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28. Thirty-microliter reactions were incubated for 40 min at 28°C and contained 10 μl of lysate (*8*) [~20 μg of protein (2×10^8 cell equivalents)] in 25 mM tris-HCl (pH 8.0), 10 mM MgOAc, 10 mM KCl, 3 mM ATP, 0.5 mM dithiothreitol, 0.16% Triton X-100, and 1.0 mM EDTA. All reactions contained 1 pmol of A6/TAG substrate and 10 pmol gA6[14] except as indicated in the figure legends. Reactions were stopped by the addition of 300 μl of NET-2[150 mM NaCl, 50 mM tris-HCl (pH 7.5), and 0.05% Nonidet P-40] and 1 μg of glycogen, extracted with phenol, and precipitated with two and one-half volumes of ethanol and one-tenth of a volume of 3.0 M NaOAc. Reverse transcriptions were done with radiolabeled A6-RT oligonucleotide at 48°C with Superscript II (Bethesda Research Laboratories) as described (*26*) but contained nucleotides at a final concentration of 0.4 mM dA, 0.4 mM dC, and 0.1 mM ddT. For RNA sequencing reactions, either 0.4 mM dA, 0.1 mM dC, 1.0 mM ddC, and 0.1 mM ddT, or 0.1 mM dA, 1.0 mM ddA, 0.4 mM dC, and 0.1 mM ddT was substituted as the nucleotide mix. Samples to be treated with terminal transferase were incubated with 0.5 μg of ribonuclease A for 30 min at 37°C, extracted with phenol, and precipitated with two and one-half volumes of ethanol and one-tenth of a volume of 3.0 M KOAc. Deoxynucleotidyl transferase reactions were incubated overnight at 37°C and contained 10 mM dTTP, 30 units of terminal deoxynucleotidyl transferase (TdT) (Bethesda Research Laboratories), 1× TdT buffer (Bethesda Research Laboratories), and primer extension products. Samples were run on gels containing 20% acrylamide, 8 M urea, and 1× TBE [8.9 mM tris-base, 8.9 mM boric acid, and 0.2 mM EDTA (pH 8.0)].
29. We thank P. Myler for suggesting the use of terminal transferase to distinguish dideoxy- and deoxy-terminated primer extension products and M. Ares, R. Braun, J. A. Steitz, D. Toczyski, and members of the Stuart laboratory for comments on the manuscript. Supported by NIH grant GM 42188 to K.S., who is also a Burroughs Wellcome Scholar of Molecular Parasitology.

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Identification of the *ron* Gene Product as the Receptor for the Human Macrophage Stimulating Protein

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Macrophage-stimulating protein (MSP) is a member of the hepatocyte growth factor–scatter factor (HGF-SF) family. Labeled MSP bound to Madin-Darby canine kidney (MDCK) cells transfected with complementary DNA encoding Ron, a cell membrane protein tyrosine kinase. Cross-linking of ^{125}I-labeled MSP to transfected cells (MDCK-RE7 cells) and immunoprecipitation by antibodies to Ron revealed a 220-kilodalton complex, a size consistent with that of MSP (80 kilodaltons) cross-linked to the β chain of Ron (150 kilodaltons). The binding of ^{125}I-labeled MSP to MDCK-RE7 cells was inhibited by unlabeled MSP, but not by HGF-SF. MSP caused phosphorylation of the β chain of Ron and induced migration of MDCK-RE7 cells. These results establish the *ron* gene product as a specific cell-surface receptor for MSP.

Macrophage-stimulating protein (MSP) is an 80-kD disulfide-linked serum protein that induces the responsiveness of murine peritoneal resident macrophages to chemoattractants (*1*). MSP also acts directly as a chemoattractant for resident macrophages (*2*), causes shape change of macrophages (*1*), stimulates macrophage ingestion of complement-coated erythrocytes (*3*), and inhibits expression of inducible nitric oxide synthase mRNA in endotoxin- or cytokine-stimulated macrophages (*4*). MSP is synthesized in a biologically inactive form (pro-MSP) that can be cleaved to an active disulfide-linked heterodimer by specific serine proteases that include coagulation factors XIIa and XIa, plasma kallikrein (*5*), and also the glandular kallikreins [nerve growth factor–γ and epidermal growth factor binding protein (*6*)]. MSP is structurally related to HGF-SF (*7*, *8*).

