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Ron is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP

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RON, a cDNA homologous to the hepatocyte growth factor (HGF) receptor gene (MET), encodes a putative tyrosine kinase. Here we show that the RON gene is expressed in several epithelial tissues as well as in granulocytes and monocytes. The major RON transcript is translated into a glycosylated single chain precursor, cleaved into a 185 kDa heterodimer (p185RON) of 35 (α) and 150 kDa (β) disulfide-linked chains, before exposure at the cell surface. The Ron β-chain displays intrinsic tyrosine kinase activity in vitro, after immunoprecipitation by specific antibodies. In vivo, tyrosine phosphorylation of p185RON is induced by stimulation with macrophage stimulating protein (MSP), a protease-like factor containing four 'kringle' domains, homologous to HGF. In epithelial cells, MSP-induced tyrosine phosphorylation of p185RON is followed by DNA synthesis. p185RON is not activated by HGF, nor is the HGF receptor activated by MSP in biochemical and biological assays. p185RON is also activated by a pure recombinant protein containing only the N-terminal two kringle of MSP. These data show that p185RON is a tyrosine kinase activated by MSP and that it is a member of a family of growth factor receptors with distinct specificities for structurally related ligands.

Key words: growth factor receptors/HGF/MET oncogene/MSP/RON tyrosine kinase

Introduction

Receptor tyrosine kinases play a critical role in the control of cell growth and differentiation mediating the response to extracellular signals such as peptide hormones and growth factors (Schlessinger and Ullrich, 1992; Fanti et al., 1993). The common architecture of these molecules includes an extracellular ligand binding domain, a hydrophobic membrane spanning domain and a large cytoplasmic portion which carries the catalytic function. All receptor type tyrosine kinases share a conserved catalytic domain which is found in combination with a range of different extracellular domains (Hanks et al., 1988; Yarden and Ullrich, 1988). The latter activate the catalytic domain in response to the binding of specific extracellular ligands.

There are several sub-families of receptor tyrosine kinases; ligands have only been assigned to a few of these. For others, the receptor function has been postulated on the basis of structural similarities to known receptors (for a review see Hunter and Lindberg, 1994).

We previously showed that the protein encoded by the MET proto-oncogene (p190MET) is the prototype of a family of tyrosine kinase receptors on the basis of specific features. These include: (i) the heterodimeric subunit structure (Giordano et al., 1989a), (ii) two neighbouring tyrosine residues in the kinase domain, responsible for regulation of the enzymatic activity upon autophosphorylation (Ferracini et al., 1991; Longati et al., 1994), (iii) a bidentate docking site in the C-terminal tail which mediates high-affinity interactions with multiple SH2-containing signal transducers (Ponzetto et al., 1994).

The physiological role of p190MET was unveiled when it was identified as the receptor for hepatocyte growth factor (HGF) (Bottaro et al., 1991; Naldini et al., 1991a,b). HGF is a large protein, made of an α-chain, containing four kringle domains, and a disulfide-linked β-chain, endowed with strong homology with serine proteases, but devoid of catalytic activity (Miyazawa et al., 1989; Nakamura et al., 1989). HGF controls an array of complex biological functions, including proliferation, motility and differentiation of epithelial cells (Gherardi and Stoker, 1991).

Recently the MET gene family acquired two new putative members, after the isolation of the cDNA for avian c-sea (Huff et al., 1993) and for human RON (Ronsin et al., 1993). The deduced amino acid sequences show significant homologies with the Met protein and include the above listed structural features of the HGF receptor. Interestingly, two independent reports pointed out the existence of one protein which is related to HGF. This protein, called 'HGF-like' (Han et al., 1991) or macrophage stimulating protein (MSP; Skeel et al., 1991; Yoshimura et al., 1993) shares with HGF the overall four kringle/protease-like structure.

In this paper we show that RON encodes an heterodimeric transmembrane glycoprotein (p185RON), expressed in a variety of tissues, mainly of epithelial origin, and in monocytes. p185RON is endowed with intrinsic tyrosine kinase activity that is stimulated by MSP.

