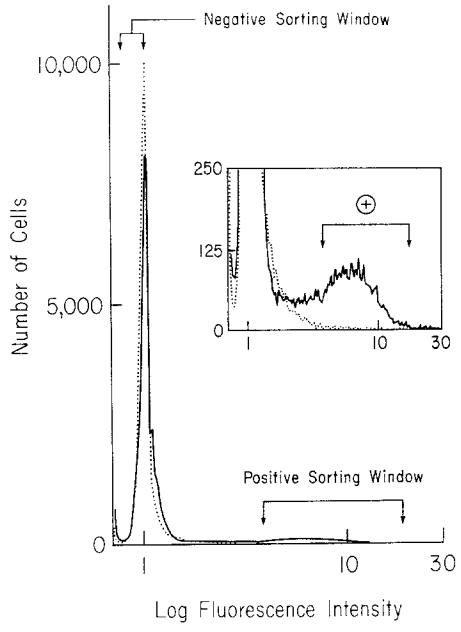

Figure 3. Fluorescence-Activated Flow Cytometry of Cat Peritoneal Exudate Cells Using a Monoclonal Antibody to a v-*fms*-Coded Epitope

Cells from a thioglycolate-induced inflammatory exudate (Experiment 1 of Table 1) were reacted with monoclonal antibody SM 2.6.3 (solid line) or with an isotype-matched control (dashed line) in the presence of 3% cat plasma and sorted. Approximately 10% of the total cells showed positive fluorescence with SM 2.6.3 as compared to the control antibody. The inset shows the positive fluorescence profile on an expanded scale.

et al., 1985a). To compare the primary structures adjacent to sites of phosphorylation in vitro, two-dimensional separation of tryptic phosphopeptides from gp140$^{v\text{-}fms}$ and gp170$^{c\text{-}fms}$ was performed. Figure 2 shows that five major tryptic phosphopeptides isolated from both molecules were identical. This was confirmed in a mixing experiment in which the major radiolabeled peptides comigrated in both dimensions. The five major phosphopeptides (labeled A, B, C, C′, and D in Figure 2) have been mapped in gp140$^{v\text{-}fms}$ and are designated to indicate their linear order in the polypeptide chain from the amino to the carboxy terminus. Peptides C and C′ are probably related tryptic fragments derived from overlapping amino acid sequences (C. W. Rettenmier, unpublished data). These results show that gp140$^{v\text{-}fms}$ and gp170$^{c\text{-}fms}$ are antigenically and biochemically similar molecules expressing the same enzymatic activity.

Since homogenates of unfractionated splenocytes were active in immune complex kinase reactions, we attempted to determine the differentiated phenotype of the cell(s) expressing the c-*fms* gene products. When spleen cells were fractionated by density in Percoll gradients, all of the c-*fms*-associated enzyme activity was recovered in a light density fraction (1.048–1.062 g/ml) composed primarily of granulocytes, macrophages, and large lymphoid cells, which together represented less than 5% of the total splenocytes (data not shown). Thus, only a minor spleen cell population lacking small lymphocytes and erythrocytes expressed the c-*fms* gene product. Because the population expressing gp170$^{c\text{-}fms}$ included mature phagocytic elements, we reasoned that relatively pure populations of such cells could be more readily recovered from acute inflammatory exudates. Cats were therefore inoculated intraperitoneally with irritants (thioglycolate or glycogen), and inflammatory cells were collected from the peritoneal cavity 4 days later. Table 1 summarizes the results of two representative experiments. Thioglycolate induction (Experiment 1) resulted in an inflammatory exudate consisting primarily of polymorphonuclear leukocytes, with admixed macrophages, eosinophils, and other cell types (see Figure 4A), whereas glycogen infusion (Experiment 2) resulted in an exudate containing approximately equal numbers of granulocytes and macrophages. Lysates prepared from these cells were greatly enriched for c-*fms*-coded glycoproteins, which were readily detected in the immune complex kinase reaction (Figure 1A).

The specific monoclonal antibodies used in the kinase assays are directed to amino-terminal epitopes of the v-*fms*-coded glycoprotein (Rettenmier et al., 1985b). Cells from cat peritoneal exudates were therefore examined by fluorescence-activated flow cytometry for the presence of surface epitopes related to those of the v-*fms* gene product. As shown in Figure 3 (Experiment 1 of Table 1), a significant percentage of the cells in these exudates expressed surface antigens related to the v-*fms* gene product. No positive fluorescence was detected using an isotype-matched, control myeloma protein. The fluorescence-positive and -negative populations were separated cytometrically and examined morphologically. In each experiment, the fluorescence-negative population consisted almost entirely of granulocytic cells, whereas the positive population consisted almost exclusively of mature macrophages (Figures 4B and 4C and Table 1). Histochemical staining confirmed that the fluorescence-positive cells ex-

Table 1. Fractionation of Acute Inflammatory Cells by Fluorescence-Activated Flow Cytometry

Experiment	Inducing Agent	Total Cells Recovered	Differential Counts		
			Total Cells	Fluorescence-Negative	Fluorescence-Positive
1	Brewer's thioglycolate	2×10^8	16% Macrophages 72% PMNs 10% Eosinophils 2% Others	1% Macrophages 88% PMNs 11% Eosinophils	88% Macrophages 8% PMNs 4% Eosinophils
2	Oyster glycogen	4×10^7	59% Macrophages 36% PMNs 4% Eosinophils 1% Others	7% Macrophages 82% PMNs 11% Eosinophils	90% Macrophages 9% PMNs 1% Eosinophils

Cells were recovered by lavage 4 days after intraperitoneal inoculation of the inducing agent, and sorted by fluorescence-activated flow cytometry using a monoclonal antibody (SM 2.6.3) to a v-*fms*-coded epitope. Staining of the cells was performed in the presence of 3% cat plasma to inhibit nonspecific binding of the antibodies to immunoglobulin F_c receptors. Fluorescence-positive and -negative populations were collected in 35 mm diameter culture dishes, and cytospin preparations of each fraction were stained with Giemsa. The fluorescence profiles and morphology of different cell types are shown in Figures 3 and 4, respectively. PMNs: polymorphonuclear leukocytes. Other cell types included lymphocytes and mast cells.

pressed butyrate esterase, a typical macrophage marker (4C, inset). Thus, fluorescence-activated cell sorting performed with antibodies to v-*fms*-coded molecules can be used to purify mature macrophages from mixtures of different cells. When cell homogenates from the fractionated populations of Experiment 2 were examined for immunoprecipitable c-*fms*-coded products in the immune complex kinase assay, enzyme activity was detected in 1×10^6 fluorescence-positive macrophages, whereas 5×10^6 granulocytic cells assayed in parallel lacked detectable activity. From these data, we conclude that the feline c-*fms*-coded glycoprotein is expressed at relatively high levels by mature differentiated cells of the mononuclear phagocytic lineage.

