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Point mutations in the tyrosine kinase domain release the oncogenic and metastatic potential of the Ron receptor

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Ron (the receptor for Macrophage Stimulating Protein) has never been implicated before in human malignancies or in cell transformation. In this report we show that Ron can acquire oncogenic potential by means of two amino acid substitutions—D1232V and M1254T—affecting highly conserved residues in the tyrosine kinase domain. The same mutations in Kit and Ret have been found associated with two human malignancies, mastocytosis and Multiple Endocrine Neoplasia type 2B (MEN2B), respectively. Both mutations caused Ron-mediated transformation of 3T3 fibroblasts and tumour formation in nude mice. Moreover, cells transformed by the oncogenic Ron mutants displayed high metastatic potential. The Ron mutant receptors were constitutively active and the catalytic efficiency of the mutated kinase was higher than that of wild-type Ron. Oncogenic Ron mutants enhanced activation of the Ras/MAPK cascade with respect to wild type Ron, without affecting the JNK/SAPK pathway. Expression of Ron mutants in 3T3 fibroblasts led to different patterns of tyrosine-phosphorylated proteins. These data show that point mutations altering catalytic properties and possibly substrate specificity of the Ron kinase may force cells toward tumorigenesis and metastasis.

Keywords: metastasis; ron; signal transduction; tumorigenesis; tyrosine kinase

Introduction

Oncogenic activation of receptor tyrosine kinases (RTKs) can occur through point mutations in the extracellular, transmembrane, and catalytic domain, resulting in ligand-independent activation. Examples of activating mutations directly affecting the structure of the TK domain are those found in the tyrosine kinases Kit and Ret. The Kit mutation, which causes human mast cell leukaemia (Furitsu *et al.*, 1993; Tsujimura *et al.*, 1994) and human mastocytosis (Longley *et al.*, 1996), consists of the substitution of a conserved aspartic acid residue located in subdomain VII of the kinase with a neutral residue (Hanks *et al.*, 1988). In the case of the Ret receptor, a genetic alteration first

identified in patients with the inherited cancer syndrome Multiple Endocrine Neoplasia type 2B (MEN2B), converts a methionine residue in subdomain VIII of the kinase into threonine (Carlson *et al.*, 1994; Hofstra *et al.*, 1994). Interestingly, the residues mutated in Kit and Ret are highly conserved among the receptor tyrosine kinases, but have different identities in the cytosolic, non-receptor tyrosine kinases (Hanks *et al.*, 1988). It has been shown that both mutations subvert the substrate specificity of the respective kinases (Santoro *et al.*, 1995; Piao and Bernstein, 1996).

Ron is the receptor for MSP (Macrophage Stimulating Protein; Gaudino *et al.*, 1994; Wang *et al.*, 1994). When activated, Ron exerts a wide spectrum of biological activities on target cells of different origin: epithelial cells (Collesi *et al.*, 1996; Medico *et al.*, 1996), keratinocytes (Wang *et al.*, 1995), neuroendocrine cells (Gaudino *et al.*, 1995; Willett *et al.*, 1997), osteoclasts (Kurihara *et al.*, 1996), and haematopoietic cells (Iwama *et al.*, 1996; Banu *et al.*, 1996). Recently it has been demonstrated that *Ron*^{-/-} mice display a deregulated inflammatory response (Correll *et al.*, 1997). Ron mediates mitogenic and apoptotic responses, as well as motility by signalling through a multi-functional docking site, conserved in the evolutionary related receptors Met and Sea (Ponzetto *et al.*, 1994; Iwama *et al.*, 1996; Wang *et al.*, 1996).

A number of RTKs are constitutively activated by gene rearrangements which cause fusion of their kinase domains with N-terminal dimerization motifs (for a review see Rodrigues and Park, 1994). The Ron homologue Met is converted into its transforming counterpart Tpr-Met, by rearrangement with Tpr sequences providing two leucine zippers (Cooper *et al.*, 1984; Park *et al.*, 1986). We have previously shown that a Tpr-Ron chimera induces constitutive activation of the Ron kinase but does not convert it into an oncogene. This is most probably related to the low intrinsic catalytic efficiency of the Ron kinase which results in lesser stimulation of MAPK/Erk 2 compared to Tpr-Met (Santoro *et al.*, 1996).

