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The unique insert of cellular and viral *fms* protein tyrosine kinase domains is dispensable for enzymatic and transforming activities

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The receptors for colony stimulating factor-1 (CSF-1), platelet derived growth factor and the c-*kit* protein tyrosine kinase (PTK) contain within their catalytic domains a stretch of 60-100 residues, largely unrelated in sequence, with no counterpart in other PTKs. Of the 64 amino acids within this kinase insert, 58 were deleted from the mouse CSF-1 receptor by oligonucleotide-directed mutagenesis. The mutant CSF-1 receptor was not markedly affected in its kinase activity, post-translational processing or its ability to induce autocrine transformation of NIH 3T3 mouse fibroblasts. Similarly, retention of kinase and transforming activities were observed following deletion of part or all of the kinase insert from the v-*fms* oncoprotein. The c- and v-*fms* kinase inserts were probed using monoclonal and polyclonal antibodies and were found to be highly antigenic. Two monoclonal antibodies raised to the v-*fms* cytoplasmic domain both recognized epitopes within the insert, and bound enzymatically active v-*fms* glycoproteins. These results indicate that the *fms* kinase insert is located on the surface of the protein and folds separately from the rest of the catalytic domain, but is not required for the biological activity of *fms* PTKs ectopically expressed in mouse fibroblasts. The insert may therefore play a specific function in cells such as monocytes and trophoblasts that normally express the CSF-1 receptor.

Key words: CSF-1 receptor/*fms* oncogene/kinase insert

Introduction

Transmembrane protein tyrosine kinases (PTKs) mediate the diverse biological effects of many polypeptide hormones and growth factors (Yarden and Ullrich, 1988). Different hormones, acting through their respective PTK receptors, elicit quite separate and individual physiological responses. This specificity in the biochemical properties of PTK receptors suggests that their intracellular domains have distinct targets. This might simply reflect a limited and co-ordinate expression of the receptor and target. An alternative possibility is that the various receptors have intrinsically different target specificites. Assuming that their biological activities are entirely due to their phosphorylation of cellular proteins on tyrosine, their catalytic domains might therefore have different substrate specificities.

The enzymatic domains of protein kinases have two highly conserved regions, involved in ATP-binding and phospho-transfer, that apparently interact to form the catalytic active site (Buechler and Taylor, 1988; Hanks *et al.*, 1988; Moran *et al.*, 1988). These are separated in the primary structure by a more variable element that may be involved in substrate recognition (Miller and Kaiser, 1988). A subfamily of receptors that includes the c-*fms* (CSF-1-receptor[R]) (Sherr *et al.*, 1985), PDGF-R (Yarden *et al.*, 1986) and c-*kit* (Yarden *et al.*, 1987) proteins contain a stretch of 60-100 extra amino acids, relative to other PTKs, embedded within this variable region (see Figure 1). These additional (insert) residues of the PDGF-R and c-*kit* gene product show only very weak sequence relationship to one another, and show no obvious similarity to the CSF-1-R kinase insert. However, they each possess a large proportion of proline, glycine and charged residues. The hydrophilicity of these insert sequences suggests that they might lie on the surface of the native proteins. A number of functions might be envisioned for these inserts, such as direct involvement in catalysis, modulation of ligand-stimulated kinase activity, or the recognition of cellular proteins that act as substrates for the kinase domain or that regulate intracellular transport or stability of the receptors.

To test these possibilities we are investigating the role of the kinase insert in the activities of *fms* proteins. c-*fms* and its oncogenic counterpart v-*fms*, the transforming gene of the Susan McDonough feline sarcoma virus (SM-FeSV), encode transmembrane receptors with an intracellular tyrosine kinase domain joined to an ectodomain that binds macrophage colony stimulating factor-1 (CSF-1/M-CSF) (Sherr *et al.*, 1985; Sacca *et al.*, 1986; Downing *et al.*, 1988; Lyman *et al.*, 1988). Expression of c-*fms* is restricted to trophoblasts of the embryonic placenta (Pollard *et al.*, 1987; Regenstreif and Rossant, 1989) and hematopoietic cells of the monocyte lineage (Woolford *et al.*, 1985). CSF-1 induces differentiation and growth of monocytic cells, is required for their survival in culture and promotes mature cell functions (Tushiniski *et al.*, 1982; Stanely *et al.*, 1983). While the kinase activity of gp165$^{c\text{-}fms}$ is CSF-1 dependent (Yeung *et al.*, 1987; Downing *et al.*, 1988), the v-*fms* product has acquired amino acid substitutions in its ecto-domain and at its C-terminus that induce constitutive kinase activity (Roussel *et al.*, 1988; Woolford *et al.*, 1988). We have assessed the consequences of deleting the c- and v-*fms* insets on their enzymatic and transforming activity in mouse NIH 3T3 fibroblasts.

