Migration-Stimulating Factor: A Genetically Truncated Onco-Fetal Fibronectin Isoform Expressed by Carcinoma and Tumor-Associated Stromal Cells

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ABSTRACT

Migration-stimulating factor (MSF) is a 70-kDa motogenic protein previously reported to be expressed by fetal and cancer patient fibroblasts cultured in vitro and present in the serum of breast cancer patients. A 2.2-kb full-length MSF cDNA has been cloned and shown to be a truncated isoform of fibronectin generated from its primary gene transcript by a hitherto unrecognized intron read-through mechanism. MSF cDNA is identical to the 5′ end of fibronectin cDNA, up to and including exon III-1a, and terminates in a novel 195-nucleotide 3′ sequence. This MSF unique sequence is derived from the intron immediately downstream of exon III-1a in the fibronectin gene and is not found in any previously identified “full-length” fibronectin cDNA. MSF mRNA is 1000-fold less abundant than full-length fibronectin message in fetal fibroblasts and exhibits rapid biphasic decay kinetics previously associated with oncogenes and stress response molecules. MSF recombinant protein exhibits a potent and substratum-dependent motogenic activity, with half-maximal response manifest at 0.1–1.0 pg/ml. This activity is (a) mediated by the IGD amino acid motif; and (b) not expressed by (i.e., cryptic within) full-length fibronectin. In situ hybridization and immunohistochemistry confirm that MSF is expressed by tumor-associated fibroblasts and additionally indicate that it is also expressed by carcinoma cells and tumor-associated vascular endothelial cells. MSF, as a consequence of its potent bioactivities and expression by both stromal and carcinoma cell populations, is well placed to function as an epigenetic effector promoting cancer development.

INTRODUCTION

The induction and progression of human cancers has conventionally been attributed to the step-wise accumulation of genetic lesions within the target epithelium (1). Recent years have, however, witnessed a paradigm shift in which perturbed paracrine signaling from tumor-associated stromal cell populations (e.g., fibroblast and endothelial) are increasingly recognized to make a significant contribution to tumor progression (2–5), as well as the initiating transformation process (6–8).

We have long been advocates of stromal involvement in the cancer process (9–11). Our initial observations in this regard indicated that (a) fetal skin fibroblasts migrate into three-dimensional gels of type I collagen to a significantly greater extent than adult cells (12); (b) this difference in behavior results from the secretion by fetal fibroblasts of an autocrine migration-stimulating factor (MSF), which is not expressed by their adult counterparts (13); and (c) fibroblasts obtained from patients with common human cancers resemble fetal cells in terms of both their migratory phenotype on collagen gels and their production of MSF (14–18). Subsequent reports documented the presence of detectable quantities of MSF bioactivity in the serum of greater than 90% of breast cancer patients compared with only 10% of age- and sex-matched controls (19). Apart from stimulating cell migration, MSF also up-regulates hyaluronan synthesis by target fibroblasts (20, 21) and has recently been demonstrated to be a potent stimulator of angiogenesis.5 Biochemical characterization of MSF indicated that it is a 70-kDa protein, and partial peptide sequencing revealed that it contained a region that was highly homologous with a portion of the gelatin-binding domain of fibronectin (22, 23).

Fibronectin is a modular glycoprotein (Fig. 1) consisting of a number of functional domains named on the basis of their binding affinities for other matrix macromolecules and members of the integrin family (24). Starting at its NH2 terminus, these functional domains include: Hep1/Fib1 (NH2-terminal low-affinity binding to heparin and fibrin); Cell-BD (binding to gelatin/collagen); Cell-BD (RDG-mediated binding to integrins); Hep2 (high-affinity heparin binding); and Fib2 (COOH-terminal fibrin binding site). Each functional domain consists of three possible structural modules (types I, II, and III) containing approximately 45, 60, and 90 amino acids, respectively. All type I and II modules are encoded by a single exon, whereas the majority of type III modules are coded for by two exons (designated a and b). Alternative splicing at three locations within human fibronectin pre-mRNA results in the generation of approximately 20 distinct isoforms (25, 26), some of which are preferentially expressed by transformed cells or tumors (27, 28) and are believed to contribute to cancer pathogenesis (29, 30). All of these fibronectin isoforms are “full length,” having molecular masses in the region of 250 kDa (30).

Structure-function analyses have indicated that (a) the potent motogenic activity of MSF (half-maximal activity manifest at femtomolar concentrations) is mimicked by purified preparations of the Gel-BD of fibronectin, whereas full-length fibronectin and all other of its functional domains are devoid of activity when similarly assessed in the collagen gel migration assay; and (b) the motogenic activities of both MSF and Gel-BD are matrix dependent, i.e., manifest by fibroblasts adherent to a native, but not denatured, type I collagen substrate (23, 31, 32). The functional and structural convergence of MSF with Gel-BD suggest that these two effector molecules may be related genetically.

To further our understanding of the role of MSF in cancer pathogenesis, the objectives of this study have been (a) to clone the cDNA coding for MSF; (b) to ascertain its possible relationship to the fibronectin gene; (c) to compare the function of recombinantly expressed protein coded by the cloned MSF cDNA with that of cell-produced MSF, as well as Gel-BD and native fibronectin; and (d) to document the expression of the cloned MSF message and protein by cell populations in normal adult, fetal, and tumor tissues.