We now report that introducing single point mutations, modelled on Kit and Ret in subdomains VII and VIII of the Ron kinase, results in activation of its oncogenic potential and in triggering of a strong metastatic program. These oncogenic substitutions besides activating the Ron kinase in a constitutive way also increase its catalytic efficiency. The mutations cause enhancement of MAPK (but not of JNK) signalling and change the pattern of cellular protein phosphorylation. These observations suggest that

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naturally occurring human tumours may harbour these or similar mutations in the *Ron* gene.

Results

Mutations in the tyrosine kinase domain convert *Ron* in a transforming gene

To explore the oncogenic potential of *Ron*, we inserted in its tyrosine kinase domain single amino acid substitutions (D1232V and M1254T) reproducing the oncogenic mutations found in *Kit* (D814V) and *Ret* (M918T), respectively in human mastocytosis and in MEN2B. The substitutions were inserted by site-directed mutagenesis in the *Ron* receptor and in the constitutively active non-transforming Tpr-*Ron* chimaera (Figure 1). The mutant cDNAs were subcloned into an expression vector containing the Major Late

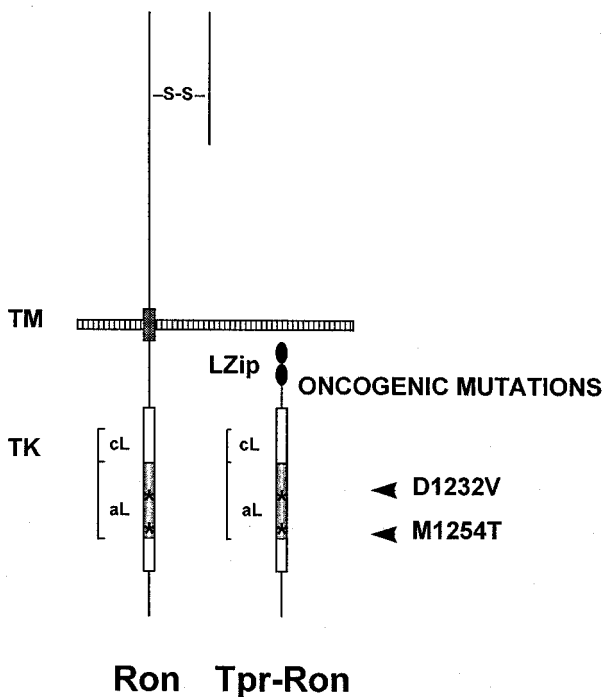


Figure 1 Schematic representation of the *Ron* receptor and the Tpr-*Ron* chimaera. The oncogenic mutations in the activation loop (shaded area) of the *Ron* tyrosine kinase domain are indicated. TM, transmembrane domain; TK, tyrosine kinase domain; LZip, two leucine zipper motifs

Adenovirus promoter and tested for *focus* forming activity in NIH3T3 fibroblasts.

While *Ron* and Tpr-*Ron* were totally ineffective on cell transformation, the D1232V and M1254T mutants were highly transforming (Table 1). The M1254T mutation induced a high number of *foci* (comparable to Tpr-Met: 830–850/10 μ g cDNA) whether it was inserted in the context of *Ron* or Tpr-*Ron*. Conversely, the D1232V mutation was less efficient in transformation when inserted in the full-size *Ron*.

Cells expressing *Ron* mutants are tumorigenic in athymic mice

To investigate the tumorigenicity of the transforming *Ron* mutants, we used stable-transfected cell lines expressing comparable levels of wild-type and mutant *Ron* and Tpr-*Ron* proteins (Figure 2a). Western blotting with phosphotyrosine antibodies revealed that while in cells expressing wild-type *Ron* there was no detectable autophosphorylation of the receptor, its mutant counterparts and all Tpr-*Ron* chimaeras were constitutively phosphorylated *in vivo* (Figure 2b).

These cells were injected subcutaneously in athymic (*nu/nu*) mice. Control cells, as well as of non-transformed cells expressing full-size *Ron*, did not induce tumour formation, within an 8 week period of observation (Table 1). In the case of Tpr-*Ron* (also non-transforming), two mice out of four developed small tumours with a long latency. In contrast, injection of cells expressing Tpr-*Ron* mutants D1232V and M1254T produced large tumours (about 20 mm in size) within 2–4 weeks in all animals tested. Consistently with their transforming ability, also the corresponding full-size *Ron* mutants (D1232V and M1254T) led to tumour formation. However in this case the latency was five weeks long. These data show that the D1232V and M1254T *Ron* mutants, besides being transforming, are also tumorigenic *in vivo*.