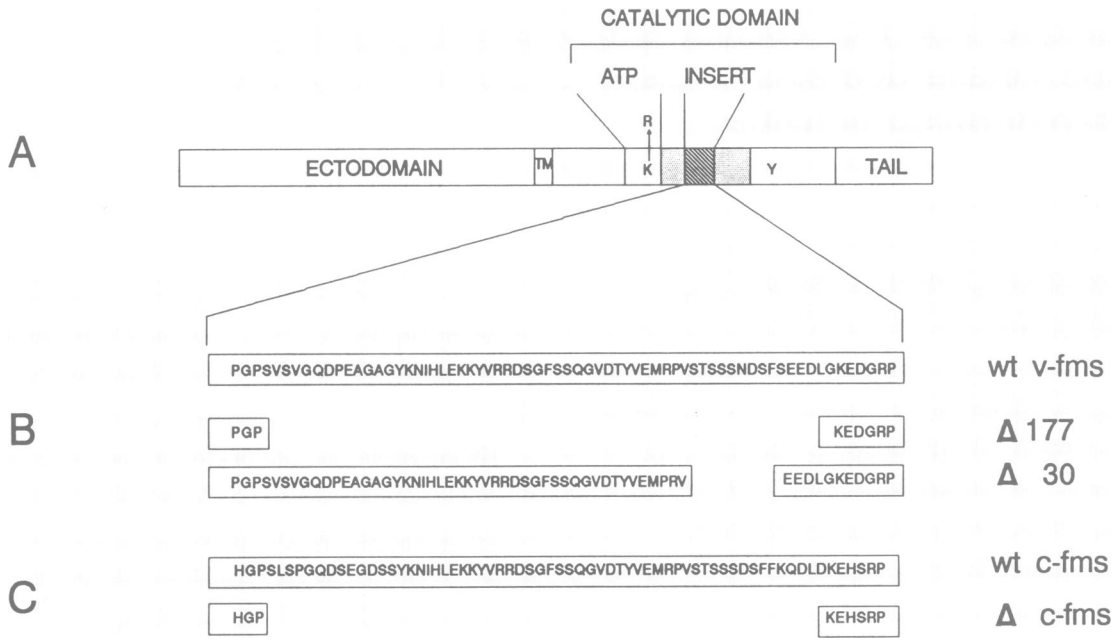

Fig. 1. *fms* proteins and insert deletions. (**A**) Schematic diagram of *fms* encoded proteins showing the ligand binding domain (ECTODOMAIN), the transmembrane domain (TM), the catalytical domain and the COOH terminal tail (TAIL). Structural differences between c-*fms* and v-*fms* proteins are not illustrated. The catalytic domain is further divided into two highly conserved regions involved in ATP binding (ATP) and phosphotransfer (a highly conserved tyrosine residue equivalent to $p60^{v\text{-}src}$ Tyr-416 is indicated Y). These two regions are separated by a more variable region (speckled box) and the insert region (striped box). The Lys (K) to Arg (R) mutation at residue 613 is also shown. (**B**) The amino acid sequence of the v-*fms* insert region is shown in one letter code. The two deletion mutants are also shown. (**C**) The amino acid sequences of c-*fms* and Δc-*fms* inserts are shown.

Results

Transforming and kinase activities of mutant c-fms glycoproteins

The coding sequence for the mouse CSF-1-R, derived from a c-*fms* cDNA (Rothwell and Rohrschneider, 1987), was placed into an SV40-based expression vector and transfected into mouse NIH 3T3 fibroblasts in conjunction with pSV2neo. Cell lines were selected for resistance to G418 and analyzed for c-*fms* expression by Northern blot anlaysis (data not shown). NIH 3T3 cells expressing mouse c-*fms* RNA were metabolically labeled with [^{35}S]methionine, lysed and immunoprecipitated with anti-c-*fms* antiserum. The antiserum specifically recognized an immature c-*fms* glycoprotein precursor ($gp130^{c\text{-}fms}$) and the fully glycosylated c-*fms* protein ($gp165^{c\text{-}fms}$) (Figure 2). Similar results were obtained with Western blotting using the same antiserum (Figure 3A, lane 2). These cells were also shown to express high affinity receptors for ^{125}I-labeled mouse CSF-1 (data not shown). Both $gp130^{c\text{-}fms}$ and $gp165^{c\text{-}fms}$ became autophosphorylated on tyrosine in immune complex kinase reactions following immunoprecipitation from transfected NIH 3T3 cells (Figure 2). Using these assays, no c-*fms* gene products were identified in parental NIH 3T3 cells.

All cell lines which expressed c-*fms* were morphologically transformed (see Figure 4B). In contrast G418^r cell lines from the same transfections which failed to express c-*fms*, or control lines transfected only with pSV2neo, retained a flat morphology equivalent to parental NIH 3T3 cells. The ability of mouse c-*fms* to transform NIH 3T3 cells was not unexpected, since fibroblasts, including NIH 3T3 cells, express CSF-1 (Stewart and Lin, 1978; Rollins *et al.*, 1988) and the introduction of the CSF-1 receptor would therefore close an autocrine loop. Autocrine transformation of BALB/c mouse fibroblasts by mouse c-*fms* has also been observed (Rohrschneider *et. al.*, 1989).

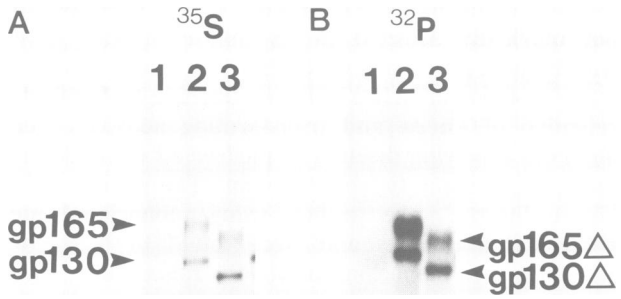

Fig. 2. Expression and kinase activity of wild-type and mutant CSF-1 receptors. (**A**) Cells were labeled with [^{35}S]methionine and lysates were immunoprecipitated with antisera 4069-B2. (**B**) Unlabeled cells were immunoprecipitated with antisera 4069-B2 and incubated with [γ-^{32}P]ATP to assess kinase activity. Lane 1, NIH 3T3 cells; lane 2, NIH 3T3 cells expressing wild-type mouse c-*fms*; lane 3, NIH 3T3 cells expressing the Δc-*fms* mutant. The mobilities of the immature (gp130) and mature (gp165) c-*fms* glycoproteins are indicated. The corresponding Δc-*fms* products are designated gp130Δ and gp165Δ.

Having established a biological assay for mouse c-*fms*, we deleted the codons for c-*fms* residues 669−747 using