Antisera to a Recombinant v-*fms*-Coded Protein Precipitate the Mouse c-*fms* Gene Product

We suspected that the feline c-*fms* gene product might represent a hematopoietic growth factor receptor expressed on macrophages, but we could not confidently assay possible ligands for receptor binding because the biological activities of CSFs and interleukins are often species-specific. Since many of the candidate growth factors have been purified and assayed in murine systems, we first attempted to prepare antisera that would cross-react with the product of the mouse c-*fms* gene and identify a protein analogous to feline $gp170^{c\text{-}fms}$. A portion of the v-*fms* gene encoding the distal 321 amino acids of the amino-terminal domain, the 26 amino acid transmembrane segment, and the complete 406 amino acid carboxy-terminal domain was excised from the molecularly cloned, biologically active SM-FeSV provirus (Donner et al., 1982) and cloned into an inducible pBR322 expression vector. A recombinant v-*fms*-coded protein of 81 kd (here designated $bp81^{v\text{-}fms}$) was purified from bacterial extracts and used to immunize rabbits. After three immunizations, each of four inoculated animals was found to synthesize antibodies that precipitated the v-*fms* product at titers equal to or higher than those previously obtained by immunization of rats with syngeneic SM-FeSV-transformed tumor cells (Ruscetti et al., 1980).

The rabbit antiserum to the recombinant v-*fms*-coded product precipitated three v-*fms*-specific glycoproteins from SM-FeSV-transformed cells metabolically labeled with [^{35}S]methionine (Figure 5A). These three products are routinely detected at equivalent levels in SM-FeSV-transformed cells using either polyvalent rat antisera or specific monoclonal antibodies to v-*fms*-coded epitopes. The largest species of 180 kd represents the cotranslationally glycosylated polyprotein ($gP180^{gag\text{-}fms}$) that contains amino-terminal residues encoded by the FeSV *gag* gene and carboxy-terminal sequences specified by v-*fms*. Proteolysis removes the *gag*-coded portion of the polyprotein and generates a v-*fms*-coded glycoprotein, $gp120^{v\text{-}fms}$, which is the predominant form detected in transformed cells (Barbacid et al., 1980; Ruscetti et al., 1980). A small proportion of these molecules undergoes modification of its N-linked oligosaccharides in the Golgi complex, and appears as a glycoprotein of higher apparent molecular weight ($gp140^{v\text{-}fms}$) (Anderson et al., 1982; 1984). Only the latter form of the glycoprotein is detected at the cell surface (Anderson et al., 1984; Roussel et al., 1984; Manger et al., 1984). Figure 5B shows that the rabbit anti-$bp81^{v\text{-}fms}$ sera were also active in an immune complex kinase assay performed with the precipitated v-*fms*-coded glycoproteins. In each case, $gP180^{gag\text{-}fms}$, $gp120^{v\text{-}fms}$, and $gp140^{v\text{-}fms}$ were phosphorylated in vitro; phosphoamino acid analysis confirmed that these molecules were radiolabeled exclusively on tyrosine residues (data not shown). To determine if the rabbit antisera would detect the c-*fms*-coded glycoproteins as well, immune complex kinase reactions were carried out using lysates of cat peritoneal exudate cells. Both $gp170^{c\text{-}fms}$ and $gp130^{c\text{-}fms}$ were detected in amounts similar to those detected using monoclonal antibodies (Figure 5C).

The rabbit antiserum to $bp81^{v\text{-}fms}$ cross-reacted with c-*fms*-coded molecules from other species, and in this respect differed from previously prepared immunological reagents. Several mouse macrophage cell lines were metabolically labeled with [^{35}S]methionine, and the lysates were incubated with the rabbit antisera. Figure 5D shows that each of three mouse macrophage cell lines ex-