MATERIALS AND METHODS

Cells and Reagents. The MRC5-SV2-immortalized human fetal lung fibroblast line was obtained from the European Tissue Type Collection. Early

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passage diploid human fetal skin fibroblasts were obtained from the Cytogenetics Department, Ninewells Teaching Hospital, University of Dundee. Postnatal and adult fibroblast lines were established and cultured in our laboratory as described previously (12). Experiments were performed with cells at passages 10–18.

Fibronectin functional domains (Fib1/Hep1, Gel-BD, and Hep2) were obtained from Sigma: the MSF COOH-terminal decapacitation synthetic peptide was purchased from Dr. G. Bloomberg (University of Bristol, Bristol, United Kingdom). Antibodies were obtained from the following sources: antifibronectin NH2-terminal domain (MAB1936; Chemicon); antifibronectin gelatin-binding domain (MAB1892; Chemicon); antifibronectin cell-binding domain (MAB1937; Chemicon); antifibronectin Hep-2 domain (PSF3 SC18827; Santa Cruz Biotechnology); and anti-Xpress tag (Invitrogen). Rabbit polyclonal antibody was raised against a synthetic peptide containing the MSF-specific COOH-terminal decapeptide sequence. The Ni-HRP His Probe kit was purchased from Pierce and Warriner.

MSF Cloning Strategy. mRNA extracted from the MSF-producing MRC5-SV2 line was used to prepare a library using ZAP-cDNA Synthesis Kit (Stratagene). cDNA from this library was amplified using a degenerate primer capable of annealing to the Gel-BD homologous peptide sequence found in MSF (5'-TATGCACTTGTTGACAGACA-3') and a second primer that annealed to the library vector (5'-GCCGCCCTCAGGATTTTTTTTTTT-3'). Because MSF had been previously shown to have a molecular mass of 70 kDa, only amplicons smaller than that predicted for fibronectin were cloned in the pCRII vector (Invitrogen). A 1.2-kb fragment was eventually identified that was homologous to fibronectin cDNA extending downstream from the Gel-BD apart from the 3' 195 bp, which were not present in any previously identified cDNA.

This unique sequence was digoxigenin labeled by PCR using the primers 5'-CAAGACACCTGTTGGCAACATTGCGCTGAGCCTGACCC/GCTGAGCCTGACCC-3' and 5'-TCACTGACTCAAGTACATTATCTGCCAGATCAGTTC-3' according to the manufacturer's instructions (Boehringer Mannheim) and used to probe the MRC5-SV2 cDNA library. Phage dilutions of the library were plated on XL1-Blue MRF (Stratagene) and duplicate plaque lifts were plated on XL1-Blue MRF 

Quantification of MSF and Fibronectin mRNA Using Real-Time PCR. The MSF-specific reaction amplified a sequence spanning fibronectin exon I-9 and the unique MSF 3' terminus (primer pair, 5'-GACAGCTGGCAACATTGCGCTGAGCCTGACCC/5'-TCACTGACTCAAGTACATTATCTGCCAGATCAGTTC-3'). The fidelity of each PCR was confirmed by cloning and sequencing. Plasmids containing these cloned amplicons were used as quantification standards. One microgram of total RNA was reverse transcribed using an oligo(dT) primer in a total volume of 20 μl according to instructions (Promega). One microliter of cDNA sample or appropriate standard was used in each PCR. The reaction mixture included reaction buffer (final MgCl2 concentration, 3 mM) and SYBR Green I (both BioGene Ltd), 1 unit of Hot Rod Taq polymerase (ABgene), 1:125 dilution of TaqStart antibody (Sigma), and 5 μl of each oligomer in a volume of 10 μl. All reactions were performed and analyzed using the LC24 LightCycler and associated LC2.1 software (Idaho Technologies, Salt Lake City, UT). Amplification fidelity was assessed by detailed melt analysis and gel electrophoresis.

Manufacture of MSF Constructs and Expression of Recombinant Protein. 3T3 cells were transfected with pCDNA3.1 or pCDNA4 vectors (Invitrogen) containing the cloned MSF sequence and selected using G418. MSF was also expressed in BL21 pLySs bacteria using the prSET vector (Invitrogen). In addition to wild-type MSF, a mutant insert was generated in which the exon I-7 and I-9 IGD amino acid motifs were reversed to DGI. These sequences were generated by PCR and cloned into the vector using appropriate restriction sites. Insert fidelity was confirmed, in all cases, by sequencing. All expression vectors add NH2-terminal His and Xpress epitope tags to facilitate detection and purification of recombinant protein.