Although MSP and HGF-SF both affect cell motility and morphology, the target cell specificities of the two proteins are different. MSP acts on resident macrophages (*1*, *2*), whereas HGF-SF affects epithelia and endothelia (*9*, *10*). These differences strongly suggest that the two proteins bind to distinct cell membrane receptors. The receptor for HGF-SF is the product of the proto-oncogene c-*met* (*11*). It is synthesized as a single chain precursor, which is subsequently cleaved to yield a disulfide-linked heterodimer (with a 40-kD α chain and an 150-kD β chain) with an intracellular protein tyrosine kinase domain (*12*). The receptor for MSP is not known. Recently, the *ron* gene, a member of the c-Met receptor family, was cloned from a human foreskin keratinocyte complementary DNA (cDNA) library (*13*). Comparison with c-Met suggests that the *ron* gene product is also a membrane-spanning disulfide-linked heterodimer with intracellular tyrosine kinase activity. Transcripts of the *ron* gene were found in a human transformed keratinocyte cell line and in normal human lung. The genes encoding human MSP (*3*) and Ron (*13*) are both located on the short arm of chromosome 3 (3p21), a region of frequent deletion or mutation in small cell lung and renal carcinoma (*14*). The genes encoding both HGF-SF and its c-Met receptor are located on chromosome 7q (*15*, *16*). The location of ligand and receptor on the same chromosome, and the structural similarities between MSP and HGF-SF, suggested that the ligand for Ron might be MSP (*13*).

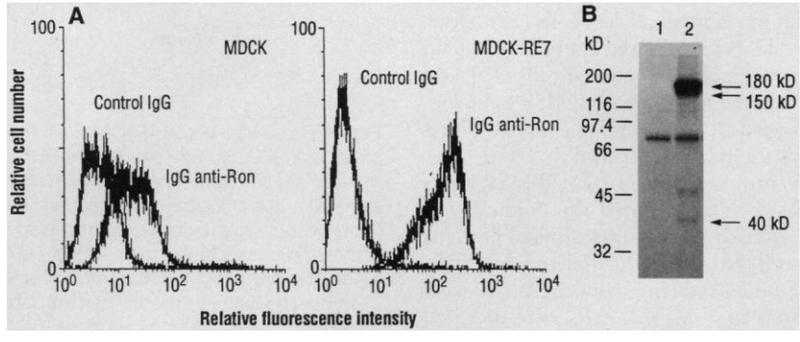

Fig. 1. Expression of the Ron receptor on MDCK-RE7 cells. (**A**) Detection by immunofluorescence with rabbit antibody to the extracellular domain of Ron (anti-Ron). (**B**) Immunoprecipitation of Ron from MDCK-RE7 cells with rabbit antibody to a COOH-terminal peptide of the Ron β chain (lane 2). Lane 1 shows the normal IgG control.

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We transfected Madin-Darby canine kidney (MDCK) cells with human *ron* cDNA (*17*) and obtained a clone with high Ron expression (MDCK-RE7), which was confirmed by detection of the protein on the cell surface by fluorescence-activated cell sorting analysis (Fig. 1A). The small fluorescence shift in MDCK control cells was not investigated. After immunoprecipitation with antibodies to the COOH-terminal peptide of the Ron β chain, a 180-kD band was detected after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Fig. 1B). This band probably represents the uncleaved single chain form of Ron (pro-Ron), which has a calculated molecular size of 190 kD, based on the cDNA sequence. We also detected 40-kD and 150-kD bands, which correspond to the α and β chains of Ron, respectively. About 40% of Ron was in the disulfide-linked heterodimeric form.