Results

Cloning and analysis of the RON cDNA

In an effort to identify new members of the MET receptor family, we performed a reverse-transcriptase polymerase chain reaction analysis on RNAs extracted from different cell lines and tissues, following the method of Raz
Activation of the Ron receptor by MSP

Fig. 1. (A) Schematic representation of the open reading frame of the RON cDNA, isolated from the GTL-16 cell line. The consensus sequence for the proteolytic cleavage generating the αβ-heterodimer (KRRRR), the transmembrane (tm) and the tyrosine kinase (tk) domains are indicated. The base pairs 884–932 are deleted in the variant ΔRON. (B) Schematic representation of the mature heterodimeric Ron protein. α, light chain of 35 kDa. β, heavy chain of 150 kDa. The number and the location of the disulfide (-S-S-) bond(s) are unknown. The conserved critical tyrosine residues of the cytoplasmatic domain are indicated.

et al. (1991). Degenerate oligonucleotide primers designed according to sequences situated in the subdomains VI and X of tyrosine kinases (Hanks et al., 1988) were used. Sequences of the several clones obtained were compared with the EMBL Database. One of the cDNA fragments (384 bp in length) matched the sequence of RON, a newly identified homologue of human MET (Ronin et al., 1993). Using this as a probe, full-length cDNA for RON was obtained from a library derived from the GTL16 gastric carcinoma cell line (Giordano et al., 1993). The cDNA showed complete identity with the previously reported RON sequence (Ronin et al., 1993). A variant diverging by an in-frame 49 amino acid deletion in the extracellular domain, was also identified. The boundaries of the deletion were located between positions Glu883–Tyr884 and positions Gln932–Val933 (Figure 1).

Expression of the RON gene
The expression of RON was examined in a number of human tissues and cell lines by Northern blot analysis of total RNA, using a cDNA probe encompassing the entire coding sequence. RNAs from human tissues were prepared from fresh samples harvested from organ donors. A 5.0 kb mRNA was the major detectable RON transcript; a 2.0 kb mRNA was also observed. As shown in Figure 2, significant levels of mRNA were found in colon, skin, lung and bone marrow. Among blood cells, granulocytes were strongly positive whilst lymphocytes were negative. Adherent monocytes showed a detectable expression of RON mRNA. In a panel of cell lines, the expression of RON was studied at both RNA and protein level. Epithelial cell lines derived from gastric, pancreatic and mammary carcinoma and the hematopoietic cell lines HL-60 and K562 were positive (Figure 3A and B).

Structure and biosynthesis of the Ron protein
The RON cDNA was expressed in COS-1 cells under control of the adenovirus late promoter. The translation product was analysed using antibodies raised against a GST fusion protein containing 41 C-terminal amino acids. In Western blots, Ron antibodies reacted with two proteins (Figure 4A). One showed an apparent molecular mass of 185 kDa (p185RON) when Western blots were performed under non-reducing conditions. After reduction this molec-

![Figure 2](Image)
exposed at the cell surface, as shown by domain-selective cell surface biotinylation (Crepaldi et al., 1994b). In COS-1 cells overexpressing the transfected RON cDNA a fraction of uncleaved protein was found at the cell surface (Figure 6).

**The Ron protein is a functional tyrosine kinase**

The intracellular portion of the Ron β-chain contains canonical motifs identifying a putative tyrosine kinase domain (Hanks et al., 1988; Ronsin et al., 1993). In order to assess whether this molecule is indeed a functional kinase, recombinant p185RON, immunoprecipitated from COS-1 cells, and native p185RON, immunoprecipitated from GTL-16 cells, were incubated with [γ-32P]ATP in the presence of Mn ions. It is known (Yarden and Ullrich, 1988; Naldini et al., 1991c) that under these conditions the antibody induced dimerization activates tyrosine kinase receptors leading to autophosphorylation, even in the absence of a specific ligand. p185RON autophosphorylated on tyrosine on its β-chain (Figure 7). Phosphorylation occurred on tyrosine residues, as demonstrated by alkali resistance and by phosphoamino acid analysis (data not shown). The tyrosine phosphatase inhibitor sodium ortho vanadate, added to the cells 10 min before lysis, enhanced the autophosphorylation of p185RON.