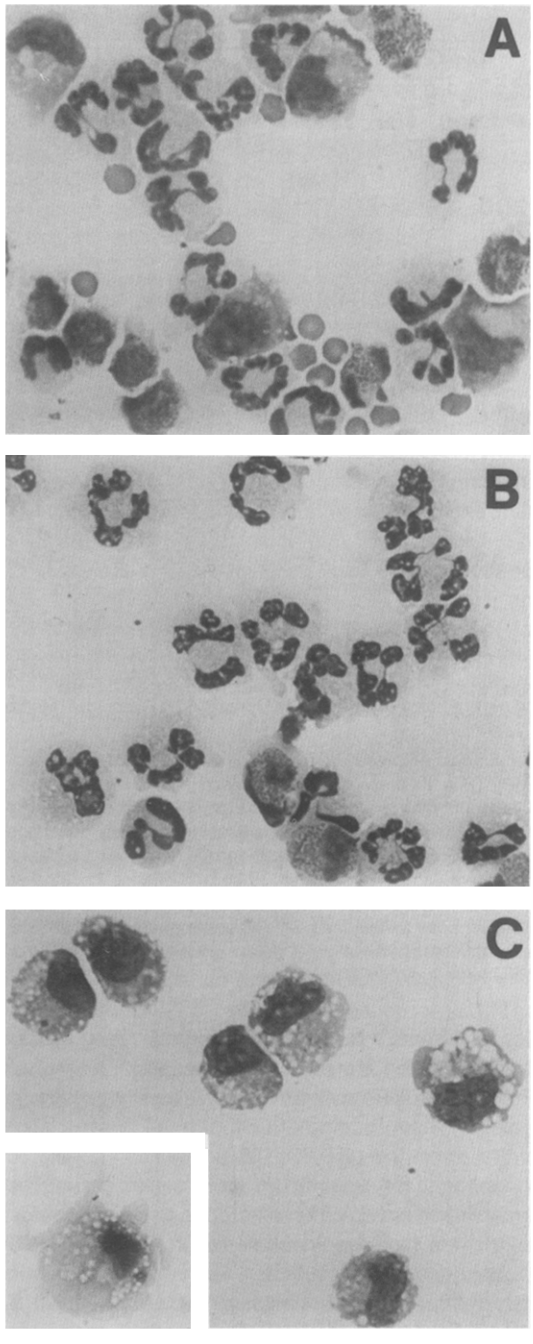

Figure 4. Morphology of Cat Peritoneal Exudate Cells

A stained cytospin preparation of unfractionated cells from Experiment 1 (Table 1) is shown in A. The cells consist of polymorphonuclear leukocytes (PMNs), macrophages, eosinophils, and erythrocytes. Sorted fluorescence-negative PMNs and eosinophils (B) and fluorescence-positive macrophages (C) were derived from the experiment shown in Figure 3. Quantitative differential counts for each population appear in Table 1 (Experiment 1). The inset in C shows positive histochemical staining for butyrate esterase.

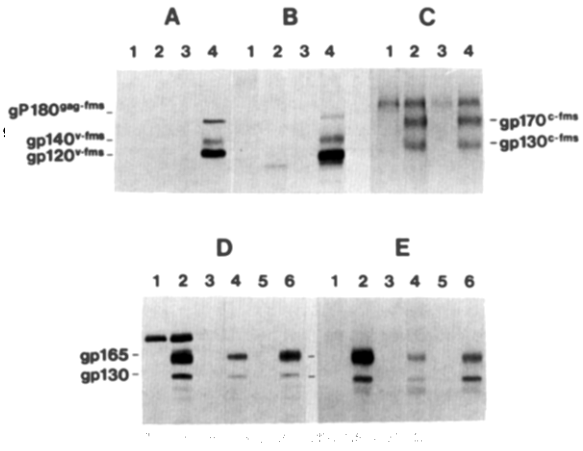

Figure 5. Characterization of Rabbit Antisera to a Recombinant bp81$^{v\text{-}fms}$ Polypeptide

(A) Proteins metabolically radiolabeled with [^{35}S]methionine from untransformed mink CCL64 cells (lanes 1 and 2) and from an SM-FeSV-transformed subclone (lanes 3 and 4) were precipitated with preimmune (lanes 1 and 3) or immune (lanes 2 and 4) rabbit antiserum to bp81$^{v\text{-}fms}$. The positions of gP180$^{gag\text{-}fms}$, gp120$^{v\text{-}fms}$, and gp140$^{v\text{-}fms}$ are noted in the left margin.

(B) Immune complex kinase assays performed with mink cell lysates and a rabbit antiserum to bp81$^{v\text{-}fms}$. The cells and immune reagents are ordered as in A.

(C) Immune complex kinase assays performed with immunoprecipitates from extracts of cat peritoneal exudate cells elicited by thioglycolate inoculation. The precipitates were generated using a control myeloma protein (lane 1), a mixture of monoclonal antibodies (SM 2.6.3 and SM 5.15.4) to v-*fms*-coded epitopes (lane 2), preimmune rabbit serum (lane 3), and rabbit antiserum to bp81$^{v\text{-}fms}$ (lane 4). The positions of gp170$^{c\text{-}fms}$ and gp130$^{c\text{-}fms}$ are noted in the right margin.

(D) Proteins from mouse macrophage cell lines metabolically-labeled with [^{35}S]methionine and precipitated with rabbit antiserum to bp81$^{v\text{-}fms}$. Lanes 1, 3, and 5 show results with preimmune serum, and lanes 2, 4, and 6 with the immune serum. The cell lines examined included P388D1 (lanes 1, 2), IC-21 (lanes 3, 4), and BAC1.2F5 (lanes 5, 6). The positions of the mouse 165 kd and 130 kd proteins are indicated in the left margin.

(E) Immune complex kinase assays performed with mouse cell lysates and a rabbit antiserum to bp81$^{v\text{-}fms}$. The cells and immune reagents are ordered as in D.

pressed a major antigenically cross-reactive polypeptide of about 165 kd. Lesser amounts of a 130 kd protein were also detected; this form is apparently an immature glycosylated precursor of the 165 kd polypeptide. As previously described for the cat c-*fms*-coded glycoproteins (Rettenmier et al., 1985a), the major 165 kd polypeptide was sensitive to neuraminidase digestion and resistant to endoglycosidase H, whereas the minor 130 kd protein exhibited a reciprocal pattern of enzyme sensitivity (data not shown). The 165 kd and 130 kd glycoproteins were detected in macrophage cell lines that were either dependent (BAC1.2F5) or independent (P388D1, IC-21) of the mouse mononuclear phagocyte growth factor, CSF-1, for proliferation in culture. Both P388D1 and BAC1.2F5 have been shown to express high affinity CSF-1 binding sites (Guilbert and Stanley, 1980; Morgan and Stanley, 1984); IC-21 has not been similarly tested. When assayed in the kinase reaction, immunoprecipitates prepared with lysates of these mouse macrophages also contained an associated kinase activity that phosphorylated the mouse 165 kd and 130 kd polypeptides (Figure 5E). As shown in Figures 6A–6C, the immune complex kinase reaction yielded phosphotyrosine as the phosphorylated amino acid in the 165 kd and 130 kd glycoproteins from both CSF-1-dependent and -independent cells. Thus, the glycoproteins precipitated from mouse macrophage cell