Specific binding of ^{125}I-labeled MSP to MDCK-RE7 cells (*18*) approached a plateau within about 60 min (Fig. 2A) and was dependent on concentration (Fig. 2B). Nonspecific binding was a small fraction of the total, and a plateau of specific binding was reached with about 3 nM MSP. Labeled MSP did not bind to untransfected MDCK cells or NIH 3T3 cells transfected with c-Met (*19*), the receptor for HGF-SF (*20*). The absence of MSP-specific binding to cells expressing the HGF-SF receptor is consistent with our observations that MSP has no in vitro action on HGF-SF target cells (*10*).

Because MSP contains four kringle domains in its α chain and kringle structures are thought to participate in macromolecular interactions (*21*), we determined if HGF-SF or plasminogen—proteins with kringles and high sequence similarity to MSP—could compete with MSP for the Ron binding site. Neither of these proteins inhibited binding of ^{125}I-labeled MSP to MDCK-RE7 cells (Fig. 2C). We conclude that both c-Met and Ron are specific receptors for their respective ligands.

The proto-oncogene c-*sea* also encodes a receptor tyrosine kinase protein similar to Ron, and its mRNA is highly expressed in chicken blood mononuclear cells (*22*). However, ^{125}I-labeled MSP did not bind to human peripheral blood mononuclear cells (*19*), and these cells did not respond to MSP in biological assays (*2*). Therefore, it is unlikely that c-Sea is the receptor for MSP.

To confirm that ^{125}I-labeled MSP binds to Ron, we characterized the binding sites by cross-linking (*23*). Cross-linked proteins from cell lysates were analyzed by SDS-PAGE under reducing conditions. No visible bands from MDCK cells were detected by autoradiography (Fig. 3A). Several bands were seen in lysates from MDCK-RE7 cells. The 220-kD band was of the expected size of MSP (80 kD) cross-linked with the β chain of Ron (150 kD). Unlabeled MSP inhibited formation of complexes of ^{125}I-labeled MSP with Ron in a concentration-dependent manner. The less intense band seen at 260 kD could be MSP cross-linked with the Ron αβ chain heterodimer. The 95-kD band is probably the 53-kD MSP α chain linked with the Ron α

