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Identification of the Ligand-Binding Regions in the Macrophage Colony-Stimulating Factor Receptor Extracellular Domain

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The c-*fms* gene encodes the receptor for the macrophage colony-stimulating factor (M-CSF), and its extracellular domain consists of five immunoglobulin-like subdomains. To identify which of the five immunoglobulin-like regions are involved in ligand binding, we polymerase chain reaction-cloned five segments of the extracellular domain of the murine c-*fms* gene, each starting with the normal initiation codon and containing successive additions of the immunoglobulin-like subdomains. These protein segments are designated A, B, C, D, and E and contain, from the N-terminal end, either one, two, three, four, or all five immunoglobulin-like subdomains, respectively. Each segment was expressed as a secreted soluble protein from a baculovirus expression vector in Sf9 insect cells. In addition, segments A, B, C, and E were produced as soluble alkaline phosphatase fusion proteins, as was a segment containing only the fourth and fifth immunoglobulin domains. These segments of the Fms extracellular domain were used to assess M-CSF binding by competition radioimmunoassays, plate binding immunoassays, and immunoprecipitation analyses. The results indicated that the first two N-terminal immunoglobulin-like domains did not interact with M-CSF but, in combination with the third immunoglobulin-like domain, provided high-affinity M-CSF binding. The fourth and fifth immunoglobulin-like domains near the cell membrane did not exhibit M-CSF binding and may inhibit interaction of M-CSF with the first three immunoglobulin domains. These results suggest that the three N-terminal immunoglobulin-like domains constitute the high-affinity M-CSF binding region and that the fourth and fifth immunoglobulin-like domains may perform functions other than ligand binding.

The macrophage colony-stimulating factor (M-CSF) has been identified as the key lineage-specific hematopoietic growth factor that acts directly on monocytes/macrophages and their progenitor cells to stimulate their survival, proliferation, differentiation, and mature cell functions (20). M-CSF is a disulfide-linked homodimer synthesized as a transmembrane molecule and proteolytically processed to produce the soluble form (19, 30, 31). Both membrane-bound and soluble M-CSF can stimulate target cell growth, and the biologically active portion of the ligand is contained in the amino-terminal 150 amino acids of the approximately 550-amino-acid full-length form (16, 49). Structural studies of M-CSF have indicated that the disulfide-bonded dimer configuration is essential for activity but that the presence of carbohydrate on the molecule is not needed.

The receptor for M-CSF is a member of the tyrosine kinase class of growth factor receptors and is identical to the protein product of the c-*fms* proto-oncogene (Fms) (40). Fms is an integral transmembrane glycoprotein and possesses an extracellular ligand-binding domain containing five predicted immunoglobulin-like repeats with 8 to 12 consensus N-linked carbohydrate attachment sites, a hydrophobic transmembrane segment, and an intracellular tyrosine kinase domain interrupted by a stretch of hydrophilic amino acids called the kinase insert (reviewed in reference 32). These general structural features are shared by several other members of the tyrosine kinase class of growth factor receptors, including both α and β platelet-derived growth factor receptors, the c-*kit* proto-oncogene, several fibroblast growth factor-type receptors, the Flk receptors, and the *Drosophila torso* gene product (5, 41, 47, 52). In addition, Fms is a member of a broader class of receptor molecules defined by the existence of immunoglobulin-like structural subdomains within the extracellular region of the receptor (58). This group includes receptors containing multiple immunoglobulin-like subdomains such as NCAM, ICAM (15), the membrane-bound form of immunoglobulin M, the CD4 antigen, the interleukin-1 receptor (42), and the α and β subunits of the T-cell receptor. Those receptors with a single immunoglobulin-like subdomain such as the α and β subunits of the interleukin-6 receptor, interleukin-7 receptor, LIF (leukemia inhibitory factor) receptor, and the granulocyte CSF receptor also can be considered members of this group (2, 7, 8, 11).

Signal transduction and other effector functions of Fms initiate with M-CSF binding to a single high-affinity site in the extracellular domain of each of two Fms molecules (reviewed in reference 59). This M-CSF-induced dimerization of the receptor activates the cytoplasmic tyrosine kinase domains with subsequent transphosphorylation of adjacent receptor molecules on four primary tyrosine residues (28, 51). Three of these tyrosine residues are situated within the kinase insert region at amino acid positions 697, 706, and 721 of the murine Fms. The fourth tyrosine is found in the main kinase domain at position 807. These phosphorylated tyrosines act as affinity sites for binding SH2-containing proteins that propagate the downstream signalling events. The receptor and ligand are ultimately transferred to lysosomes for degradation and termination of the signal.

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An oncogenic version of Fms also has been characterized as the product of the v-*fms* oncogene of both the Susan-McDonough and Hardy-Zuckerman-5 strains of feline sarcoma virus. The viral Fms proteins are fully transforming and differ from cellular Fms by deletions and substitutions at the C-terminal tail of the kinase domain and point mutations in or near the immunoglobulin-like domain IV of the extracellular portion on the receptor (for a review, see reference 32). This single immunoglobulin-like subdomain does not contain a disulfide bond which the other immunoglobulin-like subdomains possess. The activating mutations in this subdomain render Fms M-CSF independent, suggesting that these mutations may induce a conformational change similar to that caused by M-CSF binding. The exact structures participating in ligand binding, potential conformational changes, and receptor dimerization are, however, unknown.

On a broader functional scale, both M-CSF and Fms exhibit tissue-specific distributions that suggests this growth factor-receptor combination regulates both hematopoietic cell development and placental growth and differentiation (1, 27, 29). Purified M-CSF is active in stimulating production of macrophages and is expressed on bone marrow stromal cells that nurture macrophage development. The importance of M-CSF in macrophage development has been demonstrated in the M-CSF-deficient *op/op* mouse, in which administered M-CSF can correct the osteoporosis and macrophage deficiencies (56, 57, 61). These studies indicate a direct role of this ligand in macrophage formation and also in production of bone-remodeling osteoclast cells. M-CSF is also expressed on epithelial cells of the uterine endometrium under control of female sex steroids (25). In the early stages of pregnancy, the receptor for M-CSF is found in the dicidual cells around the developing egg cylinder and later in the trophoblast cells of the developing placenta. This distribution of M-CSF and its receptor in these tissues suggests they play a role in placental growth and development, and this notion is supported by the fact that *op/op* female mice have impaired fertility.

All the above biological functions of Fms are initiated and/or maintained by the primary interaction of M-CSF with the extracellular domain of Fms, yet little is known about the exact sites involved in this interaction. We have therefore begun to characterize the structural and functional features of the Fms extracellular domain and present here our initial analysis of the immunoglobulin-like subdomains that compose the single high-affinity M-CSF binding site.

MATERIALS AND METHODS

Reagents, cells, and viruses. Grace's insect medium, *Spodoptera frugiperda* (Sf9) cells, *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA, and baculovirus transfer vectors (pVL 1392/1393 and pBluebac) were purchased from Invitrogen. Serum-free medium and methionine-free Grace's insect medium were from JRH Biosciences. [^{35}S]methionine was obtained from ICN Biomedicals, and Na^{125}I was the product of Du Pont. The enhanced chemiluminescence detection kit was from Amersham. The silver staining kit was purchased from Bio-Rad. Endoglycosidase H, *O*-glycosidase and *N*-glycosidase F were from Boehringer Mannheim. QuantAffinity Epoxide-Glass beads were supplied by Rainin. Purified murine M-CSF was the generous gift of E. R. Stanley (Albert Einstein, Bronx, N.Y.). Centriprep concentrator was from Amicon. Lentil lectin Sepharose 4B was purchased from Pharmacia. A monoclonal antibody against murine alkaline phosphatase (AP) was obtained from Medix Biotech, whereas the rat polyclonal antibody raised against murine Fms was made in our laboratory and described previously (33). The rabbit polyclonal antibody to murine Fms was obtained after multiple injections (subcutaneous) of New Zealand rabbits with Rab-9 cells (ATCC CRL 1414) expressing the murine c-*fms* protein from the pZEN retroviral vector (33). Sf9 cells were grown at 27°C in Grace's medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml. The source and growth of FD(c-*fms*) cells were described previously (33).