**The Ron protein is phosphorylated on tyrosine in response to MSP**

The above data show that p185RON is a tyrosine kinase with putative receptor functions. As discussed, the protein shares structural similarities with the HGF receptor. Interestingly, one protein related to HGF has been recently identified. This molecule was called ‘HGF-like’ or MSP (Han et al., 1991; Skeel et al., 1991; Yoshimura et al., 1993).

To investigate the possible functional relationship between p185RON and MSP, we challenged cells expressing RON with recombinant MSP. Tyrosine phosphorylation of the p185RON β-chain was assessed by Western blotting with anti-phosphotyrosine antibodies of immunoprecipi-
Ron protein. Western analysis on anti-Ron immunoprecipitates from RON-transfected COS-1 cells, lysed 24, 48, 72 or 96 h after transfection. The blot was performed as in Figure 3B. At one day after transfection only the p170RON precursor is detectable. In the following hours an increasing amount of mature Ron β-chain accumulates. The mature form is predominant 4 days after transfection. As in Figure 4A, the α-chain is not visible, because of the specificity of the antibodies.

Fig. 5. Biosynthesis of the Ron protein. Western analysis on anti-Ron immunoprecipitates from RON-transfected COS-1 cells, lysed 24, 48, 72 or 96 h after transfection. The blot was performed as in Figure 3B.

That recombinant MSP is secreted as single chain precursor (Yoshimura et al., 1993). By analogy with what has been described for the HGF precursor (Naldini et al., 1992), in vitro maturation of MSP into the biologically active αβ heterodimer was obtained by serum-dependent proteolytic cleavage (see Materials and methods; Wang et al., 1994). Recombinant MSP stimulated tyrosine phosphorylation of the p185RON β-chain in both RON-transfected COS-1 cells (Figure 8A) and in T47D cells (Figure 8B). The medium from mock-transfected 293 cells neither induced p185RON phosphorylation in T47D cells nor altered the basal p185RON phosphorylated status in COS-1 transfectants.

p185RON is not cross-activated by HGF

Given the structural similarities between p185RON and the HGF receptor on the one hand, and between MSP and HGF on the other, their possible cross-reactivity was investigated. No effects on tyrosine phosphorylation of p185RON was observed after HGF treatment of cells expressing either the native or the transfected receptor (Figure 8A and B). The lack of cross-reactivity was confirmed in biological assays. It is known that MSP stimulates a chemotactic response to C5a in macrophages (Yoshimura et al., 1993). HGF, also known as ‘scatter factor’, stimulates cell motility of epithelial cells (Naldini et al., 1991b; Weidner et al., 1991; Giordano et al., 1993). In a Boyden chamber, supernatants containing recombinant MSP were found to stimulate monocyte migration, but were ineffective on A549 epithelial cells. Conversely, recombinant HGF stimulated migration of A549 but not of monocytes (Figure 9). Moreover, preincubation of monocytes with HGF had no inhibitory effect on MSP activity. Similarly, MSP did not interfere with HGF stimulation of A549 cells (data not shown).

p185RON phosphorylation by MSP is followed by DNA synthesis

Tyrosine phosphorylation of growth factor receptors activates basic intracellular signalling mechanisms, which transduce growth factor signals to the nucleus. We tested DNA synthesis in T47D epithelial cells, where MSP induces specific tyrosine phosphorylation of p185RON. Confluent and quiescent monolayers of T47D were incubated with the conditioned medium of mock-
Fig. 8. Ron is phosphorylated on tyrosine in response to MSP. RON-transfected COS-1 cells (A) or T47D cells (B) were stimulated with culture media of cells transfected either with an empty vector (EMPTY) or with a vector containing MSP or HGF cDNA. Cell lysates were immunoprecipitated with Ron antibodies; immunocomplexes were Western blotted and probed with monoclonal phosphotyrosine antibodies (anti P-Tyr) or with anti-Ron antibodies and visualized by ECL. MSP-induced tyrosine phosphorylation of the Ron β-chain is detectable both in Ron transfected COS-1 cells and in T47D cells expressing the endogenous protein. Tyrosine phosphorylation of the uncleaved precursor (uncl.) is only observed in COS-1 cells overexpressing RON.

