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

Giovanni Gaudino[1], Antonia Follenzi, Luigi Naldini, Chiara Collesi, Massimo Santoro, Kathleen A.Gallo[2], Paul J.Godowski[2] and Paolo M.Comoglio

Department of Biomedical Sciences and Oncology, University of Torino Medical School, 52 corso M. D'Azeglio, 10126, Torino, Italy and [2]Department of Cell Genetics, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA.

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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 ($p185^{RON}$) 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 $p185^{RON}$ 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 $p185^{RON}$ is followed by DNA synthesis. $p185^{RON}$ is not activated by HGF, nor is the HGF receptor activated by MSP in biochemical and biological assays. $p185^{RON}$ is also activated by a pure recombinant protein containing only the N-terminal two kringles of MSP. These data show that $p185^{RON}$ is a tyrosine kinase activated by MSP and that it is 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 type protein 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; Fantl *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 ($p190^{MET}$) 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 $p190^{MET}$ 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 ($p185^{RON}$), expressed in a variety of tissues, mainly of epithelial origin, and in monocytes. $p185^{RON}$ 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

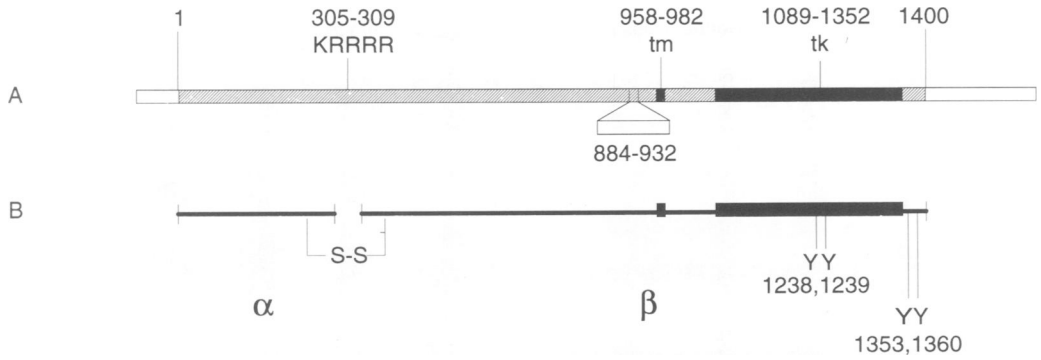

Fig. 1. (**A**) Schematic representation of the open reading frame of the *RON* cDNA, isolated from the GTL-16 cell line. The consensus sequence for the proteolytic cleavage generating the αβ-heterodimer (KRRRR), the transmembrane (tm) and the tyrosine kinase (tk) domains are indicated. The base pairs 884–932 are deleted in the variant Δ-*RON*. (**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* (Ronsin *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 (Ronsin *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 ($p185^{RON}$) when Western blots were performed under non-reducing conditions. After reduction this molecule shifted to an apparent molecular mass of 150 kDa. A second molecule with an apparent molecular mass of 170 kDa (pr170) showed the same electrophoretic mobility under both non-reducing and reducing conditions. Metabolic labelling with [^{3}H]glucosamine (Figure 4B), followed by anti-*Ron* immunoprecipitation, showed that $p185^{RON}$ is an heterodimer of two chains linked by disulfide bonds: β, of 150 kDa and α, of 35 kDa.

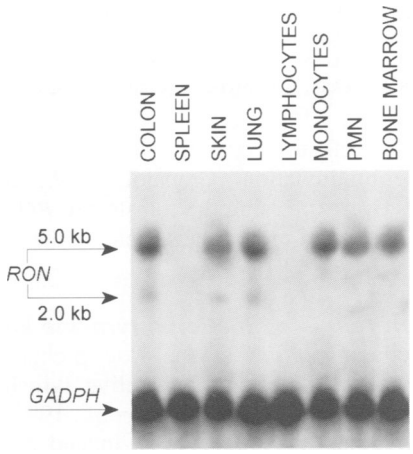

Fig. 2. Northern analysis of *RON* expression in normal human tissues. Twenty micrograms of total cellular RNA were loaded per lane. A major 5.0 kb transcript is evident and a 2.0 kb form is also detectable in most samples. A probe for the housekeeper gene glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used to evaluate the amount of mRNA transferred to filters. The Northern blot was exposed for 60 h. The molecular mass of the hybridizing bands was estimated using ribosomal RNAs as internal standards.