Construction of recombinant baculovirus transfer vectors. The expression vector pZen(c-*fms*) containing the murine full-length c-*fms* cDNA has been previously described (33). The murine c-*fms* extracellular domain and its domain truncation cDNAs were constructed and amplified from the full-length c-*fms* cDNA by polymerase chain reaction (PCR) with the same upstream primer (primer 1 in Fig. 1 and below) and the various downstream primers listed below (refer also to Fig. 1): primer 1, 5′-ACTG**GCTAGC**TATAAATATGGAGTTGGGGCCTCCT-3′; primer a, 5′-GTCA**GCTAGC***TCA*CACTGTCACCTCCTG-3′; primer b, 5′-GTCA**GCTAGC***TC**A*CTCCAATTTTATCTG-3′; primer c, 5′-GTCA**GCTAGC***TCA*CTGCAAGAGGCTCTG-3′; primer d, 5′-GTCA**GCTAGC***TCA*TGTAACACTGACCTC-3′; and primer e, 5′-GTCA**GCTAGC***TCA*CTCATCGGGGAGCTGCTT-3′. The underlined nucleotides in each primer represent the hybridizing portion, and the nucleotides in boldface type specify the *Nhe*I restriction site. The upstream primer (primer 1) contains the c-*fms* ATG translation start codon, and the adjacent seven 5′ untranslated nucleotides (TATAAAT) are identical to those upstream of the baculovirus polyhedrin gene start site. Very poor expression resulted if these seven nucleotides were derived from the corresponding GC-rich c-*fms* 5′ untranslated sequence. The codon specifying the translational stop is in italics within the downstream primers. After 4 min of preheating at 94°C, each of the 30 PCR cycles was composed of denaturation, 1 min at 94°C; annealing, 2 min at 50°C; and synthesis, 4 min at 72°C. The 100-μl PCR mixture contained 10 pmol of each primer, 10 ng of pZen(c-*fms*) vector, 1.25 mM deoxynucleoside triphosphate, PCR buffer (0.5 M KCl, 0.1 M Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.1% gelatin) and 2.0 U of *Taq* polymerase. Each truncated c-*fms* cDNA segment was amplified in two independent PCRs in order to resolve possible nucleotide misincorporation during the PCR. Clones from both PCRs were carried through to the Sf9 expression step. The resultant PCR cDNAs were digested with *Nhe*I and ligated into the similarly digested recipient pBluebac vector. The correct orientation of each insert was confirmed by restriction mapping.

The expression pBluebac M-CSF truncation vector was generated by subcloning 500 bp of M-CSF cDNA. An *Nhe*I-*Nhe*I M-CSF fragment containing the extracellular domain was constructed by PCR and inserted into the *Nhe*I site of pBluebac vector, as mentioned above.

Transfection and isolation of recombinant virus. Recombination of the pBluebac constructs with wild-type AcNPV baculovirus was achieved by cotransfecting Sf9 cells in a six-well plate (2×10^6 cells per well) with a mixture of 2 μg of recombinant baculovirus vector and 1 μg of wild-type AcNPV DNA by the calcium phosphate precipitation technique (44). For the isolation of recombinant truncated c-*fms* and M-CSF expressing pBluebac baculoviruses, Sf9 cells in six-well plates (10^6 cells per well) were infected with 10-fold serial dilutions of the cotransfection culture supernatant and

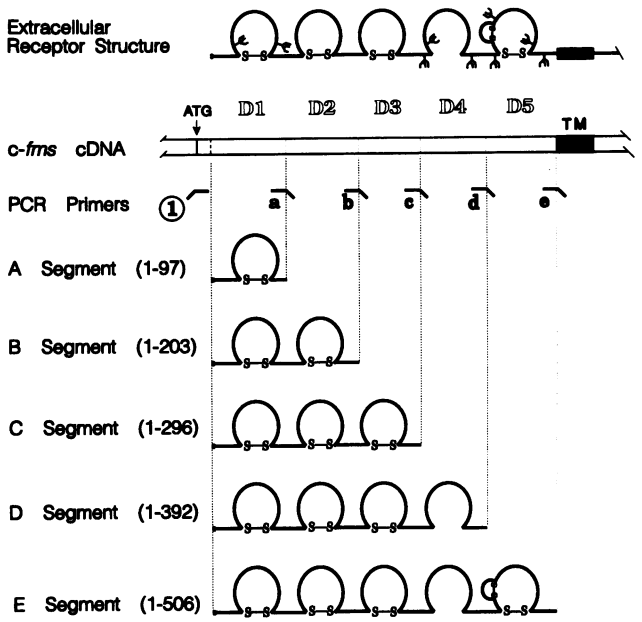

FIG. 1. Construction of the murine Fms extracellular domain segments for expression as soluble proteins from a baculovirus vector in Sf9 cells. The upper line of the diagram shows the predicted protein structural features of the murine Fms extracellular domain with the cytoplasmic domain truncated just after the transmembrane (TM) region. The individual immunoglobulin-like subdomains are designated D1 to D5, and intrasubdomain disulfide bonds (S-S) along with all nine potential sites for N-linked carbohydrate addition (small branched structures) are indicated. The c-*fms* cDNA is also shown with the ATG start codon for translation. The vertical dashed line between the ATG codon and the region encoding subdomain D1 represents the point where the protein hydrophobic leader sequence is cleaved. The cDNA regions encoding protein segments of the Fms extracellular domain designated A to E were PCR cloned by using a common upstream primer (designated primer 1) and a different downstream primer for each clone (designated primers a to e). The exact sequence for each primer is given in Material and Methods, as are the methods for cloning each segment into the pBluebac expression system and production of the soluble proteins from Sf9 insect cells. The amino acids contained in the individual soluble segments are listed in parentheses after the segment (cleavage of the leader would remove the N-terminal 19 amino acids), and the predicted structures of each protein segment of the Fms extracellular domain are shown in the lower part of the figure.

subsequently overlaid with 0.6% low-melting-point agarose containing β-D-galactoside (150 μg/ml). Six to seven days postinfection, the recombinant plaques stained blue and agar plugs containing single recombinant plaques were picked with a Pasteur pipette. Usually, three or four rounds of plaque assays were used to produce the purified recombinant baculovirus, and each purified recombinant baculovirus stock was made to yield titers of 10^8 PFU/ml. The expression of the recombinant proteins was screened by radioimmunoprecipitation assay.

Radiolabelling and immunoprecipitation. Sf9 cells (10^6 per well in six-well plates) were infected with appropriate recombinant baculovirus or wild-type AcNPV. Three days postinfection, the cells were incubated for 30 min at 27°C in 1 ml of methionine-free Grace's medium and metabolically labelled with 80 μCi of [^{35}S]methionine per ml in the same medium for 4 h. After labelling, the supernatant was harvested and the cells were solubilized in RIPAE buffer (10). The [^{35}S]methionine labelled proteins in the supernatant and cellular detergent extract were immunoprecipitated by using rabbit anti-Fms antiserum as previously reported (10). The immunoprecipitates were then washed once with RIPAE buffer, once with $MgCl_2$ buffer (1 M $MgCl_2$, 10 mM Tris-HCl [pH 7.5]), and once with Nonidet P-40 lysis buffer and analyzed on sodium dodecyl sulfate (SDS)–7.5 or 12% polyacrylamide gels under reducing conditions.

Time course assay and immunoblotting. Sf9 cells seeded into six-well plates at a density of 2×10^6 cells per well were infected with recombinant baculovirus, and the medium was replaced 2 h postinfection. The culture supernatants were harvested at 24, 48, 72, 96, and 120 h postinfection, immunoprecipitated with rabbit antibody raised against murine Fms, and electrophoresed on an SDS-polyacrylamide gel. The proteins were electrophoretically transferred to nitrocellulose membrane. The nitrocellulose was blocked with 4% nonfat dry milk in phosphate-buffered saline (PBS) buffer–0.3% Tween 20 and subsequently incubated at 4°C overnight with rat antibody raised against murine Fms. Following extensive washing with the same PBS buffer, the nitrocellulose was treated with enhanced chemiluminescence detection mixture according to the manufacturer's procedure and exposed to XAR-5 film.