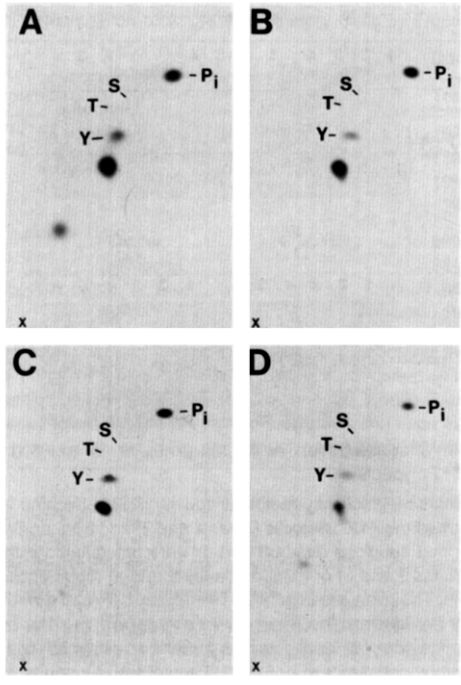

Figure 6. Phosphoamino Acid Analyses of Murine Phosphoproteins Specifically Precipitated with Antiserum to bp81$^{v\text{-}fms}$

The 165 kd (A) and 130 kd (B) glycoproteins precipitated from the murine macrophage cell line P388D1, and the 165 kd glycoprotein (C) derived from the BAC1.2F5 cells, were phosphorylated in immune complex kinase reactions, separated by gel electrophoresis, and eluted from gel slices. The [^{32}P]labeled proteins were hydrolyzed in acid and subjected to two-dimensional electrophoresis on cellulose-coated plates. The origin is at the lower left. The identity of the labeled phosphoamino acids was determined by superimposing the autoradiograms over ninhydrin-stained spots containing authentic phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y). The mobility of orthophosphate (P_i) is noted. The experiment in D shows similar results obtained with the BAC1.2F5 165 kd protein phosphorylated in membranes after stimulation with purified CSF-1; following phosphorylation in the presence of ligand (see Figure 7), the membranes were disrupted with detergent and the 165 kd phosphoprotein was specifically precipitated with rabbit antiserum to bp81$^{v\text{-}fms}$ prior to phosphoamino acid analysis.

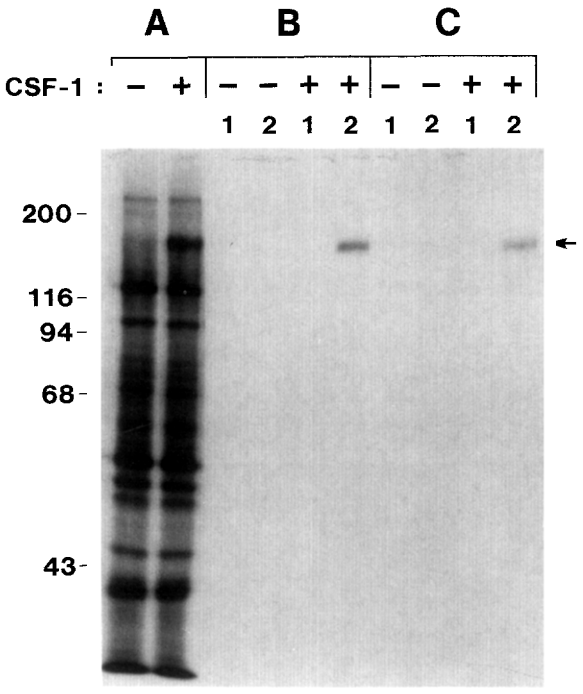

Figure 7. Phosphorylation of the Murine CSF-1 Receptor in Membrane Preparations

Membranes prepared from BAC1.2F5 cells were incubated with [γ^{32}P]ATP in the presence (+) or absence (–) of CSF-1. Equal aliquots of the membrane preparations were disrupted with detergent and subjected to either gel electrophoresis (lanes A) or immunoprecipitations using sera from two rabbits (lanes B and C). Results with control preimmune sera (lanes 1) or antisera to bp81$^{v\text{-}fms}$ (lanes 2) demonstrated precipitation of a 165 kd substrate that was phosphorylated after CSF-1 addition (arrow at right margin). The electrophoretic mobilities of protein standards of known molecular weight are indicated in kilodaltons at the left margin.

lines by antisera to bp81$^{v\text{-}fms}$ had the same enzymatic activity as the feline c-*fms* product.

The rabbit antisera directed against bp81$^{v\text{-}fms}$ did not precipitate the EGF receptor from human A431 epidermoid carcinoma cells, even though the receptor is expressed in these cells at high levels and is readily detected under these experimental conditions using a rabbit antiserum to the EGF receptor (data not shown). Moreover, rabbit anti-bp81$^{v\text{-}fms}$ did not react with the product of the v-*fes* oncogene, which is expressed in cells transformed by the Gardner-Arnstein and Snyder-Theilen strains of FeSV (Ruscetti et al., 1980). The latter control was particularly important because, among the different members of the tyrosine kinase gene family, v-*fes* and v-*fms* are predicted to share the greatest amino acid sequence homology (Hampe et al., 1982, 1984). From these data, we concluded that the rabbit antisera to bp81$^{v\text{-}fms}$ specifically detected v-*fms*- and c-*fms*-coded molecules and did not react indiscriminately with other tyrosine kinases.

Rabbit Antisera to the Recombinant v-*fms* Product React with the Mouse CSF-1 Receptor

The murine CSF-1 receptor is active as a tyrosine-specific protein kinase. Binding of CSF-1 to the purified receptor in the presence of [γ^{32}P]ATP stimulates receptor autophosphorylation specifically on tyrosine (Yeung et al., unpublished data). When macrophage membrane preparations are similarly incubated with CSF-1 at 2°C, the phosphorylation of several other membrane proteins is also enhanced (P. T. Jubinsky, Y. G. Yeung, and E. R. Stanley, unpublished data). To test whether the murine c-*fms*-coded polypeptide and the CSF-1 receptor were related molecules, membranes prepared from CSF-1-dependent BAC1.2F5 mouse macrophages, previously shown to be positive for expression of the c-*fms* gene product (Figures 5D and 5E), were incubated with [γ^{32}P]ATP in the presence or absence of purified CSF-1. When the proteins phosphorylated in vitro were electrophoretically separated in gels containing SDS, several different phosphoproteins were detected (Figure 7, lanes A). Enhanced phosphorylation was observed in the presence of CSF-1. In particular, a polypeptide with the molecular weight of the approximately 165 kd CSF-1 receptor kinase (Morgan and Stanley, 1984; Yeung et al., unpublished data) exhibited the greatest degree of CSF-1-in-