pr170 behaves as the immature precursor of the αβ heterodimer, as shown in time-course experiments after transfection of *RON* cDNA in COS-1 cells (Figure 5). After 24 h pr170 accumulated and was the only band detectable in Western blots by anti-*Ron* antibodies under reducing conditions. In the following hours the 150 kDa β-chain became progressively detectable as the amount of pr170 decreased. It should be noted that in these experiments the α-chain was not labelled since the antibodies recognize C-terminal epitopes localized only on the β-chain. In cells expressing physiological amounts of endogenous *Ron*, only the mature (cleaved) $p185^{RON}$ was

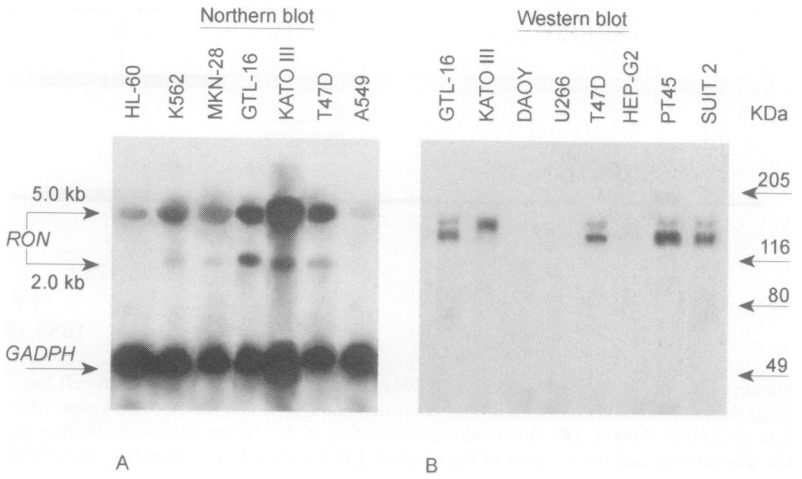

Fig. 3. Northern and Western analysis of *RON* expression. (**A**) Northern analysis of *RON* expression in a panel of assorted human cell lines (see Materials and methods). As in the case of tissues, a major 5.0 kb and a minor 2.0 kb transcript were observed. The amount of RNA transferred and the molecular mass were estimated as in Figure 2. (**B**) Western analysis on anti-*Ron* immunoprecipitates from lysates of human cell lines. Proteins were separated in reducing conditions, transferred and probed as described in Materials and methods. As described below, the major band is the *Ron* β-chain. The minor band, with higher molecular mass, is the immature single chain precursor. Positions of molecular mass markers are indicated.

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 p185^{RON}, immunoprecipitated from COS-1 cells, and native p185^{RON}, immunoprecipitated from GTL-16 cells, were incubated with [γ-^{32}P]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. p185^{RON} 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 p185^{RON}.

The Ron protein is phosphorylated on tyrosine in response to MSP

The above data show that p185^{RON} 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 p185^{RON} and MSP, we challenged cells expressing *RON* with recombinant MSP. Tyrosine phosphorylation of the p185^{RON} β-chain was assessed by Western blotting with anti-phosphotyrosine antibodies of immunoprecipitates made with specific *Ron* antiserum. The experiments were performed either on COS-1 cells, transiently expressing high levels of the recombinant protein, or on cells expressing the native protein. In *RON* transfected COS-1 cells a basal level of β-chain tyrosine phosphorylation was observed as a consequence of the overexpression (Longati *et al.*, 1994). On the other hand, in T47D mammary carcinoma cells, expressing the native p185^{RON}, the basal level of β-chain tyrosine phosphorylation was undetectable. Cells were treated with conditioned medium harvested from mock-transfected 293 cells (controls) or from cells transfected with MSP cDNA, cloned as described in Materials and methods. It should be noted