Carbohydrate analysis of the Fms proteins. Recombinant proteins metabolically labelled with [^{35}S]methionine were immunoprecipitated from cell culture supernatants with rabbit anti-Fms antiserum. The immunoprecipitate was then denatured by boiling in 1% SDS for 2 min and diluted 10-fold by addition of sodium phosphate buffer to give a final concentration of 0.02 M sodium phosphate, 0.01 M EDTA, 0.05 M Nonidet P-40, and 0.1% SDS at pH 7.2. The samples were treated overnight with 0.8 U of *N*-glycosidase F and 5 mU of *O*-glycosidase at 37°C or double digested with 0.8 U of *N*-glycosidase F and 5 mU of *O*-glycosidase for 18 h at 37°C. In addition, the denatured proteins were incubated with 30 mU of endoglycosidase H in 50 mM sodium citrate (pH 6)–0.1% SDS at 37°C for 18 h. The samples were then submitted to SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Construction and expression of c-*fms*–AP chimeras. Soluble chimeric proteins comprised of the A, B, C, and E c-*fms* extracellular domain segments fused in frame to the amino-terminal end of human placental AP were constructed. The E-AP fusion product was produced by subcloning a 1.6-kb murine c-*fms* restriction fragment (containing an introduced stop codon in place of the normal codon for amino acid 511) into the pAPtag-1 plasmid (6). The A-AP, B-AP, and C-AP fusion proteins were produced by PCR cloning the appropriate segments with a common upstream primer (5′-GGAGAG AGATTCTCACTGTCACCTCCTGTGC-3′) and the a, b, and c primers listed above, but containing six nucleotides specifying a *Bgl*II restriction site instead of the *Nhe*I site. An additional fusion protein was constructed that deleted immunoglobulin-like domains D1 to D3 (from an M13 clone by using the oligonucleotide 5′-CCTGTCATCGAGCCTGTGG TGGAGAGTGCC-3′) but retained the Fms leader sequence to facilitate translation and glycosylation. The construct was inserted into the pAPtag-1 plasmid and produced immunoglobulin domains D4 and D5 fused to AP (the protein product is termed D4, 5-AP). The pAPtag-1 plasmids containing the c-*fms* segments were cotransfected with RSV*neo* into Rat2 cells. Cells expressing the fusion proteins were isolated by screening clones for AP activity in the medium by the *p*-nitrophenyl phosphate colorimetric assay (6). Expression

of each fusion protein was checked by immunoprecipitation of [^{35}S]methionine-labelled cells, and the products were separated on a polyacrylamide gel. The protein products were of the expected sizes for the glycosylated proteins.

Expression of soluble and membrane-associated murine M-CSF in Rat2 cells. M-CSF was expressed in Rat2 cells from the retroviral vector pZen (33) as either a soluble or membrane-associated form. The soluble form was created by changing codon 150 in the murine M-CSF gene to TAA, while the membrane-associated form was made as a glycophorin A fusion protein by ligating an M-CSF gene fragment encoding amino acids 1 to 149 to the *Sac*I site at nucleotide 173 of the human glycophorin A coding region.

Partial purification of recombinant c-*fms* truncated proteins. Supernatants were collected from 250 ml of suspension culture in serum-free Grace's insect medium on the third day postinfection, precleared by centrifugation at 10,000 rpm in a Beckman GS-3 rotor for 30 min, and passed through an 8-ml lentil lectin column which had been equilibrated with 5 mM Tris-HCl–0.5% Triton X-100 (pH 7.5). The column was washed with 10 column volumes of 5 mM Tris-HCl buffer and eluted with 0.2 M methyl-α-D-mannopyranoside in the same buffer. The eluate was concentrated 10-fold with a Centriprep Concentrator and dialyzed against 20 mM Tris-HCl, pH 7.4, in 10% glycerol. The partially purified proteins were identified by immunoblotting with rabbit anti-Fms antiserum. The proteins were then subjected to SDS-polyacrylamide gel electrophoresis and silver stained, and protein amounts were quantitatively measured by densitometry with bovine serum albumin (BSA) as a standard.

Ligand-binding assays. For the immunoprecipitation binding assay, supernatant from Sf9 cells (2×10^6 cells per well in six-well plates) infected with recombinant truncated c-*fms* pBluebac baculovirus or wild-type AcNPV was harvested on the third day postinfection and dialyzed against PBS (pH 7.3) containing 3 mM polyethylene glycol prior to the binding assay. The supernatant was incubated with equal counts per minute of [^{35}S]methionine-labelled M-CSF (also expressed from a pBluebac vector in Sf9 cells) at 4°C overnight and further incubated with rabbit anti-Fms antiserum at 4°C for 30 min. The mixture was immunoprecipitated and submitted to SDS-polyacrylamide gel electrophoresis and then autoradiography.

Binding of ^{125}I-M-CSF to the receptors on the surface of FD(c-*fms*) cells was performed as follows. Purified murine M-CSF was oxidatively iodinated with carrier-free Na^{125}I by the chloramine T method as previously described (48). FDC-P1 cells expressing full-length murine Fms were suspended at 10^7 cells per ml in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 2% Wehi-3B conditioned medium. Aliquots containing 5×10^5 cells were incubated overnight with ^{125}I-M-CSF at various concentrations (1.2 to 227 pM) at 4°C in the same medium, and nonspecific binding was determined by incubation of FDC-P1 cells (devoid of Fms expression) with ^{125}I-M-CSF at the concentration of 54, 119, and 227 pM. Following three rapid washes with 1 ml (each) of cold PBS buffer (pH 7.3) containing 1% BSA, cells were lysed in 0.5 ml of sodium phosphate buffer (pH 7.3) containing 0.5% Nonidet P-40 for 30 min at room temperature, and radioactivity was measured in a gamma counter. The binding affinity was determined by Scatchard analysis (37) by using the LIGAND computer program (21).

Solid-phase binding and competition binding assays were developed in our laboratory as follows. Purified anti-AP monoclonal antibody was coated onto QuantAffinity Epoxide-Glass beads in 0.8 M phosphate buffer (pH 7) at room temperature for 3 h, and the beads were further incubated for 1 h in the same buffer containing 1% BSA at room temperature to block nonspecific binding. The E-AP fusion protein, containing AP at the C-terminal end and the entire extracellular domain of murine Fms, was coupled to the anti-AP-coated beads by incubation of anti-AP-coated beads with cell medium containing the E-AP proteins at 4°C overnight. To determine the saturation binding, the E-AP protein-coated beads were incubated with various concentrations of ^{125}I-M-CSF (1.6 to 465 pM) at 4°C, overnight in PBS buffer (pH 7.3), containing 0.1% Tween 20. The beads were rinsed with the same PBS-Tween buffer and counted in a gamma counter. The nonspecific binding was examined by incubation of the anti-AP-coated beads (lacking the E-AP fusion protein receptor) with increasing concentrations of ^{125}I-M-CSF. Also, the E-AP-coated beads were incubated with various concentrations of ^{125}I-M-CSF in the presence of increasing amounts of epidermal growth factor (10 to 300 ng/ml) or rabbit anti-Fms antiserum to further test the specific binding capacity of the E-AP fusion receptor. Competition solid-phase binding assays were performed as in the saturation binding assays, except that a single concentration of ^{125}I-M-CSF (57 pM) was used in the presence of increasing concentrations of partially purified Fms extracellular domain segments. All determinations for the competition binding assays were in duplicate, and the results are presented as the average of duplicate determinations.

A plate binding assay also was used to measure binding of ^{125}I-M-CSF to the Fms segments fused to AP. A monoclonal antibody to AP was adsorbed to the surface of 96-well plates overnight at 4°C. The wells were washed with PBS and incubated for 2 h at 4°C with 100 μl of culture medium containing the soluble AP fusion protein that had been normalized for AP activity. The wells were again washed with PBS, and then ^{125}I-M-CSF was added to each well and incubated at room temperature for 4 h. After the wells were washed, bound ^{125}I-M-CSF was removed from each well with 100 μl of 1 M HCl and counted in a gamma counter. Binding assays were repeated three times with similar results.

RESULTS

Expression of Fms extracellular domain and its truncated variants in Sf9 insect cells. Gene segments corresponding to the Fms extracellular domain and a series of truncations beginning from the transmembrane domain were cloned by PCR with the same upstream oligonucleotide primer and different downstream oligonucleotide primers, as summarized schematically in Fig. 1. Five cloned segments were inserted into a baculovirus vector, and the proteins were expressed in Sf9 insect cells. The 5′ PCR primer used to prepare each clone contained the c-*fms* initiation codon. Therefore, each of the five fragments contained the normal c-*fms* leader sequence for insertion into the lumen of the endoplasmic reticulum. This leader is cleaved 19 amino acids from the N-terminal end during processing (55), and therefore all five clones have identical amino-terminal ends but different carboxyl-terminal ends.