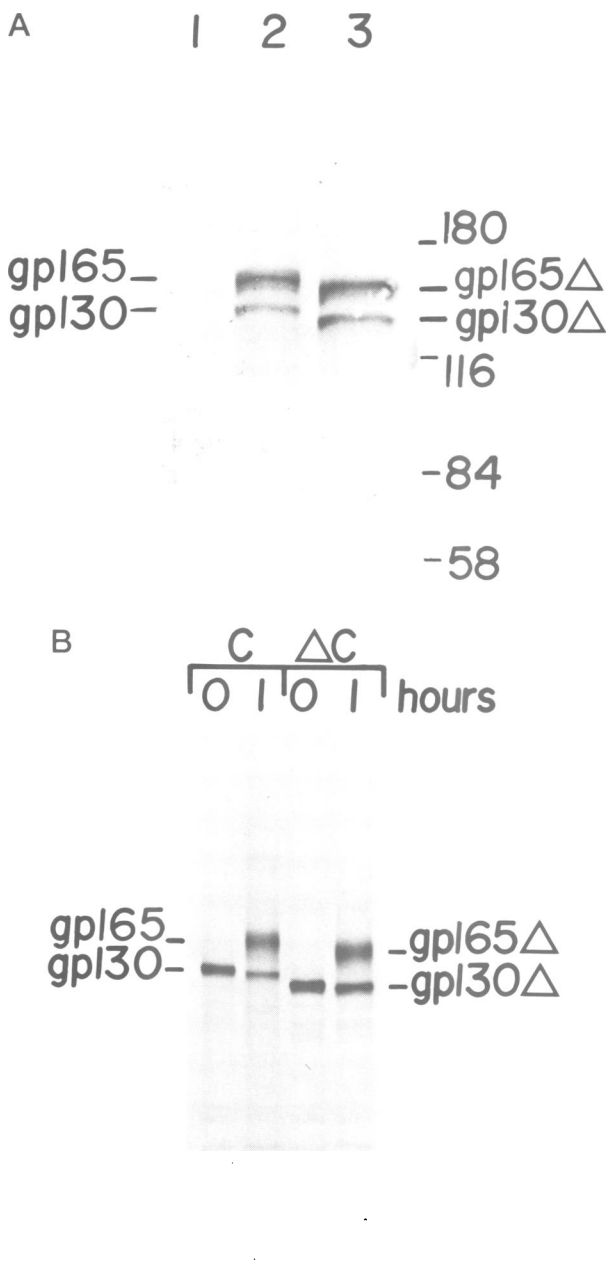

Fig. 3. Processing of wild-type and mutant CSF-1 receptors.
(**A**) Western blot analysis of NIH 3T3 cells expressing wild-type or mutant c-*fms*. Lysates from the following cell lines were subjected to Western blot analysis using antibody 4069-B2 as described in Materials and methods: lane 1, NIH 3T3 cells; lane 2, NIH 3T3 cells expressing wild-type c-*fms*; lane 3, NIH 3T3 cells expressing Δc-*fms*.
(**B**) Synthesis and post-translational processing of wild type (C) and mutant (ΔC) mouse c-*fms*. Cells were pulse labeled for 15 min with [^{35}S]methionine, and either lysed immediately (0 time point), or chased in the presence of unlabeled methionine for 1 h. Lysates were immunoprecipitated with antiserum 4069-B2.

oligonucleotide-directed mutagenesis. This deletion removes 58 residues that comprise virtually the entire insert sequence (Figure 1). The coding sequence from this c-*fms* insert mutant (Δc-*fms*) was placed into the pECE expression vector and co-transfected into NIH 3T3 fibroblasts with pSV2neo. Cell lines expressing Δc-*fms* had a highly transformed morphology (Figure 4C), and grew in soft agar with the same efficiency as cell lines expressing wild-type c-*fms* (~0.7%) (Figure 5). Soft agar colonies formed by cells expressing Δc-*fms* were smaller than those formed by cells expressing equivalent levels of wild-type c-*fms*. Δc-*fms*-transformed NIH 3T3 cells were analyzed for production of Δc-*fms* gene products by immunoprecipitation from metabolically labeled cells (Figure 2) and by Western blot analysis (Figure 3A). Both procedures detected immature and mature Δc-*fms* glycoproteins which appeared ~6 kd smaller than their wild-type counterparts, and were present in similar ratio and abundance to wild-type gp130$^{c\text{-}fms}$ and gp165$^{c\text{-}fms}$. Pulse–chase experiments showed that the half-lives of the wild-type and deleted c-*fms* proteins were similar (data not shown) and that the processing of Δc-*fms* to a mature glycosylated form was as efficient as for the wild-type protein (Figure 3B).

Immune complexes containing the Δc-*fms* glycoproteins were incubated with [γ-^{32}P]ATP in *in vitro* kinase reactions. Both forms of Δc-*fms* products became autophosphorylated on tyrosine (Figure 2). The apparent specific activity of autophosphorylation was ~50% that of the wild-type c-*fms* tyrosine kinase. This figure is probably an underestimate as two-dimensional tryptic phosphopeptide analysis of c-*fms* and Δc-*fms* autophosphorylated *in vitro* revealed at least one major spot which was missing from the Δc-*fms* digest (data not shown). The *in vitro* kinase activity of Δc-*fms* was further tested by incubating immune complexes with [γ-^{32}P]ATP in the presence of a poly(Glu,Tyr) polymer (Sadowski and Pawson, 1987). Under these conditions Δc-*fms* retained 90% of wild-type kinase activity (data not shown). The deletion of the mouse c-*fms* insert therefore had little effect on the *in vitro* tyrosine kinase activity, the intracellular processing, or the stability of Δc-*fms* glycoproteins. Since the ability of the c-*fms*/CSF-1 receptor to transform NIH 3T3 cells likely depends on appropriate interactions with CSF-1, we take the continued transforming activity of the Δc-*fms* glycoproteins as evidence for their ability to bind CSF-1 and transduce CSF-1-dependent signals.

v-fms PTKs lacking kinase insert sequences retain enzymatic and transforming activity

The *gag* sequence of the retroviral *gag–fms* oncoprotein is rapidly cleaved following synthesis, and is not required for subsequent processing or the induction of neoplastic transformation (Wheeler *et al.*, 1986). For ease of subsequent manipulation we removed the *gag*-encoded sequence of *gag–fms* as described in Materials and methods. Two deletions were made within the kinase insert of this *gag*-minus v-*fms* using oligonucleotide-directed mutagenesis (see Figure 1). The largest deletion (Δ177) removed 59 codons corresponding precisely to those excised in Δc-*fms* (the v-*fms* insert region contains an additional Asn residue at amino acid position 733 relative to c-*fms*). A smaller deletion, Δ30, removed 10 codons corresponding to a serine/threonine-rich element. Wild-type, Δ177 and Δ30 v-*fms* sequences inserted into the pECE-derived expression vector and expressed in COS-1 monkey cells encoded glycoproteins of the expected sizes (data not shown). The expression plasmids were then co-transfected into NIH 3T3 cells with pSV2neo, and transfectants were selected with G418. Cells expressing wild-type, Δ177 or Δ30 v-*fms* were morphologically transformed (Figure 4D–F). Wild-type v-*fms* encoded abundant, immature gp120$^{v\text{-}fms}$ which was only poorly processed to the mature gp140$^{v\text{-}fms}$ form, as previously described