MSP-transfected 293 cells and with the conditioned medium of cells transfected with HGF cDNA. MSP induced a significant increase in DNA synthesis, measured as [3H]thymidine incorporation (Figure 10). Again, no cross-reactivity was observed with HGF, which neither induced phosphorylation of p185RON nor stimulated DNA synthesis. It should be noted that the T47D cell line used in these experiments does not express the HGF receptor, as assessed by specific anti-Met antibodies and RT-PCR (data not shown).

p185RON is activated by the N-terminal two kringles of MSP
It has been shown previously that the binding activity of HGF to its receptor is conveyed by a functional domain located in the N-terminal portion of the molecule, including the first two 'kringles' (Matsumoto et al., 1991; Hartmann et al., 1992; Lokker et al., 1992; Lokker and Godowsky, 1993). Therefore, we tested the N-terminal region of MSP expressed as a fusion protein with a C-terminal fragment of the human IgG-1 chain (MSP–NK2). MSP–NK2 contains the first two kringles of MSP and was purified to homogeneity by affinity chromatography on immobilized protein A (see Materials and methods). As shown in Figure 11A, MSP–NK2 stimulated, in a dose-dependent manner, tyrosine phosphorylation of p185RON expressed in COS-1 cells. The effect was specific, since MSP–NK2 did not stimulate a chimeric receptor made of the intracellular tyrosine kinase domain of Ron linked to the extracellular domain of the nerve growth factor (NGF) receptor (TRK) (Figure 11B).

Discussion
In recent years new members of the protein tyrosine kinase family have been discovered at an increasing rate. Some of them have been classified as putative receptors according to the structural features deduced from their cDNAs (Schlessinger and Ullrich, 1992; Hunter and Lindberg, 1993). We isolated the same cDNA from a human gastric...
carcinoma library (Giordano et al., 1993), using a partial clone obtained by RT-PCR as a probe and degenerate oligonucleotide primers derived from the tyrosine kinase domain of the HGF receptor sequence (MET). The RON cDNA encodes a protein of 1400 amino acids which shares an overall structural homology with the HGF receptor and displays 63% sequence identity in the catalytic domain. By Northern analysis we found that Ron is expressed as a major 5.0 kb transcript in epithelial tissues and cell lines. Notably, the gene is also expressed by blood granulocytes and monocytes. The tissue distribution of this putative tyrosine kinase receptor is reminiscent of the expression pattern of the HGF receptor (Di Renzo et al., 1991; Prat et al., 1991). We show that the product of the Ron gene is a glycosylated protein of 185 kDa (p185Ron). The molecule is an heterodimer of two chains: α (35 kDa) and β (150 kDa). The two chain structure is very similar to that of the HGF receptor (Giordano et al., 1989a). We also show that, as for the HGF receptor (Giordano et al., 1989b), p185Ron is synthesized as a single chain precursor (pr170Ron), which is converted into the mature form by proteolytic cleavage. A conserved site for furin-like proteases (Mark et al., 1992) is present in the precursors of both molecules between residues 305–309 and 303–307, respectively (Figure 1). By domain-selective surface biotinylation we found that the mature heterodimeric form is delivered to the cell surface, while the unprocessed single chain precursor is not. Thus, as in the case of the HGF receptor, proteolytic cleavage of the precursor takes place before the appropriate subcellular localization (Crepaldi et al., 1994a,b). In non-physiological conditions, such as in cells overexpressing exogenous RON under control of a strong promoter, the subcellular machinery responsible for proteolytic cleavage is overloaded. This leads to the abnormal expression of uncleaved p185Ron at the cell surface. As in the case of cells overexpressing MET (Mondino et al., 1991; Naldini et al., 1991a), the uncleaved p185Ron is capable of responding to ligand stimulation.

The tyrosine kinase of p185Ron can be activated either in vitro by immunoprecipitation with bivalent antibodies, or in vivo by overexpression in transfected cells. Both conditions are known to trigger kinase activity through receptor dimerization and subsequent transphosphorylation (Honegger et al., 1988; Lammers et al., 1990; Naldini et al., 1991c). One characteristic feature of the kinase domain of p185Ron are two tyrosines at position 1238 and 1239 (see Figure 1) conserved in the HGF and in the insulin receptor (Tornqvist et al., 1987). It has been shown that these are the major auto phosphorylation sites (Ferracini et al., 1991) and are responsible for upregulation of the kinase activity in the HGF receptor (Longati et al., 1994).