Point mutations increase catalytic efficiency of the *Ron* tyrosine kinase

As previously demonstrated, the catalytic efficiency of the *Ron* kinase, expressed as a ratio between V_{\max} and K_M , was inadequate to attain the signalling threshold required for oncogenic transformation (Santoro *et al.*, 1996). To evaluate the catalytic efficiency of Tpr-*Ron* mutants, we determined their kinetic parameters, V_{\max} (app) and K_M (app), for phosphorylation of the

Table 1 Transforming activity of the *Ron* and Tpr-*Ron* mutants

cDNA	Focus-forming activity (foci/10 μ g of cDNA) ^c	No. of athymic mice with tumour formation/total number of mice ^a	Onset of tumour mass (days)	Latency (weeks)
Vector	≤ 1	0/4	–	–
<i>Ron</i>	≤ 1	0/4	–	–
<i>Ron</i> D1232V	390–405	4/4	20 (3/4)	5
<i>Ron</i> M1254T	785–810	4/4	20 (4/4)	5
Tpr- <i>Ron</i>	≤ 1	2/4 ^b	30 (2/4)	>6 ^b
Tpr- <i>Ron</i> D1232V	845–855	4/4	10 (2/4)	4
Tpr- <i>Ron</i> M1254T	800–820	4/4	6 (4/4)	2

^a10⁶ cells expressing constructs were subcutaneously inoculated into the posterior flank of female *nu/nu* mice. The mice were every week checked for tumour solid formation at the site of inoculation. Animals in which tumours did not form were observed for 8 weeks. ^bThese tumours did not reach the minimum size of 20 mm at the end of the experiments. ^cTpr-Met yields 830–850 *foci*/10 μ g cDNA in this assay

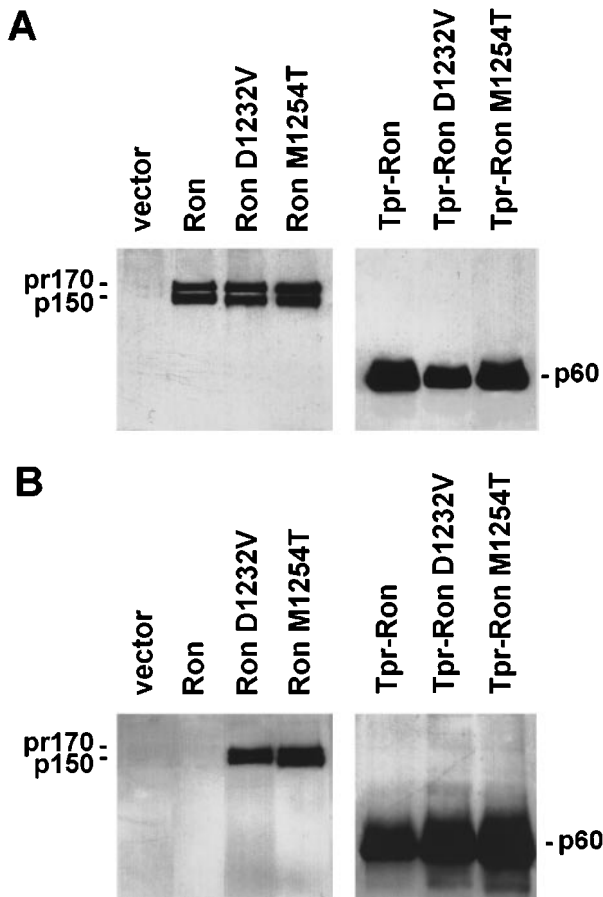


Figure 2 Expression and constitutive activation of the Ron and Tpr-Ron mutant proteins. Western analysis on anti-Ron immunoprecipitates from lysates (RIPA buffer) of the NIH3T3 cells, expressing the wild-type and mutant proteins. Proteins were separated in reducing conditions, transferred onto membranes and probed with anti-Ron antibody (a) and anti-phosphotyrosine antibody (b) as described in Materials and methods. The Ron uncleaved precursor (pr 170) and the mature form (p150) are indicated, as well as the Tpr-Ron chimaera (p60)