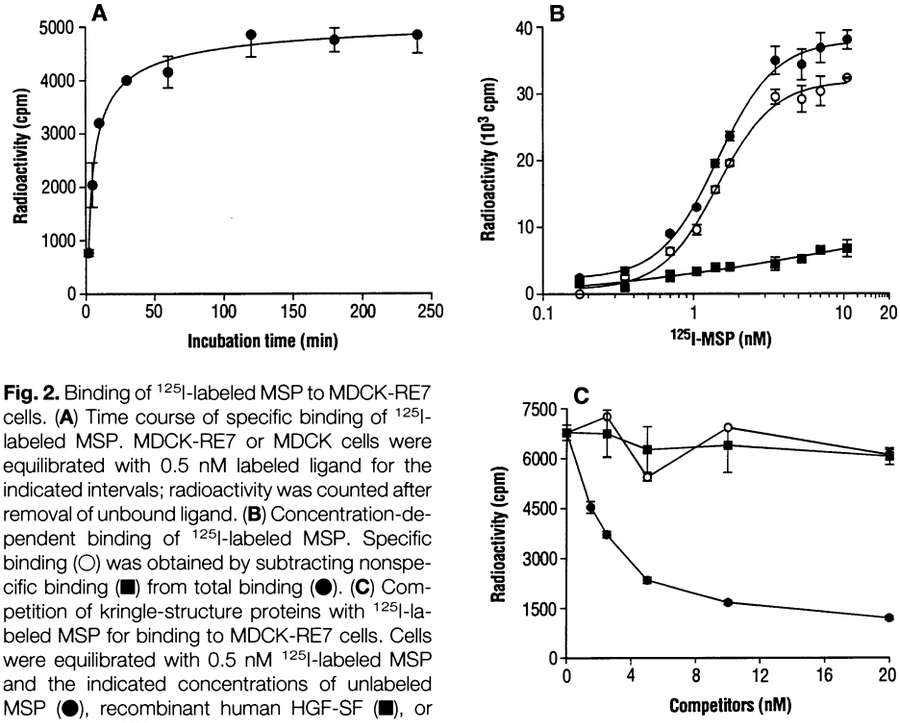

Fig. 2. Binding of ^{125}I-labeled MSP to MDCK-RE7 cells. (**A**) Time course of specific binding of ^{125}I-labeled MSP. MDCK-RE7 or MDCK cells were equilibrated with 0.5 nM labeled ligand for the indicated intervals; radioactivity was counted after removal of unbound ligand. (**B**) Concentration-dependent binding of ^{125}I-labeled MSP. Specific binding (○) was obtained by subtracting nonspecific binding (■) from total binding (●). (**C**) Competition of kringle-structure proteins with ^{125}I-labeled MSP for binding to MDCK-RE7 cells. Cells were equilibrated with 0.5 nM ^{125}I-labeled MSP and the indicated concentrations of unlabeled MSP (●), recombinant human HGF-SF (■), or plasminogen (○).

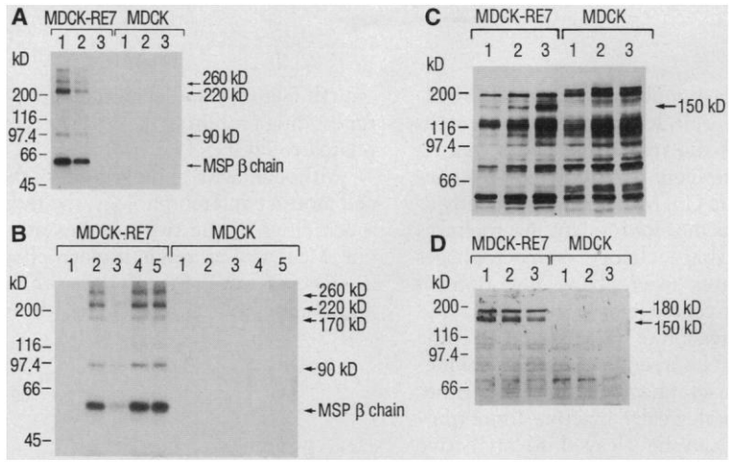

Fig. 3. Cross-linking of ^{125}I-labeled MSP to cellular proteins. (**A**) Cross-linking analysis of MSP-binding proteins on MDCK-RE7 cells. MDCK-RE7 or MDCK cells were equilibrated at 0°C for 3 hours with 1 nM ^{125}I-labeled MSP alone (lanes 1) or with 5 nM (lanes 2) or 30 nM (lanes 3) unlabeled MSP. The cells were then exposed to the cross-linking reagent and lysed. The figure is an autoradiograph obtained after SDS-PAGE (7.5% gel) of lysates under reducing conditions. (**B**) Immunoprecipitation by rabbit antibody to the Ron peptide of cross-linked protein complex. MDCK-RE7 or MDCK cells were treated with 1 nM ^{125}I-labeled MSP alone (lanes 2) or with 30 nM unlabeled MSP (lanes 3), HGF-SF (lanes 4), or plasminogen (lanes 5). After cross-linking, cell lysates were precipitated with rabbit antibody to the Ron peptide and proteins were separated by SDS-PAGE under reducing conditions. Lanes 1 are normal rabbit IgG controls. (**C**) Stimulation by MSP of tyrosine phosphorylation in MDCK-RE7 cells. Lanes 1, no stimulation; lanes 2, stimulation with 0.5 nM MSP; lanes 3, stimulation with 5 nM MSP. The arrow indicates a phosphorylated 150-kD protein. (**D**) Detection of the Ron protein by protein immunoblotting. The same membrane as in (C) was reprobed with rabbit antibody to the Ron peptide. The arrows point to a 150-kD Ron β chain and a 180-kD pro-Ron.

chain (40 kD). The 56-kD band is likely the α chain of MSP that reacted with the receptor but was not cross-linked.