Fig. 10. MSP stimulates DNA synthesis of epithelial cells. T47D cells, cultured on 24-well plates, were incubated with conditioned medium of cells transfected either with an empty vector (EMPTY), or with a vector containing the HGF or MSP cDNA. Media were used at 1:5 dilution. 3H-Thymidine incorporation was evaluated as described in Materials and methods. The data shown are the average (± SD) of replicate wells (n=3) from a typical experiment.

Fig. 11. The two N-terminal kringle of MSP stimulate phosphorylation of Ron via its extracellular domain. COS-1 cells were transfected with RON cDNA (A), or with a chimeric cDNA containing the extracellular domain of TRK fused with the intracellular domain of RON (B). Cells were stimulated with the indicated concentrations of highly purified fusion protein MSP–NK2, containing the first two N-terminal kringle domains. Controls included unstimulated cells (n.s.) and cells stimulated with nerve growth factor. Cell lysates were immunoprecipitated with Ron antibodies, Western blotted, probed with monoclonal phosphotyrosine antibodies and visualized by ECL. (A) Tyrosine phosphorylation of the Ron β-chain by MSP–NK2 is dose-dependent. As observed in Figure 8A, the uncleaved Ron protein (uncl.) is modestly phosphorylated. (B) MSP–NK2 fails to stimulate tyrosine phosphorylation of the chimeric receptor. As reported by others (Weidner et al., 1993), the Trk chimera migrates as a broad band (vertical bar). In both panels gels were run in reducing conditions.
1994). It is thus conceivable that the Ron tyrosine kinase is regulated by the same auto-catalytic mechanism operating in the HGF and insulin receptors (Lammers et al., 1990; Naldini et al., 1991c).

The high degree of homology with the HGF receptor prompted us to test the hypothesis that p185Ron could be the receptor for a recently isolated molecule closely related to HGF. This protein, called ‘HGF-like’ (Han et al., 1991) or MSP (Skeel et al., 1991; Yoshimura et al., 1993), is an heterodimer of a heavy chain of 53 kDa (α) and a light chain of 25 kDa (β). The MSP sequence includes four kringles domains, found also in HGF and in several proteins of the blood coagulation cascade. Among these, the highest homology (45% identity) has been found between MSP and HGF. Based on expression studies, the liver appears to be the main source of MSP. Both the MSP and the Ron gene map to human chromosome 3p2.1 (Han et al., 1991; Ronson et al., 1993). Co-localization also occurs in the case of the HGF and the HGF receptor (Met) genes, both located in the long arm of chromosome 7, within the region q11.2-q21.1 (Dean et al., 1985; Weidner et al., 1991; Saccone et al., 1992). The biological activity of MSP is largely unknown: the factor was named after its ability to confer responsiveness to the chemoattractant C5a to mouse resident peritoneal macrophages (Skeel et al., 1991).

We now show that recombinant MSP is able to stimulate tyrosine phosphorylation of p185Ron, whether endogenous or transfected. Moreover, MSP behaves as a mitogen in epithelial cells, where it induces p185Ron tyrosine phosphorylation. In the absence of available purified MSP, and therefore of cross-linking data, one cannot in principle exclude the possibility that MSP may interact with another molecule which in turn activates p185Ron via heterodimerization and transphosphorylation (Peles et al., 1992; Wen et al., 1992; Plowman et al., 1993). However, transphosphorylation by an unidentified receptor did not occur in the case of the Trk–Ron chimera, sharing the intracellular domain with p185Ron. Moreover, the striking similarities between Ron and the HGF receptor (Met) on one side, and between MSP and HGF on the other, strongly suggest that MSP functions as a ligand for p185Ron. Thus Met and Ron encode two members of a family of receptors for structurally-related ligands.

A variable degree of cross-reactivity among ligands for receptors of the same subfamily has been described in the case of receptors for the nine FGFs (Bellot et al., 1991; Ueno et al., 1992), PDGF A and B (Heldin and Westmark, 1989), VEGF/VPF (Devries et al., 1992; Galland et al., 1993), neurotrophins (Barbacid, 1993), EGF, TGF-α and the heregulin family (Carpenter and Wahl, 1990; Plowman et al., 1993). When we tested cross-reactivity between MSP and HGF, we found a high degree of specificity, since neither did MSP affect the kinase activity of the HGF receptor nor was MSP able to stimulate p185Ron. Moreover, the biological responses triggered by either HGF or MSP were not reciprocally stimulated or inhibited.