Dot and Western Blots. Samples of conditioned media for dot blots were concentrated by ammonium sulfate precipitation and heparin affinity chromatography, as described previously (22). For dot blots, nitrocellulose sheets (0.2 μm) were cut to an appropriate size, treated with the test protein solutions, and allowed to air dry. The blots were then blocked with Tris-buffered saline and 5% Tween-20 (pH 7.6) plus 1% (w/v) Marvel dried milk powder for 20 min at room temperature. Blots were then incubated with the indicated antibody overnight, rinsed three times with Tris-buffered saline and 5% Tween-20, incubated with secondary antibody for 2 h, washed three times in Tris-buffered saline and 5% Tween-20 at room temperature, and finally washed for 5 min with Tris-buffered saline (pH 7.6). Visualization was achieved by enhanced chemiluminescence using Supersignal (Pierce and Warriner). Proteins to be analyzed by Western blots were resolved by SDS-PAGE under reducing conditions according to the method of Laemmli (14). For analysis of resolved motogenic activity, a replicate SDS-PAGE track was cut into 5-mm slices, marked by placing it into 1 ml of 0.1 M ammonium hydrogen carbonate (pH 8.4) for 1 h, followed by thorough washing with ammonium hydrogen carbonate, and placed onto an SDS-PAGE gel. In situ Hybridization. Digoxigenin-labeled MSF riboprobes (DIG-RNA) were transcribed in forward and reverse orientation with either T3 or T7 RNA polymerase (Boehringer Mannheim) from plasmids containing the linearized MSF unique 3' sequence. An in situ hybridization protocol for detecting low-abundance message was used (35). Archival paraffin-embedded blocks of relevant tissues were obtained from the Pathology Department, Ninewells Teaching Hospital, Dundee.

8828
**Immunohistochemistry.** Paraﬁn-embedded tissue sections (6 μm) were immunostained according to standard techniques following optimization for each antibody used (36).

Functional Assays. Type I collagen was extracted overnight from rat tail tendons in 3% (v/v) acetic acid, dialyzed for 2 days against distilled water, diluted to 2 mg/ml, and used to make 2 ml collagen gels in 35-mm plastic tissue culture dishes as described previously (37). In these assays, collagen gels were overlaid with 1 ml of either serum-free MEM or serum-free MEM containing 4× the ﬁnal concentration of the test compound or conditioned medium. Conﬂuent stock cultures of ﬁbroblasts were then trypsinized, pelleted by centrifugation, and normally reseeded in MEM containing 20% (v/v) donor calf serum at 2 × 10^5 Cells/ml; and 1-ml aliquots of this inoculum were pipetted onto the overlaid gels. Considering the 2-ml volume of the collagen gel, the 1-ml medium overlay, and the 1-ml cell inoculum, this procedure gives the desired ﬁnal concentration of test molecule in MEM containing 5% (v/v) donor calf serum. Identical results were obtained under serum-free conditions. The cultures were incubated for 4 days, and the percentage of ﬁbroblasts present within the collagen gel matrix at that time was ascertained by microscopic observation of 10 randomly selected ﬁelds per gel, as described previously (37). Results are expressed as mean and SE of replicate gels for each experimental observation.

**RESULTS**

The Cloned Candidate MSF cDNA and Its Relationship to the Fibronectin Gene. A full-length cDNA (European Molecular Biology Laboratory accession no. AJ535086) was cloned from an immortalized human fetal ﬁbroblast line and judged to be a candidate for MSF on the basis of its predicted size (2.2 kb) and presence of a gelatin-binding domain (Gel-BD) coding sequence (23). Beginning at its 5′ terminus, the 2192-nucleotide (nt) candidate MSF clone, henceforth referred to as “cloned MSF,” consists of (a) a 56-nt region identical to the 5′-untranslated region (5′-UTR) of ﬁbronectin (38); (b) a 1941-nt sequence identical to the 5′ coding sequence of ﬁbronectin, extending to and including all of exon III-1a; and (c) a unique 195-nt sequence predicted to comprise a 30-nt coding sequence and a remaining 3′-UTR sequence shared by the uncloned cDNA. The deduced 657 amino acid sequence comprises (a) the intact NH₂-terminal Hep1/Fib1 functional domain of ﬁbronectin; (b) the entire Gel-BD; (c) the amino acid sequence encoded by ﬁbronectin exon III-1a; and (d) a unique 10-amino acid COOH terminus, VSIPPRNLGY (Fig. 2, A and B).

To ascertain the chromosomal location of the cloned MSF gene, a human genomic DNA PAC library was screened by two distinct PCR reactions; the ﬁrst ampliﬁed the 5′-UTR sequence shared by the cloned MSF cDNA and ﬁbronectin gene, whereas the second ampliﬁed the region spanning exon III-1a and the unique downstream sequence found in the cloned MSF cDNA. Both PCR products independently identiﬁed the same PAC clone. Restricting this clone with BamHI and subcloning led to the isolation of an approximately 20-kb genomic DNA fragment (B3-2), which reacted positively in both PCR reactions. Fluorescent in situ hybridization analysis using B3-2 as a probe identiﬁed a single hybridization site at 2q35, the chromosomal location of the ﬁbronectin gene (data not shown). Identical results were obtained with a 10-kb KpnI-generated probe, which only contained the unique MSF 3′ sequence.