The smallest truncated clone encodes the amino-terminal immunoglobulin-like domain and is designated the A segment of the M-CSF receptor. The second and third clones contain the first two and three immunoglobulin-like subdomains, respectively, and are designated the B segment and C segment. Likewise, the fourth clone encodes the first four

immunoglobulin-like subdomains, and the fifth clone encodes the entire extracellular domain including all five immunoglobulin-like subdomains of the M-CSF receptor up to the beginning of the transmembrane domain. These protein products are termed the D and E segments, respectively. The amino acids encoded in each segment are listed in parentheses in Fig. 1.

Initially, we cloned and tested expression of the E segment with an upstream PCR primer (similar to primer 1 in Fig. 1) that contained the c-*fms* 12 nucleotides 5′ of the ATG start codon. Plaques containing the recombinant baculovirus E segment were serially purified and tested for the appropriate DNA segments by both the qualitative PCR and quantitative slot hybridization (data not shown). The expression of c-*fms* E-segment protein was undetectable in either the infected Sf9 cells or the cell medium by both [^{35}S]methionine-labelled radioimmunoprecipitation and Western blotting (immunoblotting) (data not shown). This lack of expression may be attributable to the inhibitory influence of the c-*fms* G+C-rich 5′ untranslated leader sequences on the transcriptional efficiency of polyhedrin promoter or on the stability of foreign proteins, as suggested in the study of scorpion toxin (21).

To facilitate the expression of c-Fms proteins in Sf9 cells, we next constructed a new c-*fms* E segment cDNA by using an upstream PCR primer substituting the seven nucleotides (TATAAAT) upstream of the baculovirus polyhedrin ATG start site for those present in c-*fms* (see Materials and Methods). This construct was inserted into the *Nhe*I cloning site of the pBluebac transfer vector and transfected into Sf9 cells along with wild-type AcNPV DNA, and inclusion negative blue plaques were serially cloned. To identify the expression of Fms E-segment protein, Sf9 cells infected with recombinant pBluebac/Fms E-segment virus were incubated with [^{35}S]methionine for 2 h at 72 h postinfection. The labelled proteins from the cell extracts and the cell supernatants were immunoprecipitated with rabbit polyclonal antibody raised against the murine Fms extracellular domain and analyzed by SDS-polyacrylamide gel electrophoresis. As a negative control, uninfected Sf9 cells and Sf9 cells infected with wild-type AcNPV DNA were treated in a similar way. The results in Fig. 2A demonstrate that two of three clones generated the expected Fms E-segment protein at a relatively high level and that the majority of Fms E-segment proteins were detected primarily in the cell supernatants as secreted forms. The E-segment proteins were visible as three (or more) closely spaced bands on the SDS-polyacrylamide gel, with estimated sizes of 78, 72, and 69 kDa. No specific protein band was detectable in the cell extracts or in cell supernatants from either the uninfected Sf9 cells or the Sf9 cells infected with wild-type baculovirus AcNPV DNA.

These results suggest that the composition of the 5′ untranslated leader sequence just before the ATG start codon of the c-*fms* cDNA gene is crucial for the high-level expression of a foreign protein in the baculovirus expression system. The multiple discrete forms of the E-segment protein also suggest the possibility that the proteins underwent some type of posttranslational modification such as variable glycosylational processing.

The remainder of the extracellular domain segments shown in Fig. 1 were prepared with the same upstream PCR primer that gave high-level expression for the E segment (primer 1 in Fig. 1) and the indicated downstream PCR primers for terminating translation and facilitating cloning into the *Nhe*I site of the pBluebac baculovirus transfer vector. The results in Fig. 2 illustrate the relative size and

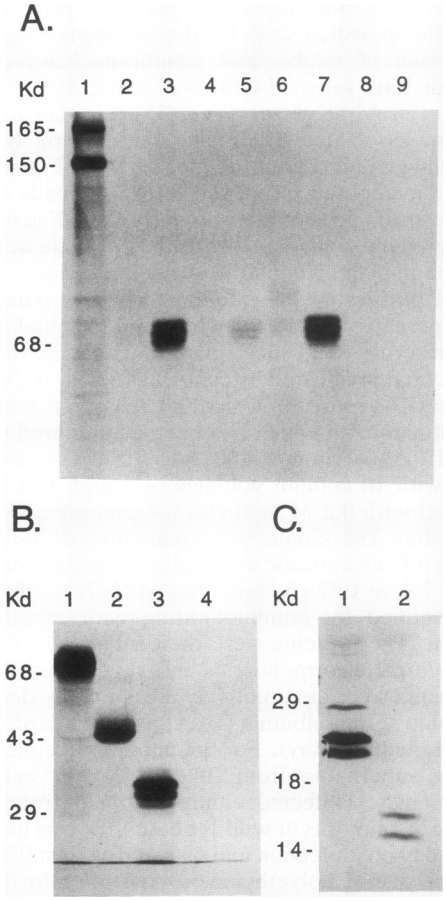

FIG. 2. Expression of Fms extracellular domain segments A to E as soluble proteins from Sf9 cells. (A) Sf9 cells were infected with the baculovirus vector containing the Fms E-segment cDNA clone. Three days after infection, the cells were labelled for 4 h with [^{35}S]methionine, and both cells and labelling medium were collected separately. Fms proteins in the cell lysate and medium were detected by immunoprecipitation with a rabbit polyclonal antibody raised against full-length murine Fms, and the immune complexes were subjected to SDS–7.5% polyacrylamide gel electrophoresis and then autoradiography. For comparison, the full-length mature (gp165^{fms}) and immature (gp150^{fms}) Fms from FD(c-*fms*) cells are shown in lane 1. Lanes 2 and 3, Fms proteins in the cell lysate and medium of pBluebac clone 1-infected cells, respectively; lanes 4 and 5, Fms proteins in the cell lysate and medium of pBluebac clone 2-infected cells, respectively; lanes 6 and 7, similar analyses for pBluebac clone 3-infected cells; lane 8, immune complex prepared from the medium of uninfected Sf9 cells; lane 9, immune complex prepared from the supernatant of control wild-type AcNPV baculovirus-infected Sf9 cells. (B) Expression of Fms protein segments E, D, and C as soluble proteins from Sf9 cells. Cells were infected and labelled, and media were immunoprecipitated with anti-Fms serum as for panel A. Lane 1, soluble Fms E-segment protein; lane 2, soluble D-segment protein; lane 3, soluble C-segment protein; lane 4, anti-Fms immunoprecipitate of medium from Sf9 cells infected with control wild-type AcNPV baculovirus. (C) Expression of Fms B- and A-segment proteins from Sf9 cells was as described above, except the immunoprecipitates were electrophoresed on a SDS–12% polyacrylamide gel. Lane 1, soluble B-segment protein; lane 2, soluble A-segment protein. The migration positions and sizes of standard proteins are indicated at the left of each gel.

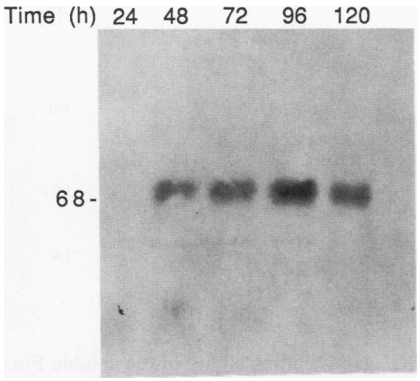

FIG. 3. Time course for soluble E-segment Fms protein expression from Sf9 cells. Sf9 cells were infected with the pBluebac Fms E-segment virus. The cell-conditioned media were harvested at 24, 48, 72, 96, and 120 h postinfection (lanes 1 to 5, respectively) and analyzed by Western blotting with rabbit anti-Fms serum.

level of protein expression achieved for each of the individual A, B, C, D, and E segments as determined by immunoprecipitation of soluble supernatants from [^{35}S]methionine-labelled Sf9 cells infected with each recombinant baculovirus. The quantity of radioactive protein expressed from each cloned segment appeared to be different; however, very similar levels of protein were calculated to be present when the autoradiogram was scanned by densitometry and the results were normalized for methionine content in each segment.