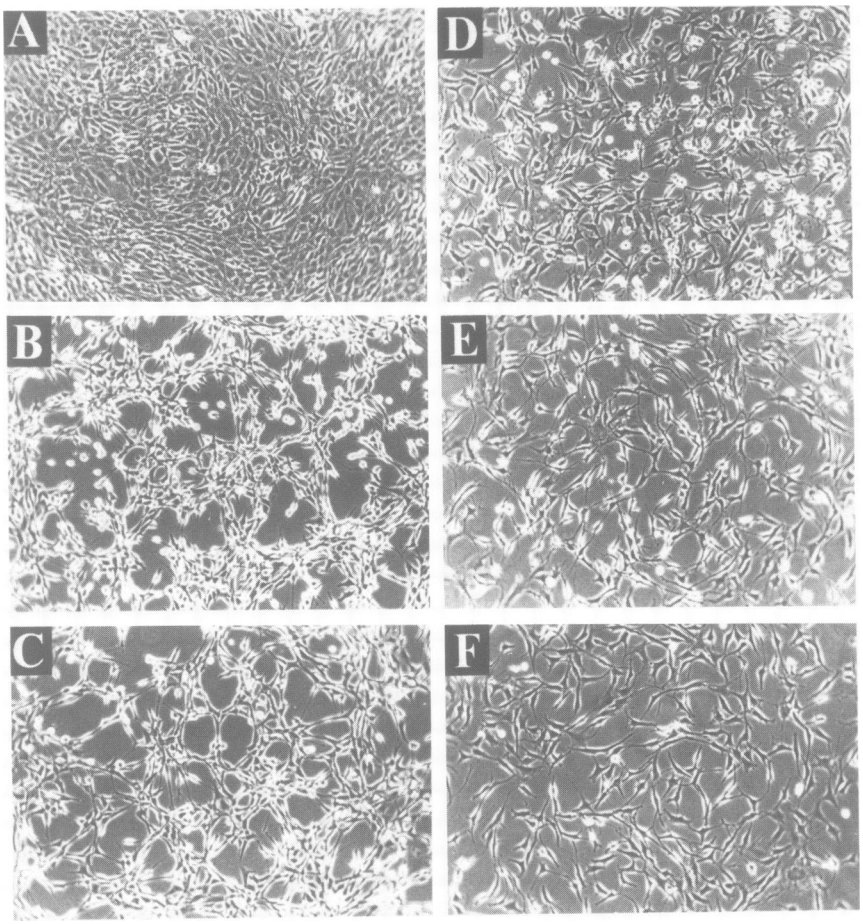

Fig. 4. Morphology of NIH 3T3 cells expressing wild-type or mutant c-*fms* or v-cfms. **(A)** NIH 3T3 cells; **(B)** wild-type c-*fms*; **(C)** Δc-*fms*; **(D)** wild-type v-*fms*; **(E)** Δ177 v-*fms*; **(F)** Δ30 v-*fms*.

(Anderson *et al.*, 1984). The Δ177 and Δ30 v-*fms* mutants encoded appropriately smaller glycoproteins, as determined by immunoprecipitation from [^{35}S]methionine labeled cell lysates with a monoclonal antibody to the v-*fms* extracellular domain (Figure 6). The wild-type, Δ177 and Δ30 v-*fms* glycoproteins were all enzymatically active, as measured by *in vitro* autophosphorylation at tyrosine (Figure 6) and the ability to phosphorylate the exogenous substrate poly(Glu,Tyr) (data not shown). Cell lines expressing Δ177 grew in soft agar with the same efficiency as wild-type v-*fms* although the colonies formed were smaller. Pulse−chase experiments indicated that the mutant proteins had half-lives similar to that of wild-type v-*fms* (data not shown). We conclude that the insert domain of v-*fms*, like that of c-*fms*, can be deleted without ablating enzymatic activity or transforming activity on NIH 3T3 cells.

Lys-613 is required for v-fms biological activity

The presumptive ATP-binding fold of protein kinases contains an invariant lysine which covalently binds the ATP analogue fluorosulfonyl benzoyl 5′-adenosine, and which is essential for catalysis (Zoller *et al.*, 1981; Barker and Dayhoff, 1982; Weinmaster *et al.*, 1986). The codon for the corresponding v-*fms* residue (Lys-613) was altered to an arginine codon by site-directed mutagenesis (see Figure 1). NIH 3T3 cells expressing the Arg-613 mutant protein were morphologically normal (data not shown). The Arg-613 gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ glycoproteins could be readily identified by immunoprecipitation of [^{35}S]-methionine-labeled cells, but such mutant proteins were unable to autophoshorylate *in vitro* (Figure 6). This result shows that, in contrast to deletion of the insert, substitution of a conserved residue in the kinase domain directly implicated in catalysis precipitates a co-ordinate loss of kinase and transforming activities.

The v-fms kinase insert is highly antigenic

In an initial analysis of wild-type and Δ177 v-*fms* gene products, we found that three separate rabbit polyclonal antisera raised against the v-*fms* cytoplasmic domain expressed in bacteria were inefficient in immunoprecipitating Δ177 glycoproteins which lack the kinase insert. This raised the possibility that the insert might be particularly antigenic. To investigate this further we used three anti-v-*fms* monoclonal antibodies. Antibody SM2.6.3 recognizes an epitope in the v-*fms* ectodomain (Rettenmier *et al.*, 1985), while antibodies 2E8 and 7F4 were raised to the bacterially synthesized v-*fms* internal domain. In each case gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ immunoprecipitated by these antibodies were active in *in vitro* autophosphorylation (unpublished results). Antibody SM2.6.3 was equally effective in immunoprecipitating wild-type, Δ30 or Δ177 v-*fms* glycoproteins (Figure 7). In contrast antibodies 2E8 and 7F4 both recognized wild-type and Δ30 gene products, but were unable to immunoprecipitate the Δ177 v-*fms* glycoprotein. This suggested that the epitope(s) for these monoclonal antibodies were contained