It has been shown that the minimal portion of the HGF molecule involved in receptor binding is located within the first two N-terminal kringles (Matsumoto et al., 1991; Hartmann et al., 1992; Lokker et al., 1992, 1994; Lokker and Godowski, 1993). Here we show that this is also the case for MSP, since a recombinant protein including only the first two kringles is active in inducing tyrosine phosphorylation of p185Ron.

This paper demonstrates that p185Ron is a tyrosine kinase receptor responsive to MSP. The intracellular signals transduced are almost completely unknown. A striking homology between p185Ron and the HGF receptor is found in the C-terminal tail, a region which is critical for signal transduction. In this otherwise divergent region of p185Ron two tyrosines are conserved, together with their surrounding residues (tyrosines 1349 and 1356 of Met, tyrosines 1353 and 1360 of Ron sequence: Figure 1). We have recently shown that phosphorylation of these two tyrosines, both in Met and in Ron generates a docking site that mediates high affinity interactions with multiple SH2-containing signal transducers. These include PI 3-kinase, phospholipase-C-γ, pp60src and the GRB-2/Sos complex (Ponzetto et al., 1994). This multifunctional docking site integrates several pathways and it has the potential of triggering diverse biological responses in target cells: growth, motility and differentiation (for a review see Comoglio, 1993). This, together with the wide spectrum of cell types expressing p185Ron, suggest that the biological function of MSP, originally confined to macrophage migration, is likely to be far more reaching.

Materials and methods

Cell lines and tissue samples

GTL16 cell line is a clonal cell line derived from a poorly differentiated gastric carcinoma line (Giordano et al., 1989a). All other cell lines were from ATCC: A549 (lung carcinoma); SU122 and PT45 (pancreatic carcinoma); K562, U266 and HL-60 (hemopoietic); KATOII (gastric carcinoma); HepG2 (hepatocellular carcinoma); DAOY (medulloblastoma); T47D (mammary carcinoma). Blood monocytes were purified from buffy coats from healthy donors by Ficoll density gradient centrifugation and adherence. Normal and neoplastic tissues were harvested from organ donors and surgical specimens, immediately frozen in liquid nitrogen, and their RNA was extracted using the single step method of extraction described by Chomczynski and Sacchi (1987).

Cloning of Ron cDNA

Total RNA (10 μg) extracted from different epithelial cell lines was used as a template for synthesis of oligo(dt)-primed double stranded cDNA, using M-MLV-RT from BRL (UK). PCRs were performed using degenerate primers based on the amino acid sequences PTVKD and QPEVC of the subdomains VI and X of tyrosine kinases (Hanks et al., 1988) and a partial cDNA clone of 384 bp was obtained. The entire Ron cDNA was isolated from a GTL16 cDNA library (Giordano et al., 1991) by plaque hybridization, using the above cDNA as a probe. Full-size Ron cDNA was subcloned in pBluescript for sequencing, performed on double stranded plasmids using the dideoxynucleotide method (Sanger et al., 1977) and T7 DNA polymerase from Pharmacia (Uppsala, S). Specific oligonucleotide primers were synthesized by standard phosphoramidite methods with a PCR-Mate 391 DNA Synthesizer (Applied Biosystem, UK). The Ron cDNA was then inserted into the pMT2 eukaryotic expression vector, which contains the major late adenovirus promoter and the SV40 origin of replication.