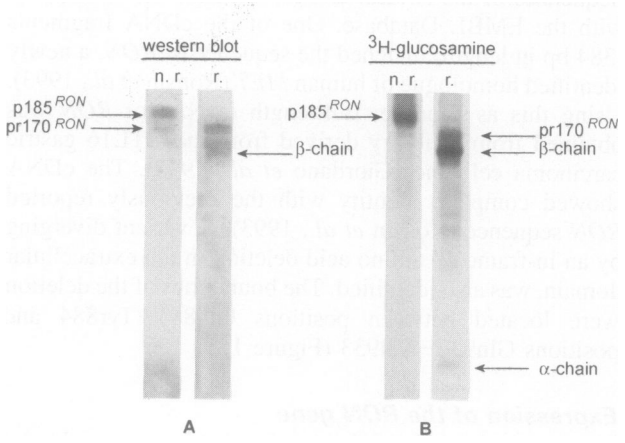

Fig. 4. Heterodimeric structure of the *Ron* protein. (**A**) Western blotting of anti-*Ron* immunoprecipitates from lysates of transfected COS-1 cells. The analysis was performed as in Figure 3B, either in non-reducing (n.r.) or in reducing (r.) conditions. (**B**) [^{3}H]Glucosamine *in vivo* labelling of *RON*-transfected COS-1 cells. Proteins eluted from immunoprecipitates made with *Ron* antibodies from metabolically labelled cells were separated by SDS–PAGE under non-reducing (n.r.) or reducing (r.) conditions. Arrows indicate the uncleaved precursor (pr170^{RON}), the mature αβ heterodimer (p185^{RON}), the 150 kDa β-chain and the 35 kDa α-chain. In the Western blot shown in (A), the α-chain is not visible, as the antibodies recognize an epitope of the β-chain.

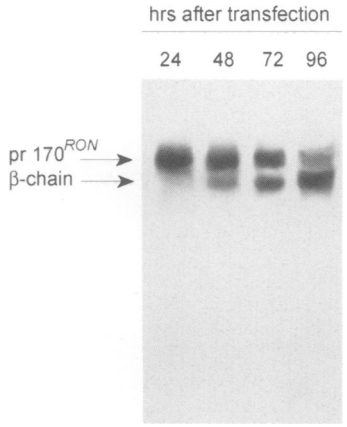

Fig. 5. Biosynthesis of the *Ron* protein. Western analysis on anti-*Ron* immunoprecipitates from *RON*-transfected COS-1 cells, lysed 24, 48, 72 or 96 h after transfection. The blot was performed as in Figure 3B. At one day after transfection only the pr170^{RON} precursor is detectable. In the following 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.

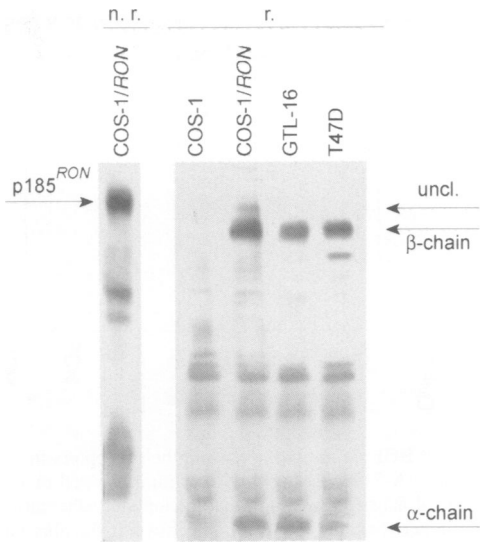

Fig. 6. Exposure of p185^{RON} at the cell surface. Mock-transfected cells (COS-1), *RON*-transfected cells (COS-1/*RON*), GTL-16 cells and T47D cells, expressing endogenous *RON*, were subjected to domain-selective cell surface biotinylation. Proteins from cell lysates were immunoprecipitated with *Ron* antibodies, separated by SDS−PAGE under non-reducing (n.r.) or reducing (r.) conditions and transferred to nitrocellulose. Biotinylated proteins were visualized by streptavidin −HRP, followed by ECL (enhanced Chemiluminescence kit). Under these conditions biotin labelled the 185 kDa heterodimer and both the 150 kDa β-chain and the α-chain (arrows). A fraction of uncleaved protein (uncl.) is labelled only in COS-1 cells overexpressing *RON*.