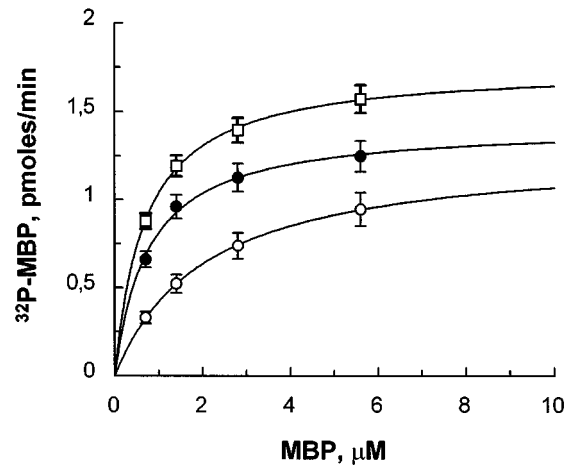
exogenous substrate MBP (Myelin Basic Protein). As shown in Figure 3, the catalytic efficiencies of the transforming mutants D1232V and M1254T were three to four times higher, compared to that of Tpr-Ron.

Interestingly, the kinetic profiles of both Tpr-Ron mutants revealed a shift to a K_M for MBP four times lower compared to that of Tpr-Ron, indicating an increase in substrate affinity (Figure 3). These data show that, in correlation with the release of its oncogenic potential, these mutations enhance the catalytic efficiency of the Ron tyrosine kinase domain, and change its affinity toward exogenous substrates.

Phosphorylation of endogenous proteins by the different oncogenic Ron mutants

To determine whether the differences in the oncogenic potential observed among wild-type and mutant Ron proteins correlated with alterations in the phosphorylation of substrates *in vivo*, we compared patterns of tyrosine phosphorylation between cell lines expressing wild-type and mutant receptors.

Immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies on lysates made with



Constructs	K_M (app) (μM)	V_{max} (app) (pmoles/min)	V_{max} (app)/ K_M (app)
Tpr-Ron	2.06 ± 0.04	1.28 ± 0.01	0.64
Tpr-Ron D1232V	0.69 ± 0.03	1.76 ± 0.02	2.55
Tpr-Ron M1254T	0.74 ± 0.08	1.43 ± 0.04	1.86

Kinase assays were carried out on immunocomplexes, in the presence of increasing concentrations of Myelin Basic Protein (MBP) and of a constant concentration of ATP, as described in the Method section. The kinase affinity for MBP [K_M (app)] and the phosphorylation maximal rate [V_{max} (app)] are indicated. The ratio V_{max} (app)/ K_M (app) represents the catalytic efficiency of the enzyme.

Figure 3 Catalytic efficiency of Tpr-Ron and Tpr-Ron mutants. Phosphorylation kinetic curves of wild-type Tpr-Ron (\circ), Tpr-Ron D1232V (\square), Tpr-Ron M1254T (\bullet) in the presence of increasing concentration of exogenous substrate (MBP, Myelin Basic Protein). Plotted values are means of triplicate determinations (error bars). The kinetic curves were calculated by using a curve-fitting program for the Michaelis-Menten equation (Grafit; Microsoft). The table indicates the kinetic parameters (V_{max} and K_M) of the mutants respect to MBP. Kinase assays were carried out on immunocomplexes, in the presence of increasing concentrations of Myelin Basic Protein (MBP) and of a constant concentration of ATP, as described in the Materials and methods section. The kinase affinity for MBP (K_M (app)) and the phosphorylation maximal rate (V_{max} (app)) are indicated. The ratio V_{max} (app)/ K_M (app) represents the catalytic efficiency of the enzyme

a buffer containing non-ionic detergent, from cells expressing the transforming mutants revealed two specific phosphoproteins, with apparent molecular masses of 100 kDa and 80 kDa. These proteins were not phosphorylated in lysates of cells expressing wild-type Tpr-Ron and Ron proteins (Figure 4a). This set of phosphorylated proteins is reminiscent of two phosphoproteins of similar molecular mass observed in fibroblasts expressing the EGF receptor carrying the MEN2B, Ret-type mutation (Pandit *et al.*, 1996). A panel of antibodies specific for known proteins in this size range, namely GAP, GAB-1, Cbl, p85, failed to react with these phosphorylated putative substrates (data not shown).