As with the E fragment, each of the segments of the Fms extracellular domain proteins detected in the Sf9 supernatant exhibited multiple size variants recognized by the anti-Fms serum. Fms A-segment protein migrated as two sharply separated bands with molecular masses of 16 and 15 kDa (Fig. 2C), while the B-segment protein appeared as a 24- and 26-kDa doublet. The Fms C-segment protein exhibited three closely spaced bands, including two major species migrating at about 32 and 33 kDa and a minor 35-kDa molecule which were visible on an SDS–7.5% polyacrylamide gel (Fig. 2B). Like Fms C-segment protein, the Fms D segment revealed three bands; two major species of 47 and 48 kDa and a minor fragment of 46 kDa. As mentioned above, the E-segment protein contained three main protein bands, but when separated on very low percentage polyacrylamide gels, five or more distinct species have been detected (data not shown).

The rate of synthesis and secretion of the Fms E protein by Sf9 cells was measured at various times after infecting Sf9 cells with recombinant pBluebac Fms E viruses. The results shown in Fig. 3 indicate that the secretion of the Fms E protein began later than 24 h postinfection and gradually accumulated in the medium during the next 24 to 48 h. After reaching the maximum at 96 h postinfection, the production of Fms E sharply decreased, coincident with cell lysis. The synthesis and secretion characteristics of Fms E protein are similar to those reported for other proteins, such as epidermal growth factor receptor, insulin receptor, and other receptors (12, 43, 54).

Analysis of carbohydrate on Fms extracellular domain segments expressed in Sf9 cells. Several glycosidases (*N*-glycosidase F, endoglycosidase H, and *O*-glycosidase) were used to structurally analyze the carbohydrate components of the Fms E-segment protein and its truncated variants produced from infected Sf9 cells. *N*-Glycosidase F removes

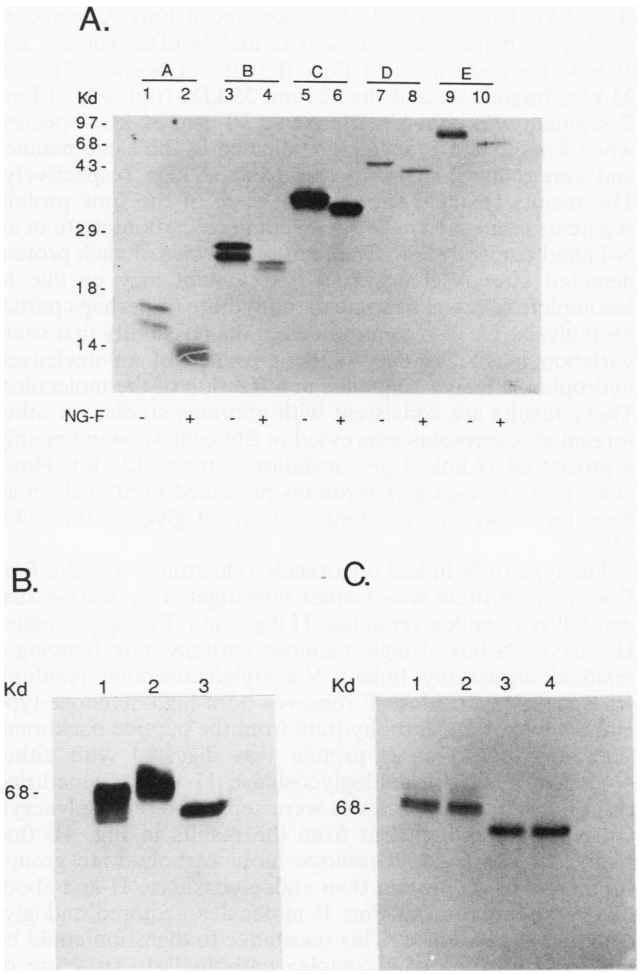

FIG. 4. Analysis of carbohydrate on the various Fms protein segments. [^{35}S]methionine-labelled proteins expressed in Sf9-infected cells were subjected to digestion with *N*-glycosidase F, endoglycosidase H, and *O*-glycosidase as described in Materials and Methods. (A) A, B, C, D, and E represent the respective Fms protein segments analyzed. Lanes 1, 3, 5, 7, and 9, undigested control samples; lanes 2, 4, 6, 8, and 10, samples digested with *N*-glycosidase F (NG-F). The molecular masses of standard proteins are given at the left of each gel in this figure. (B) The Fms E-segment protein was digested with *N*-glycosidase F (lane 1) or endoglycosidase H (lane 3). The E-segment protein in lane 2 is undigested. (C) The Fms E-segment protein was either untreated (lane 1) or incubated with *O*-glycosidase alone (lane 2), with *O*-glycosidase plus *N*-glycosidase F (lane 3), or with *N*-glycosidase F alone (lane 4).

mannose and complex N-linked oligosaccharide residues adjacent to the corresponding asparagine residue (26). Endoglycosidase H is used to remove mannose-rich, N-linked oligosaccharide chains, leaving a single GlcNAc attached to the protein (17). *O*-Glycosidase, in contrast, can selectively digest O-linked oligosaccharide associating with the serine and/or threonine residues of protein (53).

Each of the individual Fms segment proteins was labelled with [^{35}S]methionine, immune precipitated with anti-Fms antibody, denatured with 1% SDS, and incubated with the enzymes mentioned above for more than 16 h. The digestions were separated on SDS-polyacrylamide gels, and the results are shown in Fig. 4. Digestion with *N*-glycosidase F resulted in an increased mobility for all the Fms segments

(Fig. 4A). The 15- and 16-kDa doublet of Fms A segments displayed smaller size bands of 12 and 14 kDa. The 24- and 26-kDa doublet bands of Fms B segment became 21- and 23-kDa fragments, and the 32- and 35-kDa triplicate of Fms C segment was converted into two 30- and 32-kDa species, while Fms D and E segments behaved in the same manner and were reduced in size by about 4 and 8 kDa, respectively. The results strongly suggest that each of the Fms protein segments expressed in Sf9 cells contains carbohydrate in an N-linked configuration. The multiple species of each protein detected after *N*-glycosidase F treatment may be due to incomplete removal of some carbohydrate or perhaps partial proteolysis. We also cannot rule out the possibility that some variation in size is due to the presence of an uncleaved hydrophobic leader sequence in a fraction of the molecules. These results are consistent with previous studies on other foreign glycoproteins expressed in Sf9 cells showing mainly a processed N-linked glycosylation pattern (12, 46). However, other foreign glycoproteins produced in Sf9 cells may have their own characteristic pattern of glycosylation (45, 54).

The type of N-linked oligosaccharide attached to the Fms E-segment protein was further investigated by testing susceptibility to endoglycosidase H digestion. Endoglycosidase H cleaves N-linked high-mannose carbohydrate (leaving a residual asparaginyl-linked *N*-acetylglucosamine residue), whereas *N*-glycosidase F removes both high-mannose-type and complex-type carbohydrate from the peptide backbone. Therefore, the Fms E protein was digested with either *N*-glycosidase F or endoglycosidase H under denaturing conditions, and the products were separated on a polyacrylamide gel. It is apparent from the results in Fig. 4B that *N*-glycosidase F could remove more carbohydrate groups from the Fms E protein than endoglycosidase H and about half the population of Fms E molecules acquired endoglycosidase H resistance. This resistance to digestion could be due to acquisition of a complex carbohydrate structure or modification of the core mannosyl structure that serves as substrate.

The [^{35}S]methionine-labelled Fms E protein was also treated with *O*-glycosidase or cotreated with *N*-glycosidase F and *O*-glycosidase to further investigate the possibilities that the Fms E segment simultaneously underwent *O*-oligosaccharide addition on the serine and threonine residues along with the N-linked addition. The results in Fig. 4C show that the migration of three species of Fms E protein treated with *O*-glycosidase was the same as that of the Fms E protein without glycosidase digestion. The results obtained from the double digestion of Fms E with *N*-glycosidase F and *O*-glycosidase were identical to that obtained from digestion of the Fms E with *N*-glycosidase F alone (Fig. 4C, lanes 3 and 4). Considered together with the glycosidase digestion experiment, it is clear that O-linked oligosaccharide glycosylation is not involved in the processing of the Fms E-protein segment. In addition, even after treatment with endoglycosidase H or double treatment with *N*-glycosidase F and *O*-glycosidase, the Fms E fragment still exhibited two closely spaced species on gel electrophoresis. These could correspond to the protein product with and without the 19-amino-acid leader sequence cleaved from the protein. The difference in molecular weight is roughly equivalent to the size of this leader peptide; however, we have not explored this possibility further.