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 p185^{RON} β-chain in both *RON*-transfected COS-1 cells (Figure 8A) and in T47D cells (Figure 8B). The medium

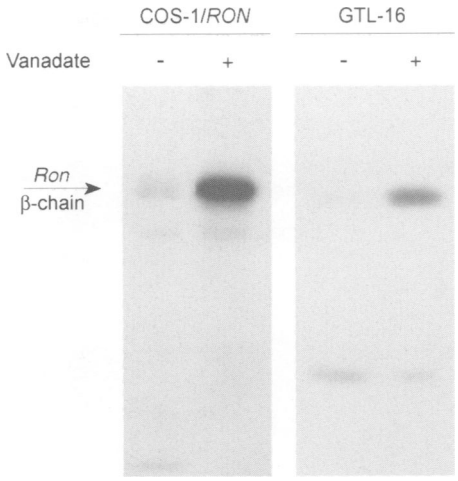

Fig. 7. *Ron* is a functional protein tyrosine kinase. Proteins from lysates of *RON*-transfected COS-1 cells or from GTL-16 cells, either treated with sodium orthovanadate (+) or untreated (−) were immunoprecipitated with *Ron* antibodies. A kinase assay was performed on immunocomplexes, as described in Materials and methods. Proteins were separated by SDS−PAGE under reducing conditions. Gels were alkali treated, dried and exposed for autoradiography with intensifying screens. A major tyrosine phosphorylated band of 150 kDa, corresponding to the β-chain of *Ron* is evident; tyrosine phosphorylation was enhanced in samples from cells pretreated with vanadate prior to extraction.

from mock-transfected 293 cells neither induced p185^{RON} phosphorylation in T47D cells nor altered the basal p185^{RON} phosphorylated status in COS-1 transfectants.

p185^{RON} is not cross-activated by HGF

Given the structural similarities between p185^{RON} 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 p185^{RON} 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).

p185^{RON} 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 p185^{RON}. Confluent and quiescent monolayers of T47D were incubated with the conditioned medium of mock- and

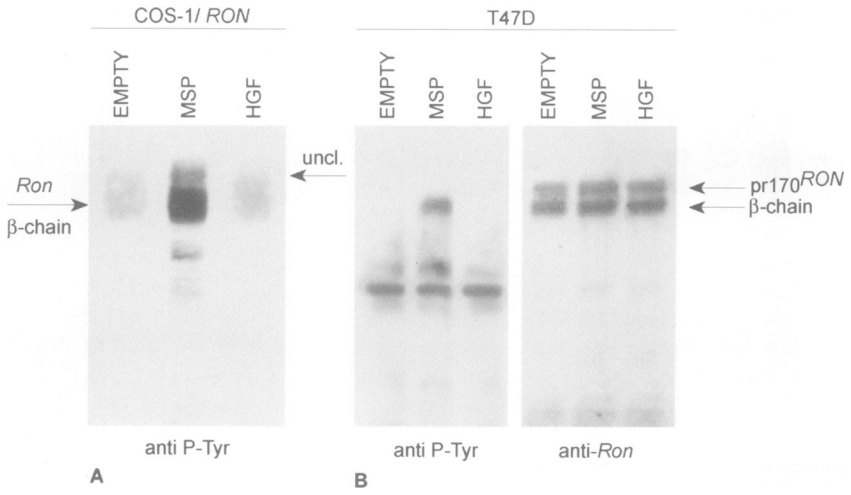

Fig. 8. *Ron* is phosphorylated on tyrosine in response to MSP. *RON*-transfected COS-1 cells (**A**) or T47D cells (**B**) were stimulated with culture media of cells transfected either with an empty vector (EMPTY) or with a vector containing MSP or HGF cDNA. Cell lysates were immunoprecipitated with *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 [^{3}H]thymidine incorporation (Figure 10). Again, no cross-reactivity was observed with HGF, which neither induced phosphorylation of p185^{RON} 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).

p185^{RON} 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 Godowski, 1993). Therefore, we tested the N-terminal region of MSP expressed as a fusion protein with a C-terminal fragment of the human IgG-γ1 heavy 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 p185^{RON}, 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).