To detect the association of Ron with specific substrates, parallel experiments were performed by immunoprecipitation with anti-Ron antibodies, followed by Western blotting with anti-phosphotyrosine antibodies. In this case lysis was performed in RIPA buffer. A tyrosine-phosphorylated protein of about 95 kDa was co-immunoprecipitated in these conditions

from cells expressing the Ron and Tpr-Ron oncogenic mutants, but not in control cells and in cell expressing full-size stimulated with MSP. Interestingly, these experiments performed in RIPA buffer reveal that the Ron and Tpr-Ron mutants were constitutively phosphorylated on tyrosine but at different levels (Figure 4b).

MAPK/Erk 2 and JNK/SAPK signalling pathways are differently activated by the Ron oncogenic mutants

Cell transformation requires activation of the Ras/MAPK signalling pathway as a mandatory step (Cowley *et al.*, 1994; Mansour *et al.*, 1994), whereas the role played by JNK/SAPK is still controversial

(Raitano *et al.*, 1995; Rodrigues *et al.*, 1997). To examine whether the mutated Ron kinase activated MAPK and/or JNK signalling, we expressed in HeLa cells MAPK/Erk 2 or JNK/SAPK together with Ron, the constitutive dimerized Tpr-Ron or their mutants. MAPK/Erk 2-HA activity upon stimulation of endogenous EGF receptor and JNK-HA activity in response to anisomycin treatment were evaluated as controls.

Tpr-Ron, which is constitutively active, induced a twofold activation of MAPK/Erk 2 relative to Ron. The D1232V mutant determined the largest increase in MAPK/Erk 2 activity (fourfold as Tpr-Ron; sixfold as Ron) over the corresponding non mutated form. The M1254T mutation induced this activity to a lesser extent (Figure 5a).

Tpr-Ron induced a fourfold increase of JNK activity, compared to Ron. In contrast to the results obtained with MAPK, the D1232V and M1254T mutations did not over-stimulate JNK activity above the level stimulated by wild-type Tpr-Ron. These results indicate that the increased catalytic efficiency of the Ron tyrosine kinase conferred by these substitutions has a selective effect on the stimulation of the MAPK pathway, but does not enhance the JNK/SAPK signalling pathway (Figure 5b).

Oncogenic activation of Ron confers a metastatic phenotype

Since the introduction of activating mutations in the kinase domain of Ron provoked a strong oncogenic response in rodent fibroblasts, we investigated whether these cells were capable to colonize lung tissues by intravenous injection into the tail vein of athymic *nu/nu* mice.

Four weeks after injection of Tpr-Ron D1232V and M1254T expressing cells, mice were sacrificed because they appeared cachectic and showed respiratory distress. Similar results were obtained with the Ron D1232V and M1254T mutants, but with longer latencies (Table 2). Upon autopsy, lymph nodes were increased in size and numerous large tumours were evident in the lung of all the animals. These tumours were particularly expanded in the case of M1254T mutants, occupying almost the entire mass of the organ (Figure 6b,c). Histopathological examination of the excised sarcomas, showed the presence of abundant vascular spaces and extensive microvascular vessels formation. Typical cell whorl structures were observed, compressing the normal alveolar tissue. These tumour masses were characterized by elongated and spindle-shaped cells with large nuclei containing condensed chromatin (Figure 6d-f).