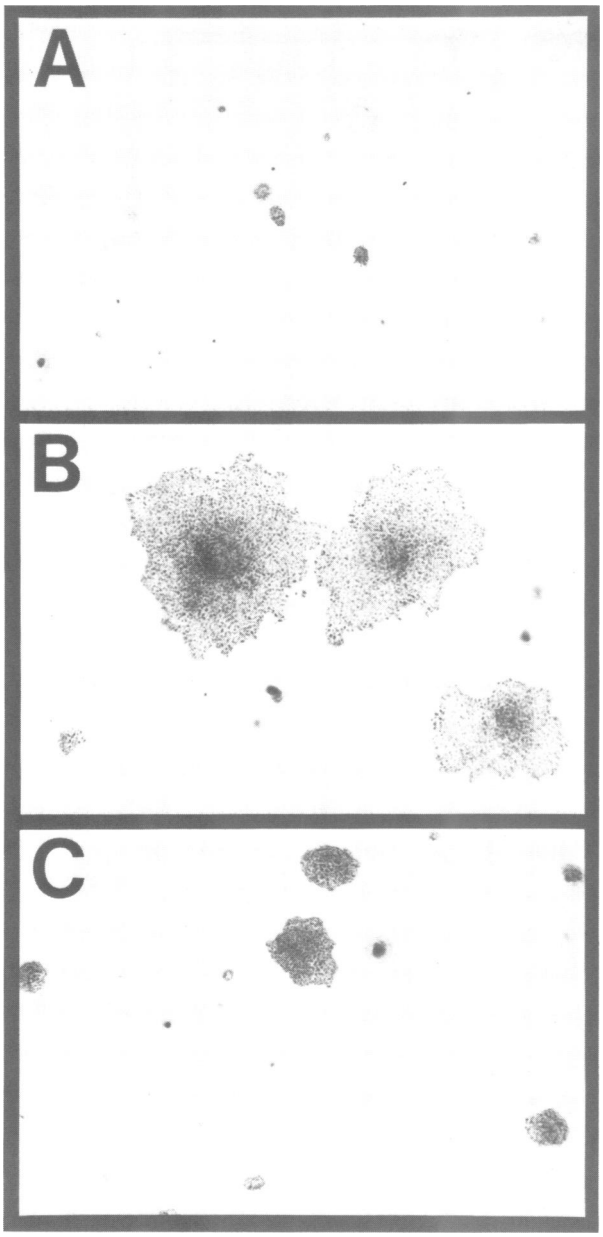

Fig. 5. Formation of colonies in soft agar. (**A**) Parental NIH 3T3 cells; (**B**) NIH 3T3 cells expressing wild-type c-*fms*; (**C**) NIH 3T3 cells expressing Δc-*fms*.

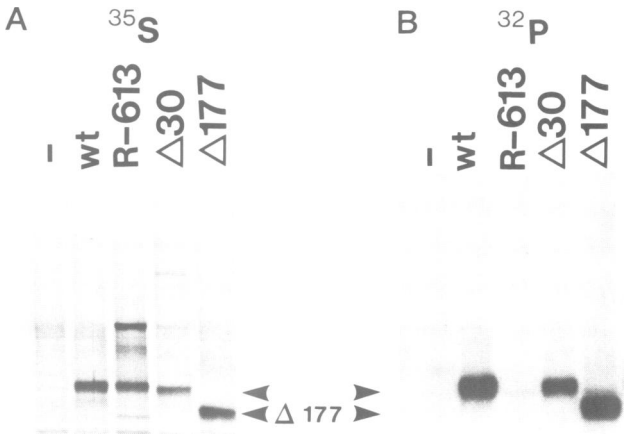

Fig. 6. Expression and kinase activity of wild-type and mutant v-*fms*. (**A**) Cells were labeled with [^{35}S]methionine and lysates were immunoprecipitated with antibody SM2.6.3. (**B**) Unlabeled cells were immunoprecipitated with antibody SM2.6.3 and incubated with [γ-^{32}P]ATP to assess kinase activity. Immunoprecipitates were from: NIH 3T3 cells (−); NIH 3T3 cells expressing wild-type v-*fms* (wt); NIH 3T3 cells expressing the Arg(613) v-*fms* mutant (R-613); NIH 3T3 cells expressing Δ30 v-*fms* (Δ30); NIH 3T3 cells expressing Δ177 v-*fms* (Δ177). Arrows indicate relevant mobilities of wild-type gp120$^{v\text{-}fms}$ or the corresponding mutant products.

within the insert sequence deleted in the Δ177 v-*fms* mutant, although not within the short insert sequences deleted in Δ30. To test this notion more rigorously the coding sequence for the v-*fms* kinase insert was isolated as a restriction fragment, inserted in-frame with the *trpE* gene of the bacterial pATH expression vectors and expressed in *Escherichia coli*. The resulting *trpE* fusion protein, which contains v-*fms* amino acid residues 662−756, carries the v-*fms* kinase insert with a few surrounding residues (Figure 8A). The 2E8 and 7F4 anti-v-*fms* monoclonal antibodies directly recognized the *trpE*−insert(v-*fms*) polypeptide on Western blots of induced bacterial lysates, but did not recognize the *trpE* polypeptide alone (Figure 8A). Thus the epitope(s) for these antibodies must lie within the kinase insert. The polyclonal anti-v-*fms* rabbit antisera that were inefficient in precipitating the Δ177 glycoproteins also reacted strongly with the bacterial v-*fms* kinase insert (data not shown). In a similar fashion the mouse c-*fms* kinase insert (residues 637−783) was expressed in *E. coli* as a *trpE* fusion protein. Two of the three polyclonal rabbit antisera, raised against the c-*fms* cytoplasmic domain, strongly reacted with the bacterial *trpE*−insert(c-*fms*) polypeptide by Western blotting (Figure 8B).

These results suggest that the kinase inserts of v- and c-*fms* are highly antigenic. The ability of anti-v-*fms* monoclonal antibodies 2E8 and 7F4 (both of which bind epitopes within the insert) to precipitate native, enzymatically active v-*fms* proteins indicate that the epitopes are located on the surface of the v-*fms* protein.

Discussion

Precise in-frame deletions within the mouse c-*fms* and feline v-*fms* coding sequences have removed part or all of the kinase insert characteristic of the *fms*/*kit*/PDGF-R family. This sequence, dubbed an insert since it has no counterpart in other PTKs, is located in a variable region of the tyrosine kinase catalytic domain. A c-*fms* mutant glycoprotein lacking kinase insert sequences retained its ability to transform NIH 3T3 mouse fibroblasts, although colony size in soft agar was reduced. The transforming activity of wild-type and mutant mouse CSF-1 receptors most probably results from autocrine stimulation by endogenous CSF-1. Thus human CSF-1 receptor, which does not bind mouse CSF-1, fails to transform NIH 3T3 cells in the absence of human CSF-1