Table 2. Precipitation of the Solubilized CSF-1–Receptor Complex by Rabbit Antiserum to bp81$^{v\text{-}fms}$

Input Antigen (cpm)	^{125}I Precipitated by Sera (cpm)				
	No Serum	Preimmune (Rabbit #1)	Immune (Rabbit #1)	Preimmune (Rabbit #4)	Immune (Rabbit #4)
^{125}I-CSF-1 alone (2.5×10^6 cpm)[a]	2326 ± 1640	3486 ± 1225	3999 ± 1416	3879 ± 670	3301 ± 732
^{125}I-CSF-1–receptor complex (22,300 cpm)[b]	270 ± 33	386 ± 81	6263 ± 511	206 ± 59	5017 ± 120

[a] Coprecipitations were performed using staphylococcal protein A. The results obtained using either preimmune or immune rabbit antisera were equivalent to those obtained without antiserum and contained ~0.1% of the input radioactivity.
[b] An equivalent amount of solubilized membrane preparation obtained from cells that had been preincubated with an excess of unlabeled CSF-1 prior to binding with ^{125}I-CSF-1 contained only 290 ± 8 cpm. There was no difference between the results obtained using immune and preimmune antiserum in immunoprecipitations performed with lysates from this sample.

duced phosphorylation. Portions of the phosphorylated membrane preparations were lysed with detergents and precipitated with rabbit antisera to bp81$^{v\text{-}fms}$. As shown in Figure 7 (lanes B and C), two different rabbit antisera to bp81$^{v\text{-}fms}$ specifically precipitated the 165 kd phosphoprotein. The same results were obtained with similar antisera from two other rabbits. In each case, phosphorylation of this protein was clearly stimulated by purified CSF-1. When the radiolabeled polypeptide was eluted from gels, hydrolyzed, and subjected to two-dimensional electrophoresis, the major amino acid phosphorylated in vitro was tyrosine (Figure 6D).

To demonstrate unequivocally that the 165 kd phosphoprotein precipitated by rabbit antisera to bp81$^{v\text{-}fms}$ was the CSF-1 receptor, we assayed the ability of the antisera to precipitate receptor–ligand complexes. These experiments are based on the stability of the CSF-1–receptor complex to the detergents used to solubilize the receptor (Yeung et al., unpublished data). ^{125}I-CSF-1 was bound to receptors on BAC1.2F5 cells by incubation at 2°C. The cells were washed to remove unbound ^{125}I-CSF-1, and membrane fractions were then prepared, solubilized with detergents, and incubated with rabbit antiserum to bp81$^{v\text{-}fms}$. As shown in Table 2, ^{125}I-CSF-1 was recovered from washed immunoprecipitates prepared with rabbit anti-bp81$^{v\text{-}fms}$ sera, but not from those prepared with nonimmune serum, and not from cells preincubated with unlabeled CSF-1. Control experiments showed that the rabbit antisera did not directly react with the purified radiolabeled hormone. These results indicated that specific receptor–ligand complexes between the 165 kd glycoprotein and ^{125}I-CSF-1 were immunoprecipitated with rabbit antiserum to the v-*fms* product. To confirm that the precipitated radioactive molecules represented intact CSF-1 molecules, the washed immunoprecipitates prepared with rabbit antisera to bp81$^{v\text{-}fms}$ were denatured and subjected to SDS gel electrophoresis in the presence or absence of a reducing agent. As shown in Figure 8, autoradiograms of these gels were essentially indistinguishable from those of similar gels prepared using purified ^{125}I-CSF-1 (see figure legend for description of the polypeptides). Together with the results shown in Figure 7, these experiments establish that the CSF-1 receptor is antigenically and functionally related to the product of the c-*fms* proto-oncogene.

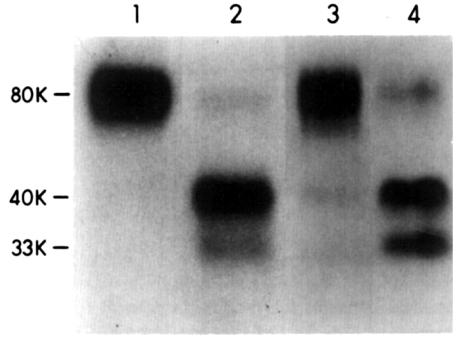

Figure 8. Precipitation of ^{125}I-CSF-1 in Receptor–Ligand Complexes Using Antiserum to bp81$^{v\text{-}fms}$

Separation of ^{125}I-CSF-1 under nonreducing or reducing conditions on 10% polyacrylamide gels containing SDS is shown in lanes 1 and 2, respectively. Lanes 3 and 4 show parallel results obtained with ^{125}I-CSF-1 recovered in complexes with the receptor using anti-bp81$^{v\text{-}fms}$ serum. Quantitative results of these experiments are summarized in Table 2. Partial amino acid sequence data are consistent with identity of the two polypeptide chains of dimeric CSF-1 (J. E. Strickler, A. Boosman, K. J. Wilson, and E. R. Stanley, manuscript submitted). Previous studies indicate that the approximately 40 kd and 33 kd polypeptides observed after reduction are derived from dimeric molecules of approximately 80 kd and 66 kd, respectively (Das and Stanley, 1982). The broad band at 80 kd contains a small proportion of the 66 kd dimer. It is unclear whether the polypeptides differ only in their degree of glycosylation.