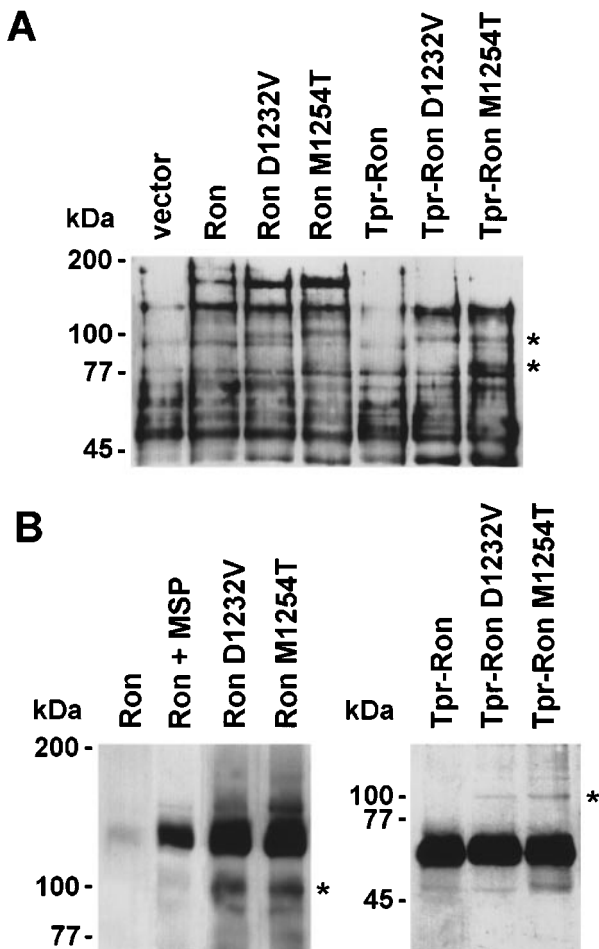


Figure 4 Tyrosine phosphorylation of endogenous proteins in NIH3T3 cells expressing the Ron and Tpr-Ron oncogenic mutants. (a) Cell lysates, made in the presence of non-ionic detergent from vanadate pre-treated cells, were analysed by immunoprecipitation and Western blotting with monoclonal anti-phosphotyrosine antibody as described in Materials and methods. (b) Co-immunoprecipitation of substrates. Cell lysates (RIPA buffer), from vanadate pre-treated cells were immunoprecipitated with anti-Ron antibody and Western blotted with phosphotyrosine antibody. Ron-expressing NIH3T3 cells (Ron) were stimulated with culture media of cells transfected with an empty vector or with a vector containing MSP cDNA (Ron + MSP; Gaudino *et al.*, 1994). Proteins extracted from cells expressing wild-type Ron and its mutants (left panel) were separated on 5% SDS-PAGE, while those from cells expressing Tpr-Ron and its mutants (right panel) were separated on 8% SDS-PAGE. Tyrosine-phosphorylated protein bands corresponding to putative Ron oncogenic substrates are indicated on the right

Discussion

Receptor tyrosine kinases (RTKs) become oncogenic through a variety of mechanisms - including over-expression, point mutations, partial deletions and gene rearrangements resulting in constitutive activation of the catalytic domain (Aaronson, 1991; Sawyers and Denny, 1994). Oncogenic conversion has been reported for the Ron homologues, Met and Sea. A rearranged form of Sea has been identified as the transforming