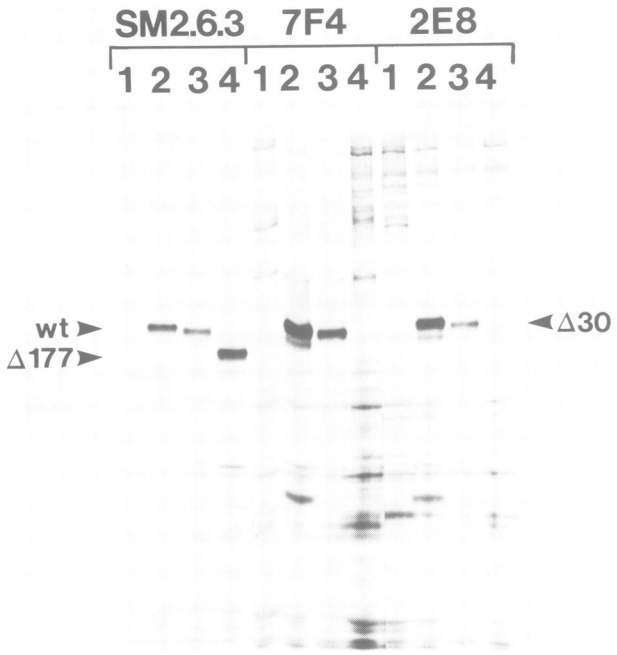

Fig. 7. Immunoprecipitation of wild-type and mutant v-*fms* proteins by monoclonal antibodies. Cells were labeled with [^{35}S]methonine and lysates were immunoprecipitated with monoclonal antibodies SM2.6.3, 7F4 or 2E8. **Lane 1**, NIH 3T3 cells; **lane 2**, NIH 3T3 cells expressing wild-type v-*fms*; **lane 3**, NIH 3T3 cells expressing Δ30 v-*fms*; **lane 4**, NIH 3T3 cells expressing Δ177 v-*fms*. Arrows indicate mobilities of wild-type gp120$^{v\text{-}fms}$ or the corresponding Δ30 or Δ177 products.

(Roussel *et al.*, 1987). The mutant CSF-1 receptor is also very similar to the wild-type glycoprotein in its *in vitro* tyrosine kinase activity, processing to a higher molecular weight form and stability. We can therefore conclude that 58 residues of the kinase insert can be removed from the CSF-1-R without markedly affecting its kinase activity, glycosylation, interaction with CSF-1 or recognition of intracellular targets involved in transformation of NIH 3T3 cells. We also found that deletion of the v-*fms* kinase insert did not seriously impair its kinase activity or transforming activity on NIH 3T3 cells. As for c-*fms*, growth in soft agar was less robust following removal of the insert. These results are in marked contrast to the v-*fms* Arg-613 mutant which only differs from the wild-type oncoprotein by a conservative substitution of Arg for Lys, and hence by the replacement of the Lys-613 Σ-amino group by a guanidinium group. This mutant glycoprotein was inactive as a kinase and was non-transforming.

These results suggest that the insert does not fold as an integral part of the catalytic domain, but may instead form a loop on the surface of the protein. Support for this hypothesis is provided by the finding that the v-*fms* and c-*fms* kinase inserts are highly antigenic. Anti-v-*fms* monoclonal antibodies whose epitope(s) are contained within the kinase insert immunoprecipitate non-denatured, catalytically active gp120$^{v\text{-}fms}$. The accessibility of these epitope(s) argues that the insert is located on the surface of the *fms* tyrosine kinase domain. This conclusion emphasizes the modular construction of protein kinases.

If the *fms* kinase insert is not essential for any property

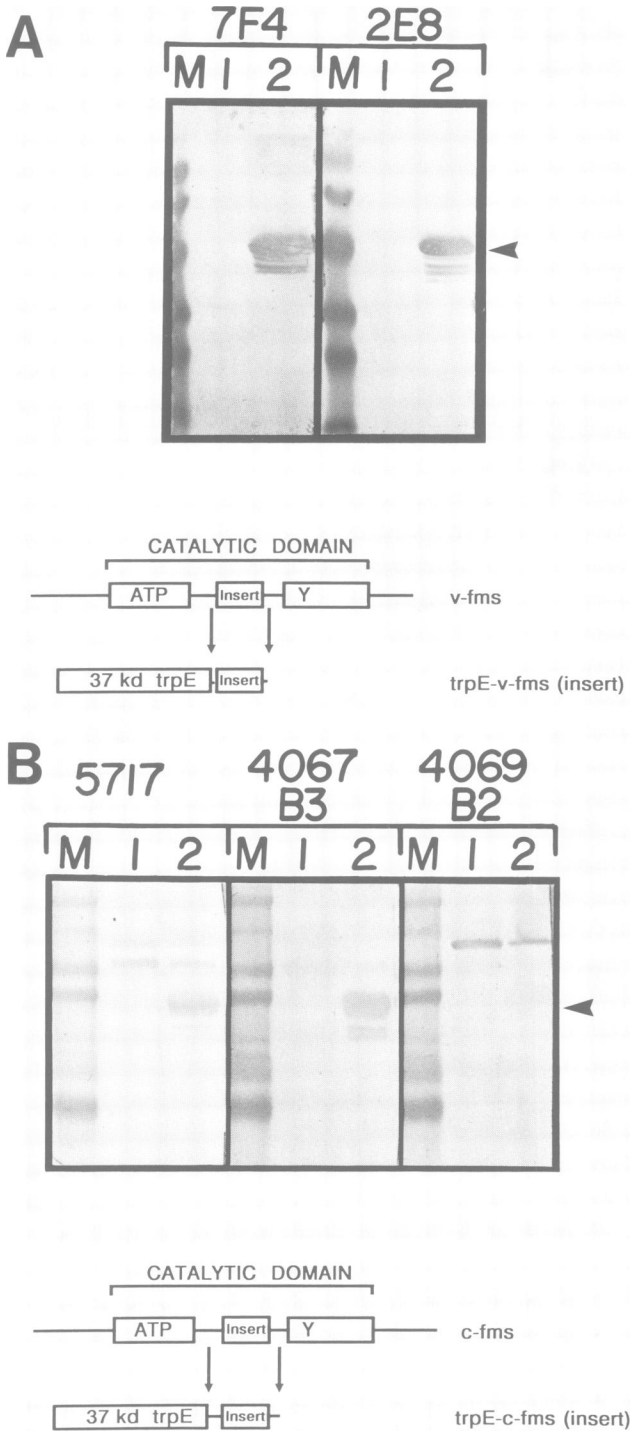

Fig. 8. Western blot analysis of *fms* inserts expressed in isolation as *trpE* fusion proteins. **(A)** Lysates from induced *E.coli* expressing the following proteins were subjected to Western blot analysis: lane 1, 37 kd *trpE* fragment; lane 2, *trpE*−v-*fms*(insert) fusion; lane M, mol. wt markers (180, 116, 84, 58, 48.5, and 36.5 kd). Protein bound to nitrocellulose was probed with either the 7F4 or 2E8 anti-v-*fms* monoclonal antibodies as described in Materials and methods. **(B)** Lysates from induced *E.coli* expressing the following proteins were subjected to Western blot analysis: lane 1, 37 kd *trpE* fragment; lane 2, *trpE*-c-*fms*(insert) fusion. Protein bound to nitrocellulose was probed with partially purified antibodies from antisera 5717, 4067-B3 or 4069-B2. Protein was electrophoresed through a 15% SDS−polyacrylamide gel, transferred to nitrocellulose and probed with antibodies as described in Materials and methods. The structures of the *trpE*−*fms* fusion proteins are illustrated below the relevant Western blots, and their mobilities are indicated with an arrow.