Discussion

The biochemical and topological properties of v-*fms*-coded glycoproteins previously suggested that the c-*fms* gene might code for a cell surface receptor (Anderson et al., 1984; Roussel et al., 1984; Manger et al., 1984; Rettenmier et al., 1985b). Using cell separation procedures, populations of mature cat macrophages isolated from acute inflammatory exudates and from spleen were shown to express high levels of the c-*fms*-coded glycoprotein, which were specifically detected either in the immune complex kinase reaction or by fluorescence-activated flow cytometry. Since CSF-1 is the only mononuclear phagocyte-specific growth factor described to date, the cellular distribution of the c-*fms*-coded glycoprotein suggested that it could represent the CSF-1 receptor. By producing rabbit antisera to a recombinant v-*fms*-coded product and testing mouse macrophage cell lines for the presence of precipitable kinase activity, we

detected an antigenically cross-reactive glycoprotein of approximately 165 kd that was active as a tyrosine kinase and was similar in size to the feline c-*fms* product. This glycoprotein was shown to represent the murine CSF-1 receptor by two criteria. First, in assays performed with membrane preparations, the 165 kd protein was phosphorylated in vitro in the presence of purified CSF-1 and was specifically precipitated. Second, receptor–ligand complexes formed at the cell surface were recovered from membranes after detergent lysis, precipitated with the rabbit antiserum, and shown to contain ^{125}I-CSF-1. These data indicate that the mouse c-*fms* gene product and the CSF-1 receptor are closely related, if not identical, proteins. Definitive evidence that these are the same molecules will require comparison of the amino acid sequences of the CSF-1 receptor and the murine c-*fms* gene product.

CSF-1 is a lineage-specific hematopoietic growth factor required for the survival, proliferation, and differentiation of cells of the mononuclear phagocyte series (precursor cell → monoblast → promonocyte → monocyte → macrophage) (reviewed in Stanley et al., 1983). The response to CSF-1 is pleiotropic and varies with the mononuclear phagocyte cell type. For example, cultured precursor cells are stimulated to survive, proliferate, and differentiate, whereas differentiated, nondividing macrophages are stimulated only to survive. The proliferation and survival of primary bone-marrow-derived macrophages in culture is strictly CSF-1 dependent (Tushinski et al., 1982). Removal of the growth factor from serum-containing cultures decreases the rate of DNA synthesis by more than 100-fold (Tushinski and Stanley, 1985). CSF-1 is required in the G0/G1 phase of the cell cycle for entry of cells into S phase (Tushinski and Stanley, 1985). However, S phase cells can complete the S, G2, and M phases of the cell cycle in the absence of the growth factor (Stewart, 1980). CSF-1 stringently regulates protein turnover by increasing the rate of protein synthesis and by decreasing the rate of protein degradation (Tushinski and Stanley, 1983). Macrophages exposed to CSF-1 exhibit morphological changes similar to those of other cells exposed to growth factors. These changes include stimulation of membrane ruffling and filopodia within 1 min of CSF-1 addition, followed within 15 min by the appearance of phase lucent vacuoles (Tushinski et al., 1982; R. J. Tushinski, P. W. Tynan, C. J. Morgan, and E. R. Stanley, unpublished observations).

CSF-1 appears to play an important role in the differentiation of primitive hemopoietic cells. In the absence of other growth factors, CSF-1 stimulates precursors of mononuclear phagocytes to proliferate and differentiate. However, in the presence of other hemopoietic growth factors (hemopoietin-1 or interleukin-3), CSF-1 can stimulate the proliferation and differentiation of even more primitive cells (Bartelmez et al., 1985; Bartelmez and Stanley, 1985). These more primitive cells possess low numbers of CSF-1 receptors and are likely to be multipotent (Bartelmez and Stanley, 1985; E. R. Stanley, T. R. Bradley, A. Bartocci, D. Patinkin, and M. Rosendaal, unpublished data). Their differentiation to mononuclear phagocyte precursors is associated with a 10-fold increase in the number of CSF-1 receptors expressed per cell (Bartelmez and Stanley, 1985). Elevated expression of the CSF-1 receptor may therefore represent the earliest marker of determination of cells to the mononuclear phagocyte lineage.

Although the CSF-1 receptor is a differentiation-specific marker apparently restricted to mononuclear phagocytes and their precursors, c-*fms* transcripts have been detected in different organs including lymph nodes, liver, and brain (Rettenmier et al., 1985a) and in human tumors of various types (Slamon et al., 1984). The distribution of c-*fms* transcripts in normal tissues and in primary tumors could reflect the widespread presence of tissue macrophages. For example, the CSF-1 receptor is expressed in lymph nodes in approximately 0.5% of the total cells that correspond in their morphology and frequency to mononuclear phagocytes (Byrne et al., 1981). Similarly, phagocytic Kupffer cells in the liver are CSF-1-responsive (Chen et al., 1979). Transcripts of c-*fms* have also been detected in placenta and in choriocarcinoma cell lines (Muller et al., 1983a, 1983b). At present, it is unclear whether c-*fms* expression in these cells is characteristic of cell lineages other than mononuclear phagocytes. It is clearly of interest to determine whether these other cell types express a functional CSF-1 receptor.

Major portions of the human (Heisterkamp et al., 1983; Roussel et al., 1983) and feline (Verbeek et al., 1985) c-*fms* proto-oncogenes have already been molecularly cloned. In man, the c-*fms* locus has been assigned to the distal long arm of human chromosome 5 (Groffen et al., 1983; Roussel et al., 1983). Deletions involving this region of the chromosome have been detected in the "5q$^-$ syndrome," a pleiotropic hematopoietic disorder consisting of refractory anemia, mild myeloid hyperplasia, the presence of hyperlobulated bone marrow megakaryocytes, and peripheral thrombocytosis (Sokal et al., 1975; Kerkhofs et al., 1982). Patients with the 5q$^-$ syndrome frequently present with one abnormal and one normal chromosome 5 in their mitotic bone marrow cells, suggesting that the c-*fms* gene could be hemizygous (Nienhuis et al., Cell, September 1985, in press). Intriguingly, a high percentage of these patients eventually develop myelogenous leukemia (Wisniewski and Hirschhorn, 1983).