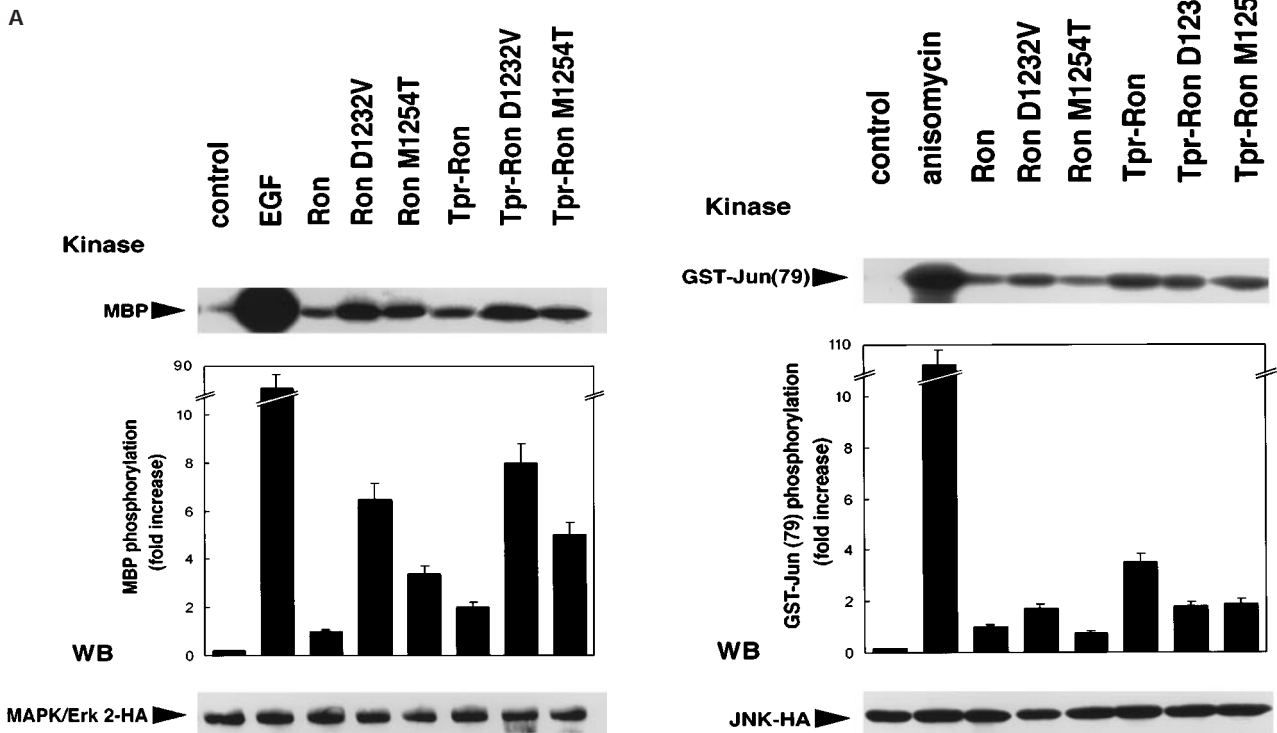


Figure 5 Effect of Ron oncogenic mutations on MAPK/Erk 2 and JNK/SAPK activity. HeLa cells were transfected with MAPK/Erk 2-HA or JNK-HA for the MAPK (a) or JNK/SAPK (b) assay, respectively, together with the empty vector or with vectors carrying wild-type or mutated proteins, as indicated. Cells were treated with 100 ng/ml EGF for 15 min (a) or with 20 µg/ml anisomycin for 20 min (b) as controls. Kinase reactions and Western blot analysis were performed on anti-HA immunoprecipitates as described in Materials and methods. Bar graph shows fold activation of MAPK (a) and JNK (b) activities in mutant transfected cells, relative to cells transfected with wild type Ron. Data represent mean ± s.d. of three independent experiments. Upper autoradiogram, showing phosphorylated exogenous substrates MBP (a) or GST-Jun(79) (b) and lower Western blot, showing the amount of immunoprecipitated HA-tagged effectors, are the results of representative experiments for MAPK and JNK assays, respectively

Table 2 Ron-mediated experimental lung colonization in athymic mice

Cell line injected	Mice with lung metastasis/ mice injected ^a	Latency (weeks)
NIH 3T3	0/4	–
Ron	0/4	–
Ron/D1232V	4/4	9
Ron/M1254T	4/4	6
Tpr-Ron	0/4	–
Tpr-Ron/D1232V	4/4	5
Tpr-Ron/M1254T	4/4	3

^aNumber of athymic mice with lung tumours/total number of mice. 10⁶ cells were injected intravenously into the tail vein of athymic nude mice, which were subsequently examined for lung colonization

component of the avian erythroblastosis S13 retrovirus (Smith *et al.*, 1989). The *Met* gene is activated by constitutive dimerization, through rearrangement with Tpr (Rodrigues and Park, 1993). To date, oncogenic counterparts of Ron have not yet been identified and no molecular alterations of the Ron receptor have been associated to human malignancies.

In the aim of identifying mutations capable of unmasking the Ron oncogenic potential, we focused our attention on two residues (aspartate in subdomain VII and methionine in subdomain VIII), highly conserved among receptor tyrosine kinases, including those of the *Met* family (Hanks and Quinn, 1991). Substitution of these residues is responsible for the

oncogenic conversion of Kit and Ret receptors (Kitayama *et al.*, 1995; Santoro *et al.*, 1995), and causes subversion of substrate specificity (Songyang and Cantley, 1995; Santoro *et al.*, 1995, Piao and Bernstein, 1996). Recently these activating mutations have been found also in *Met*, in sporadic and hereditary papillary renal carcinomas (Schmidt *et al.*, 1997; Jeffers *et al.*, 1997).