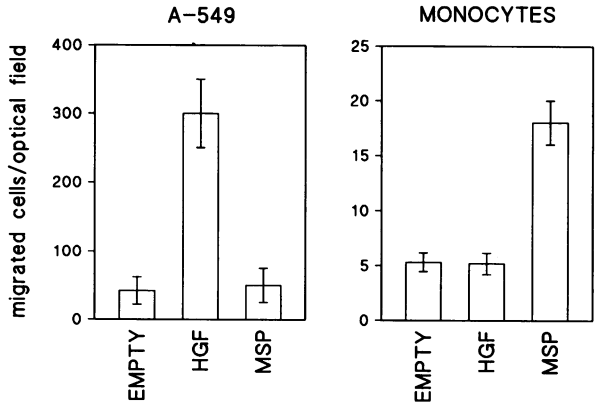

Fig. 9. MSP and HGF do not cross-activate their receptors in biological assays. A-549 cells (1.5 $\times 10^5$) or human blood monocytes, purified by Ficoll density gradient centrifugation and adherence, were plated in the upper chamber of a blind-well Boyden chamber equipped with a porous polycarbonate filter and assayed for migration. Conditioned media from cells either transfected with an empty vector (EMPTY) or containing the HGF or MSP cDNAs were tested at 1:10 dilution. For assaying the chemotactic response of A-549 cells, conditioned media were added in the bottom chamber. For assaying the stimulation of monocyte migration towards the chemoattractant (C5a) placed in the bottom chamber, conditioned media were added in the upper chamber. Both assays were performed in the presence of 5% complement-inactivated fetal calf serum. Cells which migrated to the lower side of the filter were stained and counted. Similar results were obtained with higher dilution of the culture media.

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, 1994). In several instances, the tyrosine kinase activity of the putative receptors has been demonstrated biochemically. In fewer cases the cognate ligands have been identified: Steel factor for *Kit* (Nocka *et al.*, 1990), NGF for *Trk* (Cordon-Cardo *et al.*, 1991; Kaplan *et al.*, 1991), HGF for *Met* (Bottaro *et al.*, 1991; Naldini *et al.*, 1991a) and heregulin for *HER4* (Plowman *et al.* 1993).

A putative tyrosine kinase receptor is *RON*, whose sequence has been derived from a cDNA recently cloned from a human keratinocyte cDNA library (Ronsin *et al.* 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 ($p185^{RON}$). 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), $p185^{RON}$ is synthesized as a single chain precursor ($pr170^{RON}$), 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 $p185^{RON}$ at the cell surface. As in the case of cells overexpressing *MET* (Mondino *et al.*, 1991; Naldini *et al.*, 1991a), the uncleaved $p185^{RON}$ is capable of responding to ligand stimulation.

The tyrosine kinase of $p185^{RON}$ 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 $p185^{RON}$ 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 autophosphorylation sites (Ferracini *et al.*, 1991) and are responsible for upregulation of the kinase activity in the HGF receptor (Longati *et al.*,

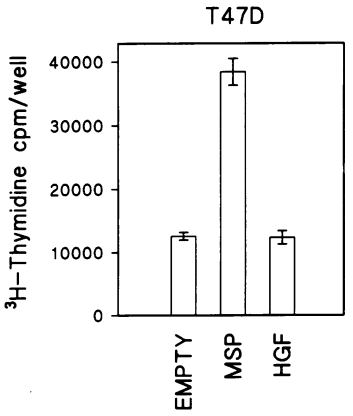

Fig. 10. MSP stimulates DNA synthesis of epithelial cells. T47D cells, cultured on 24-well plates, were incubated with conditioned medium of cells transfected either with an empty vector (EMPTY), or with a vector containing the HGF or MSP cDNA. Media were used at 1:5 dilution. ^{3}H-Thymidine incorporation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