It was noted during screening of the PAC library that the amplicon generated by the downstream PCR (i.e., that spanning ﬁbronectin exon III-1a and the MSF unique 3′ sequence) was identical in size to that generated from the cloned MSF cDNA when ampliﬁed in the same reaction. To assess their identity, relevant samples of cloned and uncloned genomic DNA, cloned and uncloned cDNA, and RNA were ampliﬁed by this reaction, and the resultant amplicons were sequenced. The data (Fig. 3) conﬁrmed that all genomic DNAs and cDNAs produced a 265-bp amplicon of identical base sequence; the RNA used to produce the uncloned cDNA failed to amplify in this reaction, thereby indicating the absence of contaminant genomic
DNA. These results demonstrate (a) that the unique MSF sequence is a genuine product of gene transcription; and (b) that it is encoded by an “intronic” DNA sequence immediately downstream of fibronectin exon III-1a. This contiguity between exon III-1a and the cloned MSF unique sequence was subsequently confirmed by published human genome project data (GI 14589768).

Taken together, these data lead to the conclusion that MSF is a truncated isoform of the fibronectin gene produced as a result of a hitherto unrecognized intronic read-through mechanism.

Abundance and Stability of Cloned MSF Compared With Full-Length Fibronectin mRNAs. The relative abundance of cloned MSF and of full-length fibronectin mRNA was assessed by real-time PCR. For this purpose, all full-length fibronectin isoforms were collectively measured by PCR amplification of cDNA containing the Hep2 domain (standard curves presented in Fig. 4, A and B). Our results indicate that the cloned MSF mRNA is approximately 1000-fold less abundant than full-length fibronectin transcripts in both the MRC5-SV2 immortalized fetal fibroblast line and all four of the diploid fetal fibroblast lines examined (Fig. 4C). To determine whether differential message stability might contribute to this observation, actinomycin D was used to inhibit gene transcription in proliferating MRC5-SV2 cells. Aliquots of cells were harvested for transcript analysis just before and up to 24 h after treatment. As indicated in Fig. 4D, levels of cloned MSF mRNA decreased over 30 times more rapidly than full-length fibronectin transcripts and displayed a biphasic decay pattern, with initial mRNA levels decreasing by half in approximately 45 min. In contrast, full-length fibronectin transcripts had not yet fallen to half their initial concentration by 24 h.

Comparison of the Motogenic Activity and Immunoreactivity of Recombidentally Expressed Cloned MSF Protein and Fetal Fibroblast MSF (ffMSF). The protein encoded by cloned MSF cDNA was expressed in both Escherichia coli and 3T3 cells. To facilitate the identification and purification of recombinant protein (rhMSF), expression plasmids were used that added both His and Xpress tags to the NH2 terminus. Amino acid sequence analysis of rhMSF by matrix-assisted desorption ionization mass spectrometry confirmed the presence of the COOH-terminal signature decapeptide. A rabbit polyclonal antibody (MSFrp2/98) and mouse monoclonal antibody (mab7.1) were raised against this MSF-specific decapeptide. The epitope recognized by mab7.1 was identified as PPRNLGY by pepscan analysis.

PAGE analysis of rhMSF revealed the presence of a single Coomassie-stained band with a molecular mass of 77 kDa (Fig. 5A, Lane a). Western blot analysis confirmed the staining of this band with antibodies to the cloned MSF COOH-terminal decapeptide and the Gel-BD of fibronectin, as well as the incorporated Xpress tag (Fig. 5A, Lanes b, c, and e). The His tag was visualized by nickel-conjugated horseradish peroxidase (Fig. 5A, Lane d). The motogenic activity of pro-rhMSF was initially determined by slicing a replicate PAGE track into 12 equal segments, each of which was eluted and assessed in the collagen gel migration assay. A single band of motogenic activity (Fig. 5A, Lane f) was revealed that aligned with the MSF protein bands.

MSF was biochemically purified from fetal fibroblasts conditioned medium (ffMSF) as described previously (32). A direct comparison between ffMSF and cloned MSF recombinant protein (both prokaryotic- and eukaryotic-expressed) revealed that they all elicited a potent biphasic motogenic response with half-maximal activity in the collagen gel migration assay manifest at 1.0–10.0 pg/ml for ffMSF and eu-rhMSF, and 0.1–1.0 pg/ml for pro-rhMSF (Fig. 5B). As reported previously for ffMSF (23, 31, 32), the motogenic activities of the recombinant proteins were also (a) completely abrogated by anti-
Collagen either native (gelatin) or denatured (gela-
tin), type I collagen (Fig. 5C, lane a) was recovered from a replicate PAGE track which was sliced into 12 equal segments, each of which was extracted in buffer and then assessed in the collagen gel migration assay as described previously (32). Shaded segment indicates gel slice yielding a greater than 3-fold stimulation of target adult fibroblasts relative to control cultures; unshaded segments were devoid of significant motogenic activity. B, comparative dose response of prokaryotic expressed candidate MSF protein (pro-rhMSF), eukaryotic expressed candidate MSF protein (eu-rhMSF), and MSF biochemically isolated from fetal fibroblasts conditioned medium (fMSF). Motogenic activity was assessed in the three-dimensional collagen gel migration assay using target adult skin fibroblasts. The control level of migration is indicated by the dotted line (mean) and shaded area (SE). The motogenic activities of the test compounds are expressed as fold stimulation relative to control (mean ± SE). C, the motogenic activities of pro-rhMSF, eu-rhMSF, and fMSF (all at 1 pg/ml) were completely abrogated by rabbit polyclonal antibody to the gelatin-binding domain of fibronectin (anti Gel-BD Ab), as well as specific monoclonal antibodies to the following fibronectin domains: cell-binding domain (Cell-BD); cell-binding domain (Cell); and the COOH terminus high-affinity heparin-binding domain (Hep-2). Comparative immunoreactivity data are presented for control-purified preparations of eu-rhMSF and full-length fibronectin (FN). C, the motogenic activity of fetal CM: total, fetal CM: bound, and fetal CM: unbound were assessed in the collagen gel migration assay and compared with results obtained with eu-rhMSF. Data are presented as fold stimulation of migration relative to control (mean ± SE). Control migration indicated by dotted line (mean) and shaded area (SE).