The target cell specificity of the McDonough strain of feline sarcoma virus offers no immediate clue to the role of the c-*fms* gene product in normal cells. SM-FeSV was isolated from a multicentric fibrosarcoma of a young domestic cat (McDonough et al., 1971) and was reported to induce fibrosarcomas when reinoculated into animals (reviewed in Hardy, 1981). The virus is able to transform both fibroblastic cell lines (e.g., NIH 3T3, NRK) and epithelial cell lines (e.g., mink CCL64) in culture, but transforms fibroblasts at considerably higher efficiency (M. F. Roussel and C. J. Sherr, unpublished data). To date, SM-FeSV has not been implicated in hematopoietic neoplasms, nor has it been shown to transform hematopoietic cells in clonal assays. If the v-*fms* gene product represents an aberrant form of the CSF-1 receptor, it must provide abnormal receptor-mediated signals, since neither fibroblasts nor epithelial cells require CSF-1 for growth. Like the v-*erb* B gene, which represents a truncated form of the EGF

receptor (Downward et al., 1984; Ullrich et al., 1984; Lin et al., 1984; Xu et al., 1984), v-*fms* could code for a protein with a constitutively active kinase domain that is hormone independent. However, the v-*fms*-coded glycoprotein appears to contain an almost complete extracellular domain (approx. 450 amino acids) which could, in principle, interact with ligand. The fact that fibroblasts produce CSF-1 (Tushinski et al., 1982) therefore raises the alternative possibility that the v-*fms* gene product binds CSF-1 or a related transforming growth factor produced by SM-FeSV-transformed target cells. If this were the case, the mechanism of viral transformation could depend on transduction of a competent receptor gene. The data argue against the hypothesis that proto-oncogenes are restricted in their expression to cells that act as targets for their viral oncogene counterparts. Instead, activated oncogenes appear to be more promiscuous in their function than their proto-oncogene progenitors. Clearly, alterations in these genes as a result of retroviral transduction can significantly affect their regulation and sites of action.

Experimental Procedures

Cells and Culture Conditions

The transformed mink lung cell line G2M (Frankel et al., 1979) contains a single copy of the SM-FeSV provirus (Donner et al., 1982). Other transformed mink cell subclones containing the Gardner-Arnstein (F3CI7) (Henderson et al., 1974) or Snyder-Theilen strains of FeSV (B3T-1) (Donner et al., 1980) were used as controls in cell sorting and immunoprecipitation experiments; the latter FeSV strains contain a different viral oncogene (v-*fes*), which is a well characterized member of the tyrosine kinase gene family (Hampe et al., 1982). Transformed cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum.

The murine macrophage cell line BAC1 was derived from adherent BALB/c × A.CA F1 murine spleen cells by transfection with origin-defective SV40 DNA. BAC1 cells are CSF-1 dependent for growth, express the Ia antigen and Fc receptor, secrete interleukin-1, produce lysozyme, collagenase, and esterase, and are capable of F_c-mediated phagocytosis (Schwarzbaum et al., 1984). The BAC1.2F5 clone, chosen because of its inability to grow in the absence of CSF-1 as well as its rapid proliferation in the presence of this growth factor (C. J. Morgan, J. W. Pollard, and E. R. Stanley, unpublished data), was used for the experiments described here. Cells were grown in α-minimal essential medium containing 15% fetal calf serum and 3000 units/ml of stage I L cell CSF-1. They were routinely passaged at a dilution of between 1:4 and 1:10 after being scraped from the culture dish with a rubber policeman.

Two other CSF-1-independent mouse macrophage lines were used for immune complex kinase assays. These included P388 D1 and IC-21, described in detail elsewhere (Walker, 1980). The cells were grown in RPMI 1640 containing 10% fetal calf serum. Both lines contain nonspecific esterase, secrete lysozyme, bear F_c and complement receptors, and are phagocytic. P388D1 expresses high affinity binding sites for CSF-1; IC-21 has not been similarly tested (Guilbert and Stanley, 1980).

Antisera and Monoclonal Antibodies

The rat monoclonal antibodies SM 2.6.3, SM 3.19.4, and SM 5.15.4 (Anderson et al., 1982; Roussel et al., 1984) are directed to v-*fms*-coded polypeptide epitopes expressed on the amino-terminal extracellular domain of the glycoprotein (Rettenmier et al., 1985b). SM 2.6.3 and SM 5.15.4 were used for immunoprecipitation and flow cytometric analysis, whereas SM 3.19.4 was used for immunoblotting of bp81$^{v\text{-}fms}$.

A recombinant v-*fms*-coded polypeptide used as an antigen was made in a pBR322-based expression vector, constructed to be identical to a previously described plasmid of others, pJL6 (Lautenberger et al., 1983). The vector contains the lambda P_L promoter, a bacterial translational start signal (Shine-Delgarno sequence), and a leader polypeptide sequence derived from the lambda CII gene. The vector has unique Cla I and Bam HI cloning sites, which were used to insert a v-*fms*-coded fragment containing 321 amino acids of the distal amino-terminal domain, the 26 amino acid membrane-spanning sequence, and the complete 406 amino acid carboxy-terminal domain. Insertion of the v-*fms* 2.5 kilobase pair Cla I–Bam HI fragment into the vector placed the oncogene coding sequences downstream of the 13 amino acid lambda CII leader sequence in an incorrect reading frame. Plasmids containing the fragment were therefore opened at the Cla I site, treated with the large Klenow I fragment of DNA polymerase, and recircularized by blunt end ligation to confer the proper reading frame predicted from the v-*fms* nucleotide sequence (Hampe et al., 1984). These manipulations abolish the Cla I site at the CII:v-*fms* junction and generate a new site for the restriction endonuclease Nru I. Transformed E. coli were screened for plasmids of the predicted size containing the novel Nru I site. These plasmids were isolated and used to transform the bacterial strain, N4830, expressing a thermolabile repressor of the lambda P_L promoter (Gottesman et al., 1980). Cells grown at 42°C, but not 32°C, synthesized a v-*fms*-coded polypeptide of 81 kd, bp81$^{v\text{-}fms}$, which was purified from the bacteria by differential salt extraction and preparative gel electrophoresis. Western blotting analysis performed using rat monoclonal antibodies (Anderson et al., 1982) confirmed that the purified polypeptide was an authentic v-*fms*-coded product. The characterization of rabbit antisera raised to this immunogen are described in the text.