Ligand-binding capacity of soluble Fms extracellular domain segments. Initially, a radioimmunoprecipitation assay was used to qualitatively assess the ligand-binding capacity

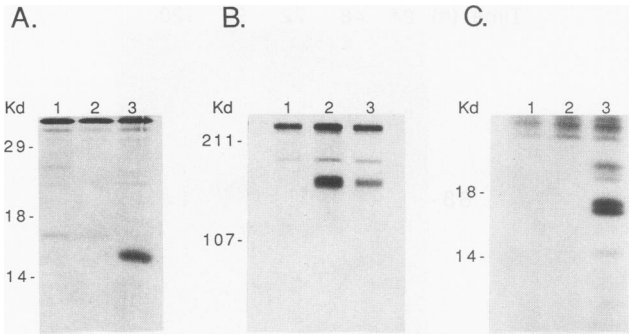

FIG. 5. The ligand-binding ability of the soluble Fms E-segment protein and a fusion protein composed of AP and the Fms E-segment protein (E-AP). (A) [^{35}S]methionine-labelled soluble murine M-CSF expressed from a baculovirus vector in Sf9 cells was incubated with Sf9 cell medium containing the soluble Fms E-segment protein. The ligand-receptor complexes were immunoprecipitated with a rabbit polyclonal antibody directed against the Fms protein and subjected to SDS–12% polyacrylamide gel electrophoresis under reducing conditions and then to autoradiography (lane 3). Cell medium from uninfected Sf9 cells (lane 1) and cell medium from Sf9 cells infected with wild-type baculovirus AcNPV (lane 2) were incubated with [^{35}S]methionine-labelled M-CSF and treated as described above. (B) The expression of a soluble E-AP fusion protein and the presence of both AP and Fms E-segment determinants on this protein were demonstrated by immunoprecipitation assay. Rat2 cells were labelled with [^{35}S]methionine, and culture medium from these cells was used to detect the soluble E-AP fusion protein. Lane 1, the control medium from Rat2 cells containing the empty plasmid APtag-1 were immunoprecipitated with a monoclonal antibody to AP; lane 2, culture medium from Rat2 cells transfected with the APtag-1 (E-segment) plasmid were immune precipitated with a monoclonal antibody to AP; lane 3, the same culture medium as in lane 2 was immune precipitated with rabbit anti-Fms serum. (C) Binding of E-AP to [^{35}S]methionine-labelled M-CSF. Cell culture medium from Katz cells expressing the soluble E-AP fusion protein was mixed with culture supernatant from [^{35}S]methionine-labelled Rat2 cells transfected with a control retroviral vector (lane 1), a retroviral vector expressing the membrane-bound form of murine M-CSF (lane 2), or a retroviral vector expressing a soluble secreted murine M-CSF (lane 3). The fusion proteins were immunoprecipitated with a monoclonal antibody to AP, and coprecipitation of ^{35}S-labelled M-CSF was detected after gel electrophoresis under reducing conditions.

of the Fms E protein produced by Sf9 cells. Unpurified Fms E protein was incubated overnight with [^{35}S]methionine-labelled murine M-CSF. The M-CSF in this case also was expressed as a soluble protein from a baculovirus vector in Sf9 cells. This M-CSF had been confirmed to have very high biological activity (620,000 U/ml) as demonstrated by bone marrow assay (data not shown). The ligand-receptor complexes were immunoprecipitated with a rabbit polyclonal antibody raised against the extracellular domain of Fms, separated on SDS-polyacrylamide gels under reducing conditions, and then autoradiographed. A specific band of 14 kDa was detected when the Fms E segment was present (Fig. 5A, lane 3) but not seen in the negative controls (lanes 1 and 2). The 14-kDa band was verified to be the M-CSF monomer by immunoprecipitation with goat anti-M-CSF serum (data not shown).

It was not possible to use the radioimmunoprecipitation assay to quantitatively study the ligand-binding capacity of the Fms E segment because the anti-Fms polyclonal antibody blocked M-CSF binding to the receptor. To circumvent this problem, we designed a solid-phase competition binding

assay. The receptor for this assay was a fusion protein composed of AP fused, in frame, to the carboxyl-terminal end of the Fms E segment. This fusion protein is termed E-AP. An E-AP receptor protein of about 70 kDa could be detected by immunoprecipitation with either a monoclonal antibody against AP (anti-AP) or polyclonal anti-Fms serum, but not by control serum (Fig. 5B). The ligand-binding ability of the E-AP receptor was subsequently determined by incubation of E-AP with [^{35}S]methionine-labelled M-CSF and immunoprecipitating with a monoclonal anti-AP antibody and subsequent SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 5C, the monoclonal antibody against AP precipitated radiolabelled M-CSF which bound to the E-AP receptor. The molecular weight of this M-CSF differed slightly from that in Fig. 5A because it was expressed in Rat2 cells. In contrast, no specific band could be detected in negative controls. These results indicate that the E-AP receptor can bind M-CSF with fairly high affinity.

The ligand-binding affinity of E-AP receptor was further evaluated by a sandwich solid-phase binding assay in which the monoclonal anti-AP antibody was covalently coupled to modified glass beads via hydroxyl and/or amino groups. The anti-AP-coated beads were then incubated with saturating amounts of the E-AP receptor, and after washing, ^{125}I-M-CSF binding activity was measured. Figure 6A shows that the specific binding of ^{125}I-M-CSF to the E-AP receptor immobilized on beads increased with increasing concentrations of M-CSF and reached saturation at above 300 pM. Nonspecific binding consistently accounted for 6% of the total binding activity. The binding of ^{125}I-M-CSF to E-AP was specific, since an unrelated growth factor, epidermal growth factor, as well as supernatants from Sf9 cells infected by the wild-type baculovirus AcNPV, did not interrupt the binding of ^{125}I-M-CSF. In addition, an antibody raised against murine Fms could specifically block the binding of ^{125}I-M-CSF to E-AP.

Scatchard analysis of M-CSF binding to the E-AP fusion receptor was analyzed by the LIGAND computer program (21) and demonstrated a single high-affinity binding site with a dissociation constant (K_d) of 24 pM (Fig. 6A, inset). The LIGAND program indicated that a two-site model also would fit the data, yielding a high-affinity binding site with a K_d of 27 pM and a small percentage (<10%) of ultra-high-affinity binding sites with a K_d of less than 1 fM. This ultra-high-affinity site may be due to cooperativity in soluble receptors binding M-CSF or may be due to inherent inaccuracy in the binding data at the extremes of the curve. The extraordinarily low K_d for the ultra-high-affinity site suggests that the latter possibility is the case.

For comparison, we examined the ligand-binding capacity of native full-length murine Fms expressed on FDC-P1 cells. The FD(c-*fms*) cells were incubated with ^{125}I-M-CSF, and the specific binding was monitored as shown in Fig. 6B. A saturation binding curve could be obtained at 100 pM M-CSF, and the full-length Fms had a single high-affinity binding site with an apparent K_d of 35 pM for ^{125}I-M-CSF, as estimated by Scatchard analysis (Fig. 6B, inset). The K_d for M-CSF binding to E-AP was similar to that of the full-length Fms. Thus, the AP Fms E-segment fusion protein appears to be a valid substitute for the full-length receptor molecule in the solid-phase receptor-binding assay.