Immunoprecipitation of proteins from cell lysates after cross-linking with rabbit antibody to a COOH-terminal peptide from Ron yielded a band with a molecular size of 220 kD (Fig. 3B). The location of this band is exactly the same as that for the band from direct cross-linking (Fig. 3A), which confirms that Ron was the membrane protein to which ^{125}I-labeled MSP is bound. We confirmed this specificity by showing that cross-linking of ^{125}I-labeled MSP was inhibited by unlabeled MSP but not by HGF-SF or plasminogen. No protein complex with ^{125}I-labeled MSP was detected from nontransfected MDCK cells (Fig. 3B).

It was anticipated that Ron, like c-Met, would be phosphorylated in response to ligand binding. Immunoblots of proteins from cell lysates probed with antibodies to phosphotyrosine showed that MSP induced tyrosine phosphorylation of a 150-kD protein in transfected MDCK-RE7 cells in a concentration-dependent manner (Fig. 3C). Stimulation of parental MDCK cells or MDCK cells transfected with the cDNA of an unrelated receptor did not cause the appearance of this phosphorylated protein. Probing of the same blot with rabbit antibody to Ron showed a band at the same position as the band of antibody to phosphotyrosine, which corresponds to the β chain of Ron (Fig. 3D). The band above the 150-kD protein is probably uncleaved pro-Ron (180 kD), which was not phosphorylated after stimulation of the cells with MSP. These findings indicate that proteolytic conversion of pro-Ron to the heterodimer is required for ligand-induced phosphorylation, which is presumably required for signal transduction.

In view of the effects of MSP on macrophage motility (*1*), we determined if MSP induced similar changes in MDCK cells transfected with the Ron receptor (*24*).

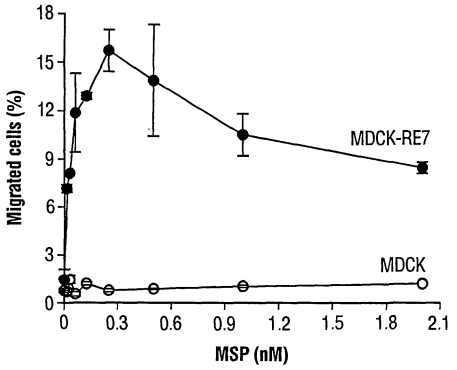

Fig. 4. Stimulation of MDCK-RE7 cell migration in response to MSP.

MSP caused migration of MDCK-RE7 cells in a concentration-dependent manner (Fig. 4). Untransfected MDCK cells did not respond to MSP.

Ron mRNA is expressed in a transformed keratinocyte line (*13*). MSP also induced phosphorylation of Ron in a primary keratinocyte line (*25*), and ^{125}I-labeled MSP bound to an immortalized keratinocyte line expressing Ron (*19*). Hitherto, the only target cell for MSP has been the mouse resident peritoneal macrophage. Evidence that the MSP receptor is also on keratinocytes leads to new areas for investigating the biological significance of MSP. MSP may function in wound healing through effects on macrophages (*6*) and keratinocytes, both of which take part in tissue repair. Finally, because Ron is structurally similar to c-Met, we should consider its oncogenic potential and the possibility that it could affect both replication and metastatic migration of cells.