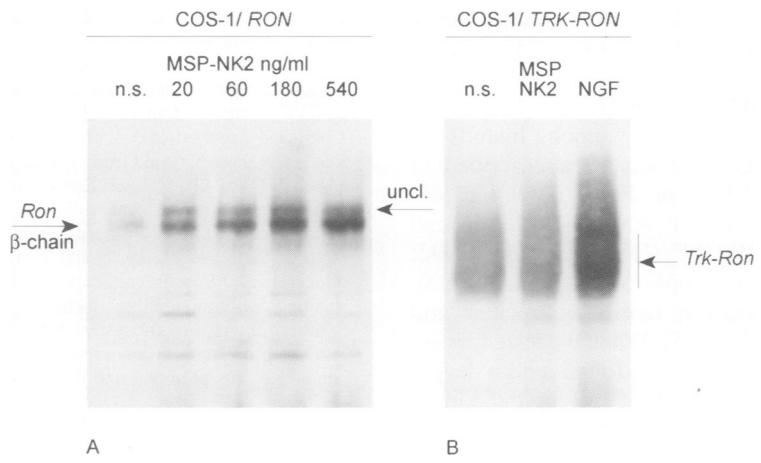

Fig. 11. The two N-terminal kringles of MSP stimulate phosphorylation of *Ron* via its extracellular domain. COS-1 cells were transfected with *RON* cDNA (**A**), or with a chimeric cDNA containing the extracellular domain of *TRK* fused with the intracellular domain of *RON* (**B**). Cells were stimulated 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 p185^{RON} 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 kringle 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; Ronsin *et al.*, 1993). Colocalization 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 p185^{RON}, whether endogenous or transfected. Moreover, MSP behaves as a mitogen in epithelial cells, where it induces p185^{RON} 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 p185^{RON} 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 p185^{RON}. 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 p185^{RON}. 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 Westermark, 1989), VEGF/VPF (Devries *et al.*, 1992; Galland *et al.*, 1993), neurotrophins (Barbacid, 1993), EGF, TGFα 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 HGF able to stimulate p185^{RON}. 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 p185^{RON}.

This paper demonstrates that p185^{RON} is a tyrosine kinase receptor responsive to MSP. The intracellular signals transduced are almost completely unknown. A striking homology between p185^{RON} 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 p185^{RON} 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, pp60$^{c\text{-}Src}$ 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 p185^{RON}, 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); SUIT2 and PT45 (pancreatic carcinoma); K562, U266 and HL-60 (hemopoietic); KATOIII (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 QPEYC 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 GTL-16 λgt11 cDNA library (Giordano *et al.*, 1993) 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′-TGCTGCTGCGGTGCCAGGTGGA-3′) and the reverse (5′-GCCACCGATCTAGAACC-TAGGAGTTCGTCCT-3′) primers for *TRK* and with the forward (5′-AGGTCCAGCGAATTCACTAAGT-

CAAGT-3′) and the reverse (5′-CCACAGTCGACGCTCCTAGGTATC-CTGC-3′) primers for *RON*. Modified *TRK* and *RON* cDNAs were recombined through the *Avr*II 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* (Giordano *et al.*, 1993) and *TRK–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 (UK). To obtain clone 5′MSP, a PCR was performed in a volume of 100 μl containing 10 μl of the RT reaction, using 1 U of Vent DNA polymerase (New England Biolabs) and 50 pmol of the forward primer (5′-CAGTGCAGCCTCCAGCCAGAA-3′) and the reverse primer (5′-CTGTACAACGCCGGATCTGGTAG-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′-AGGACGAATCCACCATGGGGTGGCTCCCACTCCTGCTGCT-TCTGACT-3′) and the nested reverse primer (5′-CCGGAATTCGAA-CTTCTGCCGGAACCCCGAC-3′). To obtain clone 3′MSP, forward primers (5′-CCGGAATTCGAACTTCTGCCGGAACCCCGAC-3′) and reverse primers (5′-ACGGAATTCCCAAGGCATATGGCATCAA-GGCT-3′) were used. The PCR products were digested with *Eco*RI, 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-γ1 heavy chain. This was accomplished by synthesizing complementary oligonucleotides (5′-GAT-CCGCAGATCGAGCGAGAATTCTGTGACCTGCCGCGGTGCGAGA CG-3′ and 5′-GTGACCGTCTCGCACCGCGGCAGGTCACAGAATT-CTCGCTCGATCTGCG-3′) which were used to link the MSP sequences through the unique *Bam*HI site in MSP to the *Bst*EII site in human IgG-g1 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 Ig-γ1 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 EBV-based expression plasmid pCIS.EBON, and stable populations of 293 cells containing these 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 pro-MSP processing to 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 $MgCl_2$, 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 Fc region of IgG1.

Antibodies

Phosphotyrosine 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 Cl_2, 100 μM DTT, supplemented with 40 μM ATP and 10 μCi [γ-^{32}P]ATP (specific activity 7.000 Ci/mM, Amersham, 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 phosphotyrosine antibodies of immunoprecipitates. Confluent, growth-arrested T47D cells, or COS-1 cells, 72 h after transfection, were treated with conditioned medium 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 Na_3VO_4) containing protease inhibitors, for immunoprecipitation.

Biological assays

DNA synthesis was assayed by [6–^{3}H]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–^{3}H]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 [^{3}H]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 chemoattractant (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 D-[6–^{3}H]glucosamine (20 Ci/mmol, 100 μCi/ml, Amersham, 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 Chemiluminescence kit (ECL, Amersham, UK).

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