Localization of the Fms ligand-binding site. The extracellular domain of Fms is predicted to contain five immunoglobulin-like subdomains, each consisting of about 100 amino acid residues (Fig. 1). To determine which of the five

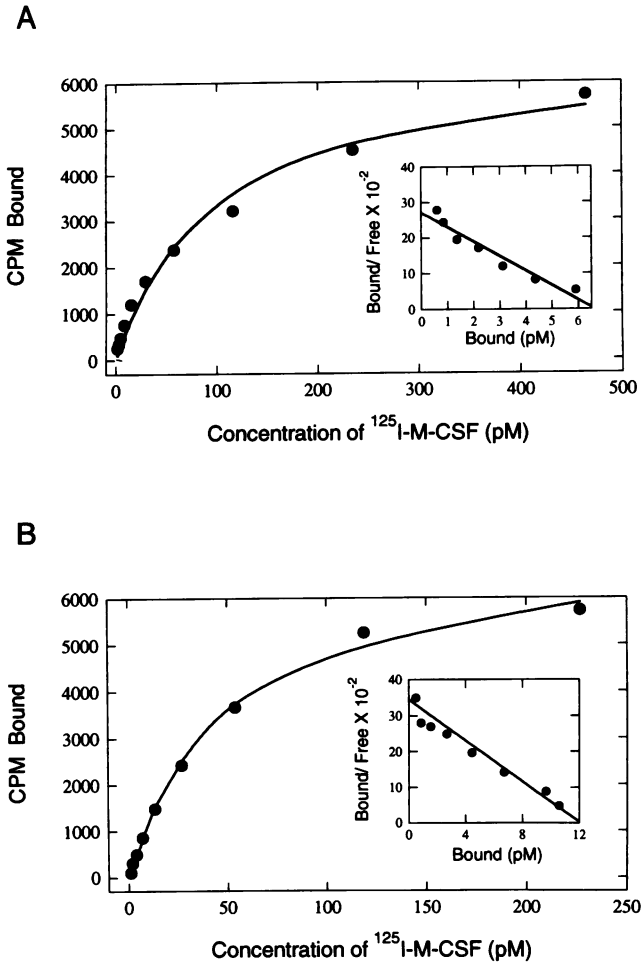

FIG. 6. Solid-phase immunoreceptor assay for measuring affinity binding of the E-AP fusion protein and full-length native Fms to ^{125}I-M-CSF. (A) Binding of purified ^{125}I-labeled M-CSF to the E-AP fusion protein conjugated to glass beads was performed as described in Materials and Methods and plotted as counts per minute (CPM) bound versus concentration of ^{125}I-M-CSF. The inset plot shows the Scatchard analysis of the specific binding of M-CSF to E-AP. Each point represents the average of three determinations. (B) Binding of ^{125}I-labeled M-CSF to full-length Fms expressed on FDC-P1 cells. Specific binding of ^{125}I-labeled M-CSF to Fms on FDC-P1 cells was tested after overnight incubation at 4°C. The inset plot shows the Scatchard analysis of the specific binding of M-CSF to Fms on the FDC-P1 cells.

immunoglobulin-like subdomains were critical for ligand binding, the five Fms truncated segments shown in Fig. 1 were tested for M-CSF binding in the competitive solid-phase assay. Each of the Fms truncated segments was first partially purified by lentil lectin chromatography, and their concentrations were assessed by silver-stained polyacrylamide gels and densitometric analysis. The competition solid-phase assay was performed by incubating the E-AP receptor-coated beads with 57 pM ^{125}I-M-CSF and increasing concentrations of Fms E-segment protein or the other truncated variants. This concentration of M-CSF was near the K_d for binding to E-AP and should give maximum sensitivity in the competition assay. Supernatants from Sf9 cells infected with wild-type baculovirus AcNPV were treated under conditions identical to those of the competition

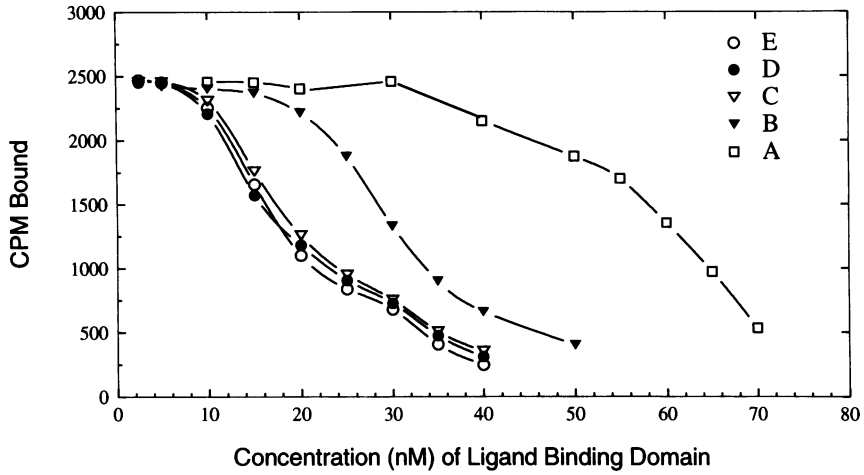

FIG. 7. Competitive solid-phase immunoreceptor assay for measuring affinity of the various Fms protein segments for M-CSF. The solid-phase assay (described in the legend to Fig. 6) used the E-AP fusion receptor protein coupled to glass beads and 57 pM ^{125}I-M-CSF in the presence of increasing concentrations of the various Fms protein segments (for details, see Materials and Methods). The Fms protein segments used for competition are listed in the upper right of the figure adjacent to the symbols for the corresponding data curve.

solid-phase binding assay and served as a negative control. The specific binding assay was based on the previous assay in which only E-AP-coated beads were incubated with 57 pM ^{125}I-M-CSF. As shown in Figure 7, Fms E segment and its truncated variants all inhibited, to some extent, ^{125}I-M-CSF binding to the E-AP receptor. The Fms C-, D-, and E-protein segments exhibited similar competition curves and competed most effectively for M-CSF binding. Half-maximal competition was achieved at a protein segment concentration of about 20 nM for the C, D, and E segments. Fms segment B competed less well with a half-maximal competition at about 32 nM. Surprisingly, the single immunoglobulin-like domain contained in the Fms A-protein segment also contained M-CSF binding activity and competed for ^{125}I-M-CSF binding to the E-AP receptor. The competition by the Fms A segment was, however, significantly less effective than the other segments and was half-maximal at greater than 60 nM. The competitive binding capacity of the truncated variants was specific, since the supernatants from Sf9 cells infected with wild-type AcNPV did not have any effect on the ^{125}I-M-CSF binding to the E-AP receptor.

Because complex interactions of the different immunoglobulin domain segments were possible with the competition experiments, we also assessed the binding of M-CSF to individual immunoglobulin domain segments produced as AP fusion proteins. The results in Fig. 8 demonstrate a rigorous test for detecting [^{35}S]methionine-labelled M-CSF binding to the individual AP fusion proteins on the basis of immunoprecipitability. Neither the A-AP nor B-AP fusion proteins bound ^{35}S-M-CSF; however, the C-AP product containing the three N-terminal immunoglobulin domains bound ^{35}S-M-CSF well. The fusion protein containing the full-length Fms extracellular domain (E-AP) also bound ^{35}S-M-CSF, but the fusion product between domains D4 and D5 and AP lacked detectable M-CSF association. The C-AP fusion protein bound 1.8 times the amount of ^{35}S-M-CSF bound by the E-AP product when the results were normalized for fusion protein content by measuring AP activity.

A second test for detecting weaker interactions between the fusion proteins and ^{125}I-M-CSF was also used. In this assay the individual AP fusion proteins were bound to an anti-AP monoclonal antibody adsorbed to the surface of a 96-well plate. Equal amounts of each fusion protein were used, on the basis of AP activity, and specific binding of ^{125}I-M-CSF measured relative to control medium from Rat2 cells not expressing a fusion protein. The results in Fig. 9 demonstrate the complete lack of M-CSF binding by the A-AP, B-AP, and D4,5-AP fusion proteins. Again, the C-AP product bound M-CSF well, and interestingly, the E-AP protein also bound M-CSF but bound significantly less than the C-AP fusion protein. This differential binding was not a function of M-CSF concentration, because the same results were obtained with amounts of ^{125}I-M-CSF differing by 10-fold (compare Fig. 9A and B).

DISCUSSION

The extracellular M-CSF binding region of the Fms molecule contains five immunoglobulin-like subdomains. The juxtamembrane subdomain (D5 in Fig. 1) forms a variable-type immunoglobulin domain, whereas the remainder form constant-type immunoglobulin-like domains. Each of these immunoglobulin-like domains, except domain D4, contains

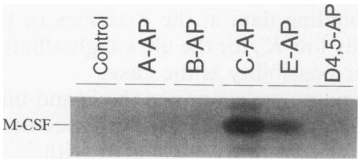

FIG. 8. Immunoprecipitation of [^{35}S]methionine-labelled M-CSF by segments of the Fms extracellular domain fused to AP. Sf9 cells expressing soluble murine M-CSF from a pBluebac vector were labelled with [^{35}S]methionine, and the supernatant was collected. Identical aliquots were mixed with equal volumes of nonradioactive Rat2 cell medium containing the AP fusion protein. A monoclonal antibody to AP was then used to immunoprecipitate the fusion proteins and any bound [^{35}S]methionine-labelled M-CSF. The immune complexes were separated on an SDS-acrylamide gel under reducing conditions. The fusion proteins A-AP, B-AP, C-AP, and E-AP refer to the Fms segments A, B, C, and E fused to AP, and D4,5-AP is composed of the D4 and D5 immunoglobulin domains fused to AP. For control immunoprecipitation, medium from Rat2 cells not expressing a fusion protein construct was used.