The immunological and functional relationship between the protein coded by the cloned MSF cDNA and fMSF was additionally assessed by affinity chromatography on type I collagen (Fig. 5D). The bound column eluate (Fig. 5C, lane c) was passed down an affinity column (Boyden chamber) assay using membranes coated with either native (collagen) or denatured (gelatin) type I collagen. Data (mean ± SE of six replicate samples) are presented as cells per high power field (hpf). Cell migration in control cultures is indicated by dotted line (mean) and shaded area (SE).

Gel-BD antibody (Fig. 5C); and (b) strictly matrix dependent when assessed in the transmembrane assay, i.e., manifest by fibroblasts adherent to membranes coated with native, but not denatured (gelatin), type I collagen (Fig. 5D).

Adherent adult fibroblast-conditioned medium. Epitope profiling additionally indicated that cloned MSF recombinant protein (positive control) and the antibody-bound fraction reacted with antibodies to Hep1/Fib1, Gel-BD, and cloned MSF, but not antibodies against Cell-BD or Hep2. In contrast, the unbound column fraction and native fibronectin (negative control) reacted with all of the anti-fibronectin domain

Fig. 5. Recombinant cloned MSF protein and MSF purified from fetal fibroblast conditioned medium express an identical substratum-dependent motogenic activity. A, PAGE analysis of cloned MSF recombinant protein expressed in E. coli (pro-rhMSF) revealed the presence of a single protein band with an apparent molecular mass of 77 kDa when visualized with Coomassie blue (Lane a); rabbit polyclonal antibody to the cloned MSF unique COOH-terminal decapetide (Lane b); antibody to the gelatin-binding domain of fibronectin (Lane c); nickel conjugated horseradish peroxidase binding to His tag (Lane d); and antibody to the Xpress tag (Lane e). Lane f, a single band of motogenic activity, also with an apparent molecular mass of 77 kDa, was recovered from a replicate PAGE track which was sliced into 12 equal segments, each of which was extracted in buffer and then assessed in the collagen gel migration assay as described previously (32). Shaded segment indicates gel slice yielding a greater than 3-fold stimulation of target adult fibroblasts relative to control cultures; unshaded segments were devoid of significant motogenic activity. B, comparative dose response of prokaryotic expressed candidate MSF protein (pro-rhMSF), eukaryotic expressed candidate MSF protein (eu-rhMSF), and MSF biochemically isolated from fetal fibroblasts conditioned medium (fMSF). Motogenic activity was assessed in the three-dimensional collagen gel migration assay using target adult skin fibroblasts. The control level of migration is indicated by the dotted line (mean) and shaded area (SE). The motogenic activities of the test compounds are expressed as fold stimulation relative to control (mean ± SE). C, the motogenic activities of pro-rhMSF, eu-rhMSF, and fMSF (all at 1 pg/ml) were completely abrogated by rabbit polyclonal antibody to the gelatin-binding domain of fibronectin (anti Gel-BD Ab). D, the motogenic response of target adult skin fibroblasts to pro-rhMSF and fMSF was also assessed in the transmembrane (Boyden chamber) assay using membranes coated with either native (collagen) or denatured (gelatin) type I collagen. Data (mean ± SE of six replicate samples) are presented as cells per high power field (hpf). Cell migration in control cultures is indicated by dotted line (mean) and shaded area (SE).

Fig. 6. Recombinant cloned MSF protein is indistinguishable from fMSF in terms of its immunoreactivity and motogenic activity. Fetal fibroblast conditioned medium (fetal: CM-total) was passed down an affinity column (Fig. 5D) and the antibody-bound fraction reacted with antibodies to Hep1/Fib1, Gel-BD, and cloned MSF, but not antibodies against Cell-BD or Hep2. In contrast, the unbound column fraction and native fibronectin (negative control) reacted with all of the anti-fibronectin domain

8831

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antibodies but did not react with the anticloned MSF antibody. Motogenic activity was present in unfractionated fetal fibroblasts conditioned medium and was enriched in the mab7.1 antibody-bound fraction. Significantly, no motogenic activity was detected in the unbound column fraction, thereby indicating that all motogenic activity in fetal fibroblast conditioned medium (previously attributed to ffMSF) is removed by absorption to an antibody raised against the MSF-specific antisense riboprobe in fetal skin (A) and adult skin (B). The tissue distribution of cloned MSF protein was directly investigated using a monoclonal antibody to the Hep-2 domain in serial sections taken from same tumor depicted in Fig. 8E revealed a distribution profile inverse to that of cloned MSF, i.e., fibronectin biological activity ascribed to the IGD motif. The involvement of these motifs in mediating the motogenic activity of cloned MSF protein was directly investigated using in vitro mutagenesis to reverse both the I-7 and I-9 IGD sequence to DGI. The recombinant mutant protein was completely devoid of motogenic activity (Fig. 7B).