of the CSF-1 receptor that has been measured in NIH 3T3 cells, what is its function? The c-*fms*, c-*kit* and PDGF-R genes likely arose following duplication of a common ancestral locus. Indeed the human PDGF-R and c-*fms* genes are adjacent on chromosome 5 (Roberts *et al.*, 1988). This evolutionary relationship is underlined by the clustering of their encoded gene products on a phylogenetic tree based on amino acid sequences of protein kinase domains (Hanks *et al.*, 1988). Nevertheless the CSF-1-R, PDGF-R and c-*kit* protein kinase inserts show little sequence identity. It is possible that the insert elements have no biological function, and have diverged accordingly. A more attractive hypothesis is that they are required for the recognition of cellular targets unique to each receptor. CSF-1 is required for the proliferation and survival of monocytes, induces myeloid differentiation, and may be important for the embryonic placenta (Tushinski *et al.*, 1982; Pollard *et al.*, 1987); PDGF induces proliferation and migration of mesenchymal cells and is involved in wound-healing (Ross *et al.*, 1985); c-*kit* is allelic with the mouse W locus, and its product presumably binds a ligand (possibly encoded by the *Steel* locus) required for the development of stem cells of the melanocyte, hematopoietic and germ cell lineages (Chabot *et al.*, 1988; Geissler *et al.*, 1988). The receptors for these ligands therefore have quite different physiological activities which may be mediated in part by their non-catalytic kinase insert regions. Thus the exposed c-*fms* insert may contact targets of the CSF-1 receptor specific to monocytes or trophoblasts, and thereby direct the specific biological effects of CSF-1. In NIH 3T3 cells, which normally do not express c-*fms*, the wild-type and mutant *fms* glycoproteins may simply function as promiscuous tyrosine kinases. During the course of this work Escobedo and Williams (1988) reported that a PDGF-R lacking a kinase insert was indistinguishable from wild-type receptor for every biochemical property tested in CHO cells in response to PDGF, except the ability to induce DNA synthesis. Since a primary physiological function of PDGF is as a mitogen, it is interesting that an insert-minus PDGF-R is specifically defective in its ability to induce DNA replication. Experiments are in progress to test the capacity of the Δc-*fms* gene to support CSF-1-induced differentiation of myeloid cells.

Materials and methods

Construction of mammalian and bacterial expression vectors

All plasmids were constructed by standard techniques. Nucleotide numbers for c-*fms* are those of Rothwell and Rohrschneider (1987), while those of v-*fms* are from Hampe *et al.* (1984).

Construction of Δc-*fms*. The murine c-*fms* expression plasmid was constructed by inserting a 3.5-kb *Eco*RI−*Xba*I fragment encompassing the complete murine c-*fms* coding region (Rothwell and Rohrschneider, 1987) into the pECE SV40-based expression plasmid (Ellis *et al.*, 1986). To construct the deletion mutant within the kinase insert of c-*fms* (Δc-*fms*) a *Hin*dIII−*Xba*I fragment (nucleotides 1118−3542) was cloned into M13mp18RF DNA. Nucleotides 2124−2298 encoding the insert domain were deleted using standard oligonucleotide-directed mutagenesis techniques described by Zoller and Smith (1987). The following mutagenic oligonucleotide was used for this purpose: 5′-CCGCTGTGCTCTTTGGGTCCATGCAGC-3′. Putative mutants were screened by plaque hybridization using the mutagenic oligonucleotide end-labeled with T4 polynucleotide kinase as the probe. Mutants were confirmed by DNA sequencing and reintroduced into the pECE-c-*fms* plasmid.

Construction of v-fms lacking gag sequences. Plasmid pSM-FeSV (Donner *et al.*, 1982) was digested with *Dra*I and a 6.4-kb fragment containing 5′ flanking mink DNA, the 5′ long terminal repeat (LTR) and the *gag*−*fms* encoding sequences was cloned into the *Sma*I site of pUC18 (Yanisch-Perron *et al.*, 1985). Digestion of the resulting plasmid (pGT26) with *Xba*I which cleaves in the pUC polylinker, and *Nhe*I which cleaves 5′ to the initiating codon of *fms* (residue 1639), removed the 5′ flanking mink DNA, the 5′ LTR and the *gag* sequences. Deletion of these sequences also left the v-*fms* coding sequences flanked by a *Bam*HI site derived from the pUC polylinker and a *Bam*HI site from the pSM-FeSV 3′ flanking sequences. This 3.3 kb *Bam*HI v-*fms* encoding fragment was subsequently cloned into the *Bgl*II site of the pECE polylinker (pGT32).

Generation of v-fms mutations. To facilitate rapid screening of the v-*fms* mutants a *Pvu*II−*Bam*HI fragment from pGT32 [which encompassed the entire SV40 promoter-v-*fms* encoding sequences and the SV40 poly(A)$^+$ signal] was cloned into the *Sma*I−*Bam*HI sites of the polylinker sequences of pGEM7Z(−) (provided by Promega Biotec). The following oligonucleotides were employed:

5′-GCCCAGGTCTTCTTCGACAGGCCTCATCTC-3′ to give Δ30;
5′-CCTCCCGTCCTCCTTAGGGCCCGGCATGGC-3′ to give Δ177;
5′-TCAGCATCCTCACAGCC-3′ to give Lys-Arg(613).

Uracil-substituted single-stranded DNA was purified from 'phagmids' recovered with helper phage M13K07 from *E.coli* strain CJ236 (Kunkel *et al.*, 1987) and mutagenized according to the procedure of Zoller and Smith (1987). Putative mutants were initially identified by colony lifts and confirmed by DNA sequencing.

Bacterial expression plasmids used to generate trpE−insert fusion proteins. A 415 bp *Hin*cII fragment corresponding to nucleotides 2010−2425 of the murine c-*fms* was cloned into the *Sma*I site of pATH-2 (Sadowski and Pawson, 1987; T.J.Koerner, personal communication) to generate a *trpE*−c-fms (insert) fusion protein. The *trpE*−v-*fms* (insert) fusion was generated by excising the v-*fms* insert as a 284 bp *Sca*I−*Xho*I fragment and cloning it into the *Sma*I−*Sal*I sites of pATH-11.