Construction of the Trk–Ron chimeric receptor

The CDNA encoding the extracellular domain (bp 1–1344) of the human high affinity NGF receptor (Trk) (Martin-Zanca et al., 1986) was fused to the sequence coding for the transmembrane and cytoplasmic regions of human Ron (bp 2903–4228). The resulting hybrid receptor (Trk–Ron) contains the ligand binding domain of the NGF receptor and the tyrosine kinase domain of Ron. The CDNA fragments for the construction of the chimeric receptor were constructed by PCR amplification, with the forward (5’–TGGTTCGCTGCAGTGCAAGTGGGA-3’) and the reverse (5’–GCCACCGAGTCAAGCC-TAGAGTCGTCCTC-3’) primers for Trk and with the forward (5’–AGGGCCAGCGAATTCTACATGAGT)
CAAGT-3') and the reverse (5'-CCACAGTGCGTCCTAGTAGTC-CTGC-3') primers for RON. Modified TRK and RON cDNAs were recombined through the AvrII restriction site. The sequence of the construct was determined as described above. The chimera was then inserted into the pmT2 eukaryotic expression vector. Transfection of the RON, MET (M.F.Di Renzo et al., 1993) and RON-RON cDNA in COS-1 cells was carried out using the DNA--calcium phosphate coprecipitation procedure (CellPhect Transfection Kit, Pharmacia).

Cloning of MSP cDNA
A cDNA encoding the full-length MSP was constructed by joining together cDNAs encoding MSP amino acids 1-340 (clone 5'MSP) and 341-711 (clone 3'MSP). These cDNAs were isolated by PCR amplification of cDNA from human liver. Total human liver RNA (10 µg) was used as a template for reverse transcription using a mixture of random hexamer and oligo(dT), using M-MLV-RT from BRL (U.K.). To obtain clone 5'MSP, a PCR was performed in a volume of 100 µl containing 10 µl of the RT reaction, using 1 µl of Vent DNA polymerase (New England Biolabs) and 50 pmol of the forward primer (5'-CAGTGCAGCTCAGCCAAGA-3') and the reverse primer (5'-CTGATCACAACGGTACGTGAC-3') and the nested forward primer (5'-CCAGGATTCCGAACGGGACAATCTGG-3') and the nested reverse primer (5'-GGCCAGATCCCGTTCGGTACCTCCTGCTGTCGCTTACG-3'). After 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 45 s) and extension (72°C, 2 min), 2 µl of the PCR were re-amplified with the nested forward primer (5'-AGGACGATCCATCCATGGGTTGCTCCACTCCTGCTGTCGCTTACG-3') and the nested reverse primer (5'-CCAGGATTCCGAACGGGACAATCTGGCCTGCTGTCGCTTACG-3'). To obtain clone 3'MSP, forward primers (5'-CCAGGATTCCGAACGGGACAATCTGGCCTGCTGTCGCTTACG-3') and reverse primers (5'-AGGACATCCATCCATGGGTTGCTCCACTCCTGCTGTCGCTTACG-3') were used. The PCR products were digested with EcoRI, purified and cloned in the vector pRK7. The sequences of six inserts amplified from separate PCRs were determined as described above. The sequences of these clones were identical to the sequence of human MSP (Yoshimura et al., 1993).

Construction of the MSP--NK2 IgG fusion protein
A partial cDNA containing the N-terminal 268 amino acids of MSP was fused to a sequence of the human IgG(1) heavy chain. This was accomplished by synthesizing complementary oligonucleotides (5'-GAT-CGCCAGATCCGAGGAATCTTGCTGAGCAGGGGAG-3' and 5'-TTGACGCTTTCTGCACTGCCGCTTGGAGA-3') which were used to link the MSP sequences through the unique BamHI site in MSP to the IgG1 heavy chain cDNA (Bennett et al., 1991). The resulting construct contained the coding sequences of amino acids 1-268 of MSP, linker sequences encoding amino acids Glu, Thr, Val and Thr, followed by the coding sequences of amino acids 216-443 of human IgG(1) heavy chain. Sequencing of the construct was carried out as described above.