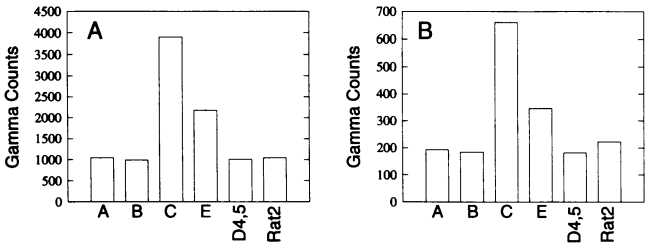

FIG. 9. Solid-phase immunoassay for detecting binding of ^{125}I-M-CSF to the AP fusion proteins. Identical units of each fusion protein (on the basis of AP activity) were bound to an AP monoclonal antibody attached to the bottom of a 96-well plate and incubated with ^{125}I-M-CSF. Bound M-CSF was detected in a gamma counter. The results in panel A were obtained with ten times the amount of ^{125}I-M-CSF used in the panel B experiment.

at least one disulfide bond contributing to the stability of these structures. To identify the M-CSF binding domains and determine their relative contribution to binding, we produced the various immunoglobulin-like extracellular domain segments as soluble proteins from a baculovirus vector in Sf9 cells or as soluble AP fusion proteins expressed in Rat2 cells from a retroviral vector. These soluble segments were then used in competitive solid-phase immunoassays to determine relative affinity for M-CSF binding, and as individual affinity reagents to detect M-CSF binding. These methods have provided results on the relative affinity of single and multiple domains for M-CSF and have additional advantages of detecting all M-CSF binding interactions, not just species-specific interactions as obtained with chimeric receptor molecules (14, 19, 60). Soluble segments for further structural and biological studies are produced in the process.

The results of the solid-phase immunoassay indicated that the extracellular domain of Fms functioned independently of the transmembrane and cytoplasmic portions in M-CSF binding. The E-AP fusion receptor exhibited a single class of high-affinity binding sites for M-CSF analogous to that reported for full-length Fms expressed on intact cells (13). The K_d value for M-CSF binding to the soluble receptor is similar to previously published results with the cloned and expressed murine c-*fms* gene (34), and close to the K_d value for Fms expressed on the FDC-P1 cells. Therefore, the solid-phase immunoassay is a valid measure of M-CSF binding. Also, the presence of the high-affinity site in the soluble receptor indicates that Fms alone constitutes the high-affinity M-CSF receptor and additional receptor subunits or host cell proteins are not necessary.

The competition immunoassay studies demonstrated that the first three amino-terminal immunoglobulin-like domains (D1 to D3) of Fms contained the complete M-CSF binding capacity. All soluble Fms segments competed to near completion for radiolabelled M-CSF binding to the E-AP fusion receptor. Domains D1 and D2 (Fms segment B) competed for M-CSF binding but with lower affinity, and surprisingly, the single amino-terminal immunoglobulin domain (Fms segment A) could also compete for M-CSF binding. Analysis of the competition data by Hill plots (not shown) indicated that multiple copies of the A and B Fms segments caused the competition for M-CSF binding to the E-AP fusion receptor. This could occur through interactions with ^{125}I-M-CSF or possibly by indirect means through interactions with the E-AP fusion receptor.

When the individual A, B, and C domains were fused to AP and directly tested for M-CSF binding by immune precipitation and plate-binding immunoassays, the A and B segments completely lacked M-CSF binding activity, whereas the C segment exhibited high affinity for M-CSF. The ability of the C-AP segment to bind M-CSF was almost twice that of the E-AP fusion fragment. This indicates that the first three N-terminal immunoglobulin-like domains of the Fms extracellular region compose the high-affinity M-CSF binding site. The D3 immunoglobulin-like domain is a major determinant of this M-CSF high-affinity binding site. The D1 and D2 domains most likely also contribute to this high-affinity binding; however, our data do not directly demonstrate that point. If the D1 and D2 domains do contribute to M-CSF binding, it is only when they are associated with the D3 domain.

M-CSF binding and subsequent activation of the Fms receptor demonstrate a strong species specificity. Human M-CSF stimulates the human, murine, and feline receptors; and murine M-CSF stimulates murine and feline (weakly) receptors, but feline and chicken M-CSF function only on Fms of their own species (37, 48, 50, 59). Amino acid sequences for murine, human, and feline Fms proteins are published (4, 34, 59), and the avian Fms sequence has been determined (3a). Sequence alignment of amino acids within each of the immunoglobulin-like domains reveals some general variation, but major differences from the consensus sequence occur within immunoglobulin domains D1 and D3. These differences generally occur in regions of the immunoglobulin domain corresponding to loops between strands of β-sheets and are in an equivalent position to those regions that have been designated CDR (complementarity determining region or hypervariable region) in antibody molecules (58). These regions function as antigen recognition sites, and within Fms these regions are likely sites for M-CSF binding and determining the species specificity.

Many receptors exhibit both low-affinity and high-affinity binding states, but the M-CSF receptor has only a single high-affinity binding state (13). Many receptors with dual binding affinity states have receptor subunits with one or both of the subunits exhibiting the low-affinity binding state, with the combined subunits supporting the high-affinity binding state (9, 23). Therefore, one explanation for the single high-affinity state in Fms is that both low-affinity and high-affinity sites are contained on the same molecule, with low-affinity interactions through single immunoglobulin domains and high-affinity interactions through multiple immunoglobulin domains. This would be consistent with the possibility that the D3 immunoglobulin domain is the major determinant of M-CSF binding but that the D1 and D2 domains may be needed as well.

Other related receptors containing immunoglobulin-like domains also have at least some ligand-binding activity within their first three N-terminal immunoglobulin domains. The PDGF receptor, like Fms, has five tandemly arranged extracellular immunoglobulin domains and determinants for isoform-specific PDGF binding are contained in the three N-terminal domains (14). Members of the FGF receptor family contain, at most, just three immunoglobulin domains in their extracellular region. The ligand must interact with one or all of these immunoglobulin domains and experiments have shown that a 50-amino-acid variable region within the third (D3) immunoglobulin domain determines ligand specificity (60). The extracellular matrix receptor, ICAM-1, also displays an array of five immunoglobulin-like domains in its extracellular portion with a diverse set of ligands binding to the first three amino-terminal domains. Distinguishable binding sites for rhinovirus and the lymphocyte function-associ-

ated antigen, LFA-1, have been characterized in the first two amino-terminal immunoglobulin domains, and an additional distinct binding site for the malaria parasite *Plasmodium falciparum* is located in this first immunoglobulin domain. Mac-1 binding maps to the third ICAM-1 immunoglobulin domain and the association is dependent on the extent of glycosylation (3, 24, and references therein).

Immunoglobulin-like domains D4 and D5 did not appear to contribute to M-CSF binding. Neither the Fms segments containing the domains D1 to D4 (Fms segment D) nor the Fms segment containing the entire extracellular domain (Fms segment E) competed to any greater degree than the Fms C segment, which contained only the first three domains. Also, the D4,5-AP fusion protein did not interact with M-CSF in either the plate binding immunoassay or immunoprecipitation assay. In fact, the presence of the D4 and D5 domains in the E-AP fusion protein may have contributed to the decreased M-CSF binding of the E-AP product compared with the C-AP protein.

The D4 and D5 immunoglobulin domains are also structurally distinct from the other immunoglobulin-like domains of Fms. Domain D4 lacks the characteristic intradomain disulfide bonds found in the other Fms immunoglobulin-like domains. Previous work has suggested that this domain may signal M-CSF binding at the distal three amino-terminal immunoglobulin domains through a conformational change (35, 59). This conformational change may be necessary for dimerization and/or transduction of information to the cytoplasmic tyrosine kinase domain. Domain D5 is classified as a variable-type immunoglobulin-like domain, whereas the others are constant-type domains (58). The Fms D5 variable-type immunoglobulin domain also lacked M-CSF binding activity. The function of this domain is unknown, but its structure and juxtamembrane location suggest it could interact with host cell surface proteins, and this possibility is currently under experimental examination.

ACKNOWLEDGMENTS

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