Cell culture and DNA transfections

NIH 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Plasmid and pSV2neo DNA (at a molar ratio of 10:1) was introduced into cells by the calcium phosphate co-precipitation technique (Graham *et al.*, 1980). At 10 h post-transfection, the precipitate was removed from the cells. Co-transfecton was followed by selection with the antibiotic G418 at 400 μg/ml. G418-resistant clones were isolated and maintained in medium without G418. Growth in soft agar was monitored as previously described (Weinmaster *et al.*, 1984).

Antisera

4069-B2 and 4067-B3 are polyclonal rabbit antisera raised against the internal 1.2 kb *Pst*I fragment of the c-*fms* cDNA clone expressed in bacteria as a *trpE* fusion protein. 5717 rabbit polyclonal antisera and mouse monoclonal antibodies 7F4 and 2E8 were raised against an internal fragment of v-*fms* expressed as fusion protein in bacteria (V.Rothwell and L.Rohrschneider, unpublished results). Rat monoclonal antibody SM2.6.3 was a gift of C.Rettenmier and has been previously described (Rettenmier *et al.*, 1985). All polyclonal antisera were partially purified by passage over BSA−Sepharose and *trpE*−Sepharose to remove non-specific contaminants and anti-*trpE* antibodies.

Metabolic labeling and immunoprecipitation of mammalian cell proteins

Normal or *fms*-expressing NIH 3T3 cells were metabolically labeled with [^{35}S]methionine for 4 h in minimal essential medium lacking methionine (Met$^-$ MEM; Flow Laboratories) plus 5% dialyzed fetal bovine serum. For pulse−chase experiments, cells were placed in Met$^-$ MEM plus 5% dialyzed fetal bovine serum for 30 min prior to the addition of [^{35}S]-methionine. Cells were incubated in the presence of [^{35}S]methionine for 15 min followed by a chase of 1 h with fresh MEM containing 20 mM unlabeled methionine. Cells were lysed in RIPA buffer (10 mM Tris−HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS) containing 0.1 mg/ml leupeptin. Cell lysates were centrifuged at 4°C for 45 min. To immunoprecipitate c-*fms* protein, cell supernatants were incubated with either 4069-B2, 4067-B3 or 5717 antisera and protein A−Sepharose CL4B (Sigma) for 2 h. v-*fms* protein was immunoprecipitated using either rat monoclonal SM2.6.3 antibody with rabbit anti-rat complexed with protein A−Sepharose CL4B or mouse monoclonals 7F4 and 2E8 with rabbit anti-mouse protein A−Sepharose CL4B. Immunoprecipitates were washed five times in 500 μl ice-cold RIPA

buffer with 15 s centrifugations between washes. This was followed by two washes in ice-cold 50 mM Tris−HCl, pH 7.5. Immunoprecipitates were resuspended in 50 μl of 1 × SDS gel sample buffer (10% w/v glycerol, 5% v/v 2-mercaptoethanol, 2.3% w/v SDS, 0.0625 M Tris HCl, pH 6.8, 0.002% w/v bromophenol blue) boiled for 2 min and analyzed by SDS−PAGE and autoradiography.

In vitro kinase reactions

fms proteins were immunoprecipitated from *fms*-expressing NIH 3T3 cells as described above. Following immunoprecipitation, protein A-precipitated immune complexes were washed two times in RIPA buffer followed by two washes in 50 mM Tris HCl, pH 7.5−1% w/v Triton X-100. Kinase activity was assayed by resuspending the immunoprecipitates in 10 μl of kinase reaction buffer (10 mM MnCl, 1% Triton X-100) to which was added 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The reaction was allowed to proceed for 10 min at 30°C and was stopped by the addition of an equal volume of 2 × SDS gel sample buffer (20% v/v glycerol, 10% v/v 2-mercaptoethanol, 4.6% w/v SDS, 0.125 M Tris HCl, pH 6.8, 0.004% bromophenol blue). The samples were boiled for 2 min and then analyzed by electrophoresis through a 7.5% SDS−polyacrylamide gel. Gels were treated with 1 M KOH for 1.5 h as described by Cooper *et al.* (1983) before autoradiography.

Western blotting

To prepare eukaryotic cell lysates for Western blot analyses normal or *fms*-transformed NIH 3T3 cells were grown to a density of ~3 × 10^4 cells/cm^2. The cells were washed twice in PBS-A (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) and immediately lysed in 0.5 ml protein sample buffer (5 mM sodium phosphate, pH 6.8, 2% v/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 0.002% w/v bromophenol blue) at 100°C. The cell lysates were incubated for a further 2 min at 100°C. They were then sheared by 10 passages through a 22 gauge needle followed by 10 passages through a 26 gauge needle. The lysates were stored at −70°C.

Bacteria for analysis of protein expression by Western blotting were grown as described by Sadowski *et al.* (1986). Following a 3 h induction bacterial cells were harvested by centrifugation, lysed in 50 μl of SDS−urea buffer (10 mM sodium phosphate, pH 7.2, 1% v/v 2-mercaptoethanol, 1% w/v SDS, 6 M urea) by vortexing for 15 min and subsequently incubated at 37°C for 15 min. Sample buffer (50 μl of 2 × SDS) was then added and the mixture centrifuged for 5 min. The supernatant was removed and heated at 100°C and 10 μl portions were analyzed on a 15% SDS−polyacrylamide gel.

Protein concentration of the cell lysates was determined by the Lowry procedure for soluble protein quantitation (Peterson, 1977). Whole cell protein (25 μg/lane) was electrophoresed through 7.5% SDS−polyacrylamide gel. Protein was transferred to nitrocellulose with a semidry electroblotter in a continuous buffer system (0.039 M glycine, 0.048 M Tris, 0.0375% w/v SDS, 20% v/v methanol). Nitrocellulose was blocked by incubation of the filters in blocking solution (PBS-A, 5% w/v milk powder, 0.02% w/v sodium azide) at 4°C for 16 h. Afterwards, the filters were gently rocked in anti-*fms* antibody (diluted 1000-fold in blocking solution) for 2 h at 22°C. Unbound anti-*fms* antibody was removed from the filters by three 20 min incubations in fresh blocking solution at 22°C. The filters were next incubated in goat anti-rabbit conjugating antibody (diluted 1000-fold in blocking solution) at 22°C for 1 h. Unbound antibody was removed using the same washing technique. Filters were rinsed in PBS-A and incubated in a buffer containing 100 mM Tris−HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 330 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml and 150 μg/ml of Nitro Blue Tetrazolium.

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