Expression and purification of MSP and MSP--NK2 cDNAs encoding MSP and MSP--NK2 were inserted into the ECV-based expression plasmid pCI.ES.BON, and stable populations were transfected, and MSP-containing plasmids were established as described (Cachianes et al., 1993). Media from control cells and MSP transfectants were treated prior to use with 5% FCS for 1 h at 37°C, in order to allow proper processing of the mature two chain form (Wang et al., 1994). For MSP--NK2 purification, serum-free conditioned media from 293 cells expressing MSP--NK2 were sterile filtered and citrate buffer (pH 6) was added to give a final concentration of 100 mM citrate. All purification procedures were performed at 4°C. The media was loaded onto a HiTrap Protein A column (Pharmacia, Uppsala, S) equilibrated with 100 mM citrate (pH 6). Bound protein was eluted in 100 mM citrate, pH 6, 3.5 M MgCl2, 2% (v/v) glycerol. Each fraction was immediately buffer-exchanged by passage through a PD-10 column (Sephadex G-25) pre-equilibrated with PBS. The fractions were pooled and concentrated. Protein concentration was determined both by anti-human Fc ELISA and by total amino acid hydrolysis. The NH2-terminal sequence of the purified, mature MSP--NK2 was confirmed by protein sequencing and protein purity and integrity were assessed by silver staining of SDS--PAGE gels as well as by Western blotting using an antibody directed against the human Fe region of IgG1.

Antibodies
Phosphorysine monoclonal antibodies were from UBI (Lake Placid, NY). Met antibodies were raised as described elsewhere (Prat et al., 1990). Ron antibodies were raised in rabbits immunized with the 41 C-terminal amino acids expressed as GST fusion protein in the prokaryotic pGEX2 expression vector. The purification of the fusion protein was performed by affinity chromatography on glutathione--agarose as described (Smith and Johnson, 1988).

Tyrosine phosphorylation assays
Immunocomplex kinase assays were performed on immunoprecipitates made with Ron antibodies from untreated and vanadate treated cells (1 mM, 10 min, 37°C), by incubation in 50 µl of 25 mM HEPES pH 7.4, 5 mM Mn Cl2, 100 µM DTT, supplemented with 40 µM ATP and 10 µCi [γ-32P]ATP (specific activity 7.000 Ci/mM, Amerham, UK) for 10 min at room temperature. Proteins were separated on SDS--PAGE. Gels were alkali treated, dried and exposed for autoradiography.

In vivo tyrosine phosphorylation was evaluated by Western blot with phosphorysine antibodies of immunoprecipitates. Confluent, growth-arrested T47D cells, or COS-1 cells, 72 h after transfection, were treated with conditioned media from 293 cells, either control or MSP-transfected, or with purified MSP--NK2 at the indicated concentrations for 10 min at 37°C. Cells were lysed in RIPA buffer (20 mM Tris--HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% DOC, 100 µM Na3VO4) containing protease inhibitors, for immunoprecipitation.

Biological assays
DNA synthesis was assayed by [6-3H]thymidine incorporation. Confluent quiescent T47D cells were exposed to increasing dilutions of the conditioned media from cells transfected with either an empty vector (EMPTY) or with a vector containing the MSP or the HGF cDNA, for 24 h, then [6-3H]thymidine (28 Ci/mmol, 1 µCi/ml) was added in fresh medium and the cultures further incubated for 18 h. DNA synthesis was evaluated by [3H]thymidine incorporation into TCA-precipitable material.

Cell migration assays were performed in blind-well Boyden chambers, as previously described (Giordano et al., 1993). Briefly, cells were plated on polycarbonate filters and incubated for 6 h (A-549) or 3 h (monocytes). Increasing dilutions of conditioned media from cells transfected with either an empty vector (EMPTY), or with a vector containing the HGF or MSP cDNA, were added in the bottom chamber, for assaying chemotaxis of A-549, or in the upper chamber, for assaying stimulation of monocyte migration towards a chemotraictant (C5a) placed in the bottom chamber. Cells attached to the upper side of the filters were then mechanically removed, whilst cells which had migrated to the lower side were fixed with ethanol, stained with toluidine blue and counted.

Other analytical procedures
Northern blot analysis on tissue and cell lines was performed as described (Di Renzo et al., 1991). Metabolic labelling with [6-3H]glucose (20 Ci/mmol, 100 µCi/ml, Amerham, UK) was performed as previously described (Giordano et al., 1989b) and labelled proteins were immunoprecipitated and visualized by SDS--PAGE, fluorography and autoradiography with intensifying screens. Domain-selective cell surface biotinylation was performed as previously described (Crepaldi et al., 1994b) and biotinylated proteins were immunoprecipitated and visualized by SDS--PAGE and Western blotting with streptavidin--HRP and enhanced Chemiluminesence kit (ECL, Amersham, UK).

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