The Expression of Cloned MSF in Tissues. The expression of the cloned MSF message by cells in tissue sections was assessed by in situ hybridization. Fetal skin (n = 3) contained numerous positive fibroblasts, as well as positive epithelial cells in the epidermis and hair follicles (Fig. 8A). Similarly processed specimens of adult skin (n = 3) displayed either weak or no staining in these tissue compartments (Fig. 8B). Control preparations of fetal skin hybridized with sense cloned MSF riboprobe were negative and devoid of background staining (Fig. 8C). The tissue distribution of cloned MSF protein was ascertained by immunohistochemistry using mab7.1. Again, positively stained fibroblasts and epithelial cells (especially apparent in the suprabasal layer) were abundant in fetal skin (Fig. 8D). In contrast, adult skin specimens were either completely negative or contained a small number of lightly stained cells (Fig. 8E).

Normal breast tissue (n = 3) revealed little evidence of cloned MSF message, with an occasional weakly positive signal associated with lobular epithelial cells (Fig. 9A). A weak positive signal was also occasionally detected in association with inter-lobular fibroblasts (Fig. 9B), but not their intra-lobular counterparts, an observation consistent with previously published in vitro results (40). In contrast, a strong signal was commonly detected in breast tumors (n = 10) with respect to both cloned MSF mRNA (Fig. 9C) and protein (Fig. 9D). The staining pattern of breast tumors was variable and heterogeneous, sometimes being homogeneously distributed throughout the section in association with carcinoma cell clusters, microvascular endothelial cells, and stromal fibroblasts (Fig. 9, C and D), whereas in other tumors, its expression was primarily confined to one cell population, e.g., the carcinoma cells (Fig. 9E), or distributed in focal “hotspots” within the tumor section (not shown). Immunolocalization of full-length fibronectin (using a monoclonal antibody to the Hep-2 domain) in serial sections taken from same tumor depicted in Fig. 9E revealed a distribution profile inverse to that of cloned MSF, i.e., fibronectin.
staining predominantly confined to the strands of stromal tissue surrounding the carcinoma cell clusters (Fig. 9F). As predicted, immunohistochemical localization of the Gel-BD domain (shared by both cloned MSF and full-length fibronectins) resulted in the staining of both carcinoma and stromal compartments (Fig. 9G).

**DISCUSSION**

**MSF Is a Truncated Isoform of Fibronectin.** A candidate full-length MSF cDNA has been cloned from a fetal fibroblast cell line. Data presented in this communication indicate that this cloned MSF (a) encodes the protein responsible for the motogenic activity previously ascribed to cell-produced MSF; (b) is generated from the fibronectin gene by read-through of the intron separating exons III-1a and III-1b; and (c) is differentially expressed by cells in fetal, normal adult, and neoplastic tissues.

Evidence supporting the conclusion that the cloned MSF cDNA encodes a protein that is functionally equivalent to cell-produced MSF include:

1. The motogenic activity of the recombinant cloned protein (rhMSF) is identical to MSF purified from fetal fibroblast conditioned medium (ffMSF) in terms of its (a) characteristic bell-shaped dose-response curve; (b) expression of half-maximal activity at femtomolar concentrations; (c) matrix dependence for manifestation of target cell response; and (d) neutralization of motogenic activity by antifibronectin Gel-BD antibodies.

2. Epitope mapping using antibodies directed to various domains of the fibronectin molecule reveal that both rhMSF and ffMSF have an identical immunoreactive profile bearing Hep1/Fib1 and Gel-BD epitopes, but lacking Cell-BD and Hep2 reactivity.

3. Antibodies directed to the unique COOH-terminal decapeptide encoded by the cloned cDNA adsorbed all of the migration-stimulating activity from fetal fibroblast conditioned medium.

A comparison of the base sequence of the cloned MSF cDNA with that of fibronectin cDNA and genomic DNA clearly demonstrates that MSF is encoded by the 5’ end of the fibronectin gene. In support of this model, our data indicate that MSF cDNA makes up the 5’ end of the cDNA sequence common to all full-length fibronectin isoforms (including the downstream portion of the 5’-UTR and the first 12 fibronectin exons, up to and including exon III-1a). MSF cDNA is distinguished at its 3’ terminus from all other fibronectin cDNA sequences by the contiguously located upstream portion of the intron separating exons III-1a and III-1b in the fibronectin gene. Fluorescent in situ hybridization analysis indicates that the MSF 3’ unique sequence hybridizes solely at 2q35, the chromosomal location of the fibronectin gene (data not shown).