Cell Separation Procedures

For fractionation of splenocytes, fresh cat spleen tissue was minced with scalpels in RPMI 1640 medium containing 10% fetal calf serum, forced through a fine mesh metal strainer, and allowed to settle by gravity for 5 min. The supernatant fluid containing single cells was passed through a 21 gauge needle and the cells were pelleted at 800 × g for 5 min. Cells were suspended at 5 × 10^8/ml and layered over Percoll gradients (Pharmacia) prepared in Hank's balanced salt solution (Gibco). One milliliter of cells was layered on each of ten 45 ml gradients containing a 5 ml bottom cushion of 70% Percoll, and a continuous 40 ml gradient from 60% to 20% Percoll. The gradients were centrifuged at 1000 × g for 20 min, and fractions containing cells were morphologically characterized in cytospin preparations stained with Giemsa. The gradients were calibrated using colored marker beads of known density (Pharmacia); the cells containing maximal c-*fms*-associated kinase activity were recovered at a density of 1.048–1.062 g/ml.

For preparation of acute inflammatory exudates, cats were inoculated intraperitoneally with 50 ml of oyster glycogen (10 mg/ml) or with 50 ml of Brewer's thioglycolate broth (Conrad, 1983), and cells were recovered by peritoneal lavage 4 days later. Between 4 × 10^7 and 2 × 10^8 cells were obtained from each animal, and consisted primarily of polymorphonuclear leukocytes and macrophages (cf. Figure 4 and Table 1). The cells were washed in DMEM containing 10% FCS and incubated with monoclonal antibody SM 2.6.3 prior to addition of fluorescein-conjugated goat antiserum to rat immunoglobulin. All incubations were performed in the presence of 3% normal cat plasma to abolish nonspecific antibody binding to F_c receptors. The cells were sorted on an Epics V flow cytometer at a rate of 500 cells/sec; dead cells labeled with propidium iodide were excluded from the analysis. Detailed procedures for fluorescence staining and flow cytometry are published elsewhere (Roussel et al., 1984).

Preparation of Macrophage Membranes

BAC1.2F5 macrophage membranes were prepared according to a procedure of Yeung et al. (unpublished). Cells were collected in phosphate-buffered normal saline (pH 7.4) containing 4 mM iodoacetic acid (IAA) and 1 mM EGTA [PBS-IAA] by scraping them from the culture dishes with a rubber policeman. They were centrifuged and collected in 5 pellet volumes of PBS-IAA. After assessing the viability by trypan blue exclusion, the cells (>90% viable) were centrifuged and resuspended in 8 pellet volumes of ice-cold hypotonic buffer (5 mM Tris-HCl [pH 8.0], 75 mM sucrose, 1 mM IAA, 0.5 mM EGTA, 10 μg/ml leupeptin [Sigma Chemicals], 0.5 units/ml aprotinin [Sigma], and 1000 units/ml soybean trypsin inhibitor Type 1S [Sigma]). Cells were allowed to swell on ice for 15 min and were disrupted in a Dounce homogenizer with a tight fitting pestle. When >90% of the cells were broken, 0.25 vol of compensating buffer (20 mM Tris-HCl [pH 7.4], 0.95 M sucrose, 26 mM

$MgCl_2$, 5 mM EGTA, 0.15 M NaCl, and 0.15 M KCl) was added to make the mixture isotonic. The homogenate was centrifuged at 2000 rpm for 45 sec after attaining speed to sediment the nuclei. The nuclear pellet was washed with a mixture of 1 part hypotonic buffer and 0.25 parts compensating buffer and recentrifuged. Pooled postnuclear supernatant fluids were layered over ice-cold 15% sucrose solution containing 0.1 M Tris-HCl (pH 7.4), 5 μg/ml leupeptin, 0.5 unit/ml aprotinin, and 100 units/ml soybean trypsin inhibitor in SW39 polyallomer centrifuge tubes (Beckman), and centrifuged at 115,000 × g for 30 min at 4°C. The membrane pellet was then resuspended in 10–20 vol of 25 mM Hepes (pH 7.4) and disaggregated using a Dounce homogenizer with a loose fitting pestle.

Assay for CSF-1-Induced Membrane Phosphorylation

Membranes (30–60 μg protein) were incubated for 8 min at 4°C in 25 mM Hepes buffer (pH 7.4) containing 10^5 units/ml of purified CSF-1, 15 mM $MnCl_2$, 8 mM $MgCl_2$, and 20 μM [γ^{32}P]ATP (3000 Ci/mmol; Amersham) in a final reaction volume of 50 μl. The reaction was terminated by addition of concentrated electrophoresis sample buffer containing SDS and loaded directly on polyacrylamide gels. Alternatively, the reaction was terminated by addition of 10 μl of 120 mM ATP, followed by solubilization in RIPA buffer (50 mM Tris-HCl [pH 7.4], containing 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 2% aprotinin) for immunoprecipitation.

Purification of Receptor–Ligand Complexes Containing Radiolabeled CSF-1

L cell CSF-1 was purified as previously described (Stanley and Heard, 1977; Stanley and Guilbert, 1981). Purity was checked by SDS gel electrophoresis under reducing and nonreducing conditions and by complexing with rabbit anti-CSF-1 antibody (Stanley and Heard, 1977; Stanley and Guilbert, 1981). CSF-1 concentration was determined by radioimmunoassay (Stanley, 1979). One unit of CSF-1 is approximately equivalent to 0.44 fmol of CSF-1 protein. The purified CSF-1 was iodinated with carrier-free ^{125}I (Amersham) with full retention of biological activity to a specific radioactivity of 400,000 cpm/ng of protein (Stanley, 1979; Stanley and Guilbert, 1981).

Cells were incubated at 2°C with 50 pM ^{125}I-CSF-1 for 2 hr. Alternatively, they were preincubated with 2.5 nM purified CSF-1 for 1 hr followed by incubation with 50 pM ^{125}I-CSF-1 for 2 hr. After incubation, dishes were washed five times with ice-cold phosphate-buffered normal saline, and the cells were scraped from plates with a rubber policeman. Membranes were purified from the cells as described above, disrupted in RIPA buffer, and subjected to immunoprecipitation using rabbit antiserum to bp81$^{v\text{-}fms}$.

Other Analytical Methods

The procedures for metabolic radiolabeling of cell lines, preparation of cell lysates, immunoprecipitation, the immune complex kinase assay, immunoblotting, peptide mapping (Roussel et al., 1984), and phosphoamino acid analysis (Rettenmier et al., 1985a) are described in detail in the references cited.

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