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17. Human *ron* cDNA (*13*) was introduced into an expression vector under control of the Rous sarcoma virus long terminal repeat, and the resulting plasmid was transfected with pSV2neo into MDCK cells. G418-resistant cells were screened for Ron mRNA or protein expression by Northern (RNA) blot and immunoprecipitation techniques. A clone with high Ron expression (MDCK-RE7) was selected. For immunofluorescence labeling, 2 × 10^5 MDCK-RE7 or MDCK cells were equilibrated in 0.5 ml of phosphate-buffered saline containing 1% fetal calf serum with rabbit antibody to the extracellular domain of Ron or normal rabbit immunoglobulin G (IgG) at 0°C for 45 min. After washing, cells were treated for 30 min with goat antibody to rabbit IgG F(ab')$_2$ conjugated with fluorescein isothiocyanate. Fluorescence intensity was analyzed by flow cytometry. To precipitate Ron proteins, we cultured 3 × 10^6 MDCK or MDCK-RE7 cells in cysteine-free Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and labeled them with 300 μCi of [^{35}S]cysteine (Amersham) at 37°C for 8 hours. Cells were then treated for 30 min with lysis buffer [50 mM tris (pH 7.4) with 1.5% Triton X-100, 150 mM NaCl, 2 mM EDTA, 100 μM vanadate, leupeptin (50 μg/ml), aprotinin (50 μg/ml), and soybean trypsin inhibitor (100 μg/ml)]. Lysates were treated with normal rabbit IgG and then equilibrated for 2 hours at 4°C with rabbit antibody to Ron peptide coupled to protein G–Sepharose. The beads were washed, suspended in sample buffer containing 2-mercaptoethanol, and heated at 100°C for 4 min. Proteins were separated by SDS-PAGE (10% gel). The gel was treated with Enlightning (DuPont), dried at 80°C, and autoradiographed at −80°C on XAR-5 film (Eastman-Kodak) with the use of intensifying screens.
18. Purified MSP (*3*) was labeled by adding 250 μCi of ^{125}I-labeled Bolton-Hunter reagent to 15 μg of MSP in 30 μl of 0.1 M borate buffer (pH 8.5). Iodinated protein was eluted from an Excellulose GF-5 desalting column (Pierce), and radioactivity was counted in a gamma counter. Specific activity of the labeled MSP was 2 × 10^5 cpm/pmol. In binding assays, 1 × 10^5 cells were equilibrated in duplicate with various amounts of ^{125}I-MSP in binding buffer [RPMI 1640 medium (pH 7.4) with 20 mM Hepes and cytochrome c (100 μg/ml)] in a total volume of 200 μl. Nonspecific binding was determined in parallel in the presence of excess MSP (30 times the concentration of labeled MSP). After equilibration for 3 hours at 0°C, cells were sedimented through an oil cushion and radioactivity was counted in a gamma counter. Values represent means ± SEM of duplicates.
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23. For cross-linking of ^{125}I-labeled MSP to its receptor, 5 × 10^6 cells were incubated with 1 nM ^{125}I-labeled MSP in 1 ml of RPMI 1640 medium at 0°C with or without unlabeled MSP, HGF-SF, or plasminogen. After 3 hours, the proteins were cross-linked with disuccinimidyl suberate. Cells were washed and lysed for 30 min in lysis buffer (50 μl). After high-speed centrifugation at 4°C for 10 min, cleared lysate proteins were either separated directly by SDS-PAGE (7.5% gel) or precipitated before SDS-PAGE with rabbit antibody to Ron as described (Fig. 1). For tyrosine phosphorylation, 3 × 10^6 MDCK-RE7 or MDCK cells in 1 ml of buffer were incubated with different amounts of MSP at 37°C for 15 min. After stimulation, cells were lysed in lysis buffer (100 μl). After SDS-PAGE and transfer to membranes, the membranes were blocked with bovine serum albumin (1%) in tris buffer (pH 7.4) with Tween 20, equilibrated overnight with 0.2 μg/ml of phosphotyrosine antibodies (4G10, UBI, Lake Placid, NY), and then equilibrated with goat horseradish peroxidase–conjugated antibody to mouse IgG. Antibody binding was detected with an enhanced chemiluminescence detection reagent (Amersham). To detect the Ron protein, we stripped the same membrane, reprobed it with rabbit antibody to Ron, and developed it as described above.
24. For an assay of cell migration, duplicate bottom wells of a multiwell chemotaxis chamber were filled with 30 μl of DMEM containing different concentrations of MSP. The interface between bottom and top wells was a type VI collagen–coated polycarbonate membrane with randomly distributed 10-μm-diameter holes. Upper wells were filled with 45 μl of MDCK-RE7 or MDCK cell suspension (1 × 10^6 cells per milliliter). After 5 hours of incubation at 37°C, the chamber was disassembled and the membranes were dried and stained. An image analyzer was used to count cells that migrated through the holes and adhered to the bottom surface of the membrane. Results were expressed as the percentage of input cells that migrated.
25. C. Ronsin, unpublished data.

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