MSF message is most likely generated from the fibronectin gene by a failure of the splicing machinery to remove the intron downstream of exon III-1a during maturation of the primary fibronectin gene transcript, with the pre-mRNA so generated subsequently cleaved and polyadenylated in response to an appropriate signal in the retained intronic sequence. Fibronectin was one of the first molecules to be characterized in terms of the alternative splicing events responsible for the generation of its various isoforms (24–26). Intron retention does not appear to feature in the normal control of full-length fibronectin expression, although this mechanism has been demonstrated to occur in other genes (41–43). Splice site selection is regulated by the complex interplay of cis-acting sequence elements and trans-acting factors (44, 45). With respect to the former, both intronic and exonic enhancer sequences in the fibronectin gene contribute to the control of alternative splicing (46, 47). However, in view of the near identity of the MSF and fibronectin coding sequences, we suggest that changes in the levels of specific trans-acting factors, as occurs in other genes (48), are more likely to be responsible for the intronic read-through postulated to generate the MSF message.

Zhao et al. (49) have identified a similarly truncated 70-kDa isoform of fibronectin in Zebrafish embryos. As is the case with MSF, this truncated fibronectin cDNA is identical to the 5’ end Zebrafish fibronectin (in this case, up to and including exon III-3) and terminates in a 3’ sequence corresponding to a portion of the immediately downstream intron. The coded protein terminates with a 20-amino acid sequence not present in any full-length Zebrafish fibronectin. Genetically truncated isoforms of fibronectin may therefore make a hitherto unrecognized contribution to normal development and pathological processes (such as cancer) across a number of phylogenetic branches of the animal kingdom.
hallmarks of stress response molecules (e.g., proto-oncogenes, such as fos) contributing to cancer progression. The instability of these cancer-associated molecules results from the presence of AU-rich instability elements in their respective 3′-UTRs (50, 51), with the resultant targeting of message for degradation by the ubiquitin-proteosome pathway (52). Significantly, an AU-rich instability element (i.e., an AUUUUA pentamer downstream of an AU-rich sequence containing a poly(U) run) is present in the 3′-UTR of MSF message.

The Possible Functional Significance of Producing a Truncated Isoform of Fibronectin. The expression of certain fibronectin bioactivities, such as binding to collagen, are dependent upon the maintenance of a native conformation (53), whereas other activities require denaturation and/or proteolytic cleavage to be manifest and are accordingly “cryptic” in the native full-length molecule (54). The motogenic activity of MSF protein does not appear to require the retention of a native conformation, because it is recoverable from a sliced PAGE track following standard sample preparation (i.e., boiling in the presence of SDS and mercaptoethanol) and manifest by prokaryotic expressed recombinant protein, which is unlikely to be folded or glycosylated in the same manner as eukaryotically expressed proteins. MSF bioactivity is not expressed by full-length fibronectins. We have reported previously that the proteolytically generated Gel-BD functional domain also exhibits a potent motogenic activity that is cryptic in full-length fibronectin (32). Similarly cryptic biological activities of other proteolytically generated functional domains of fibronectin include the stimulation of monocyte migration (55), the inhibition of cell proliferation (56), the induction of protease gene expression by adherent synovial fibroblasts (57), adipocyte differentiation (58), and an RGDS-independent mediation of cell migration (59). The structural basis of cryptogenicity has not been unambiguously ascertained but presumably involves the release of steric constraints in the native full-length molecule (54, 60).

The potent motogenic activity of MSF is indistinguishable from that of the proteolytically generated Gel-BD functional domain (32). This functional convergence provides tissue cells with two independent mechanisms of locally generating the same potent bioactivity, one by degradation of the extracellular matrix (Gel-BD) and the other by genetic control of fibronectin truncation during gene transcription (MSF). We suggest that the generation of MSF by a genetic mechanism permits tumor-associated cells to produce a single bioactive truncated fibronectin in the absence of a complex array of proteolytically generated fibronectin fragments, some of which (such as Cell-BD) are potent inhibitors of Gel-BD and MSF motogenic activities (39, 60).

The Expression of MSF by Tumor-Associated Cells: A Model of Its Potential Role in Tumor Pathogenesis. Various independent lines of evidence indicate that MSF is expressed at both the mRNA and protein levels by fetal and cancer patient fibroblasts, but not by the majority of their normal adult counterparts. These observations are consistent with previously published data from our group documenting the expression of an elevated migratory phenotype and production of MSF bioactivity by both fetal and cancer patient fibroblasts (61). Data presented in this communication additionally demonstrate that MSF is also expressed by fetal skin keratinocytes, breast carcinoma cells, and tumor-associated vascular endothelial cells. The result of an initial survey has indicated that MSF expression by carcinoma and tumor-associated stromal cells is not confined to breast cancer; but is also a feature of a wide range of common human cancers, including lung, colorectal, oral, and prostate. As discussed previously (10, 20, 61), the potent bioactivities of MSF may contribute to tumor pathogenesis by a number of mechanisms, including the stimulation of tumor cell motility, hyaluronan biosynthesis, and angiogenesis.

Full-length isoforms of fibronectin containing the alternatively spliced EDA and EDB modules have long been recognized to play an important role in cellular transformation and tumor pathogenesis (27, 28, 30). EDB-containing fibronectin promotes cell spreading and is associated with angiogenesis (29, 62). MSF is the first truncated isoform of fibronectin to be identified in tumor tissues.

Fibronectin functionality has been reported to be critically modulated by its self-association into fibrillar aggregates of “super fibronectin” (63). Cells display enhanced adhesion to super fibronectin, with a reciprocal decrease in migratory activity. Significantly, super fibronectin formation is mediated by an amino sequence coded by exon III-1b. The truncation of MSF translation at exon III-1a and the consequent absence of the super fibronectin signal may support its function as a stimulator of migration and cell-produced counterpoise to the migration-inhibiting activity of super fibronectin.

The matrix dependence of MSF is intriguing and may provide a “tissue-level” means of modulating its effect on target cells in pathological processes characterized by matrix remodeling, such as cancer. In these situations, it is possible that temporal changes in matrix composition may render adherent cells responsive or nonresponsive to MSF. The matrix dependency of MSF has an additional practical implication. The majority of published studies documenting the motogenic activity of fibronectin and its purified functional domains have used the transmembrane (Boydren chamber) assay using membranes coated with denatured collagen (i.e., gelatin). Such studies have consistently failed to demonstrate the motogenic activity of the Gel-BD and would similarly not be able to identify MSF motogenicity, which requires fibroblast attachment to a native type I collagen substratum. The collagen gel migration assay provides a means for studying cell migration within a physiologically relevant three-dimensional macromolecular matrix and has been crucial for the identification and cloning of MSF.

Dynamic and reciprocal interactions between epithelial and stromal cells are required for organogenesis during fetal development and continue to contribute to the maintenance of normal tissue structure and function in the adult (Fig. 10). These epithelial-stromal interactions are mediated by an array of diverse chemical signals, including both soluble effectors and matrix macromolecules, whose precise effect upon target cells is contextually regulated as a consequence of cross-talk between their respective signal transduction pathways, as well as other mechanisms (31). Perturbations in these interactions are now recognized to make a significant contribution to the etiology of various pathological conditions, including cancer. In keeping with the prevailing “epithelio-centric” paradigm of carcinogenesis and tumor progression, the majority of recent models speak of reactive stromal changes induced by mutation-driven perturbations in epithelial signaling (64). Other more radical models postulate that epigenetic perturbation in the production of signaling molecules by stromal cells may be sufficient (in the absence of genetic lesions) to alter epithelial cell proliferation/differentiation in a manner that promotes subsequent neoplastic transformation events (6). Our previously published data support the view that the presence of aberrant (MSF-producing) fibroblasts may precede the development of a clinically recognizable cancer (18) and therefore be capable of functioning in such a cancer-promoting capacity. In view of the iterative and dynamic nature of epithelial-stromal inter-dependence, it is clear that both cellular compartments may exhibit inductive and reactive modes of interaction. The signaling molecules produced by these cells mediate both paracrine and autocrine regulatory loops, some of which may be self-perpetuating in nature, and contribute thereby to the uncontrolled character of tumor progression.
Fig. 10. The postulated involvement of MSF in cancer pathogenesis. Dynamic and reciprocal interactions between stromal and epithelial cell populations contribute to the control of normal tissue homeostasis. Exposure to carcinogens and environmental stress agents may contribute to cancer pathogenesis by (a) induction of mutations in the target epithelial cell population (dotted red arrow); and (b) the induction of epigenetic effector molecules (both soluble factors and matrix macromolecules) which perturb normative cell-cell interactions (solid red arrows). MSF, as a consequence of its potent bioactivities and expression by both stromal and carcinoma cell populations, is well placed to function as such an epigenetic effector.

MSF, as a consequence of its potent bioactivities and expression by both stromal and carcinoma cell populations, is well placed to function as an epigenetic effector promoting cancer development. For example, its stimulation of hyaluronan synthesis (20, 21) may create a permissive/inductive microenvironmental “soil” that promotes the clonal expansion of (pre-)invasive epithelial cells, as well as their expression of a more aggressive invasive phenotype (65). In this regard, a key question relates to the control of MSF expression. Initial studies have identified several stress response molecules and genotoxins that induce a persistent “switch-on” of MSF expression by control adult fibroblasts and may therefore contribute to the regulation of its expression in cancer patients.

In conclusion, we suggest that MSF may make a significant contribution to both the initiation and progression of cancer. The cloning of MSF and the subsequent development of specific riboprobes and antibodies (both identification and function neutralizing) will allow future studies to assess the potential diagnostic/prognostic information of MSF expression profiles in cancer patients, whereas its spectrum of potent bioactivities may provide novel targets for therapeutic intervention. With respect to the latter, identification of the role played by the conserved IGD motif in mediating MSF bioactivity should provide a rational platform for the development of antagonists.

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Migration-Stimulating Factor: A Genetically Truncated Onco-Fetal Fibronectin Isoform Expressed by Carcinoma and Tumor-Associated Stromal Cells

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