In this work we tested the effects of the D1232V ('Kit-type') or the M1254T ('Ret-type') substitutions in full-size Ron and in the constitutively active Tpr-Ron chimaera. The latter was previously shown to be non-transforming according to the canonical assays on murine fibroblasts, such as *focus* forming assay, anchorage-independent growth and low serum-cell proliferation (Santoro *et al.*, 1996). Tpr-Ron chimaeras containing either the 'Kit-type' or the 'Ret-type' substitutions were capable of inducing the formation of a number of *foci* of the same order of magnitude as the well characterized Tpr-*Met* oncogene. The same results were also obtained with the 'Ret-type' full-size Ron. However only 50% of the *foci* were induced by the 'kit-type' full-size Ron.

In vivo tumorigenesis revealed differences between the two Tpr-Ron mutants, related to the latency of tumour appearance and growth. While 'Kit-type' Tpr-Ron led to tumour formation in 4 weeks, a much shorter latency of 2 weeks was observed for the Ret-type mutant. This result is particularly striking since a

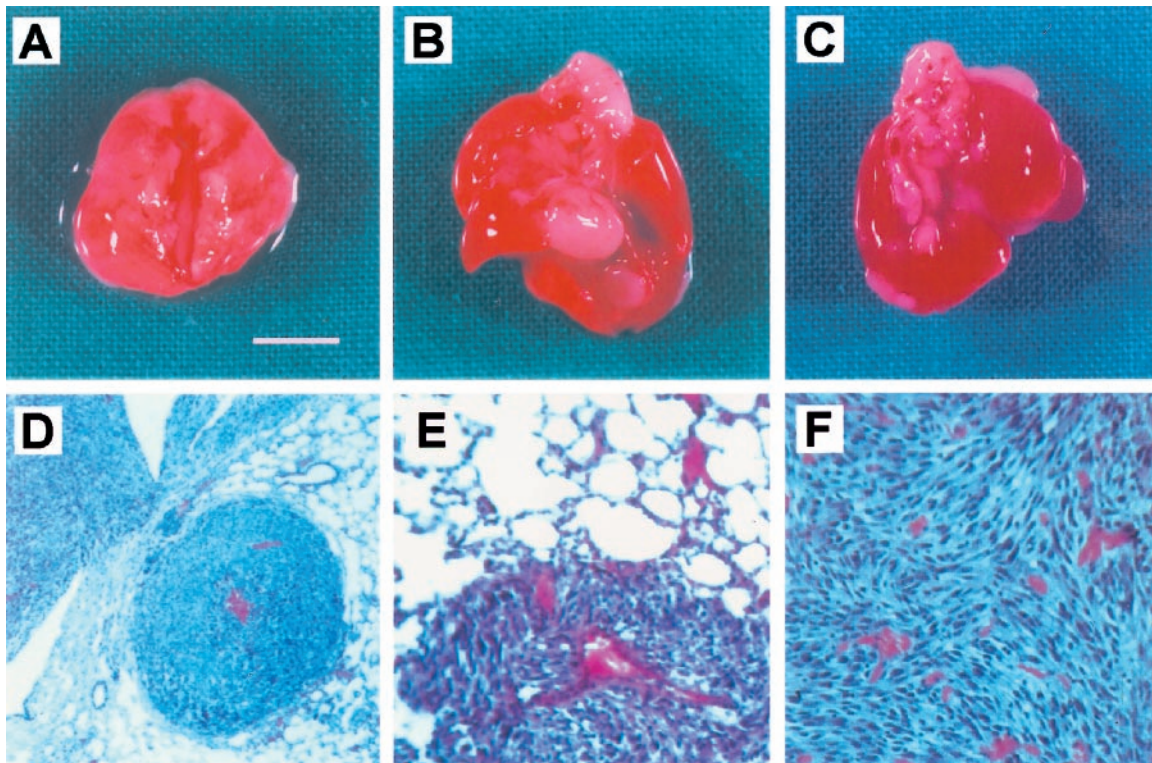


Figure 6 Histopathology of Ron-induced lung sarcomas. (a) Normal lung. (b,c) Lungs from animals injected with Ron D1232V and Ron M1254T mutants are characterized by a massive cell colonization. Scale bar: 8 mm. Lungs were paraffin-embedded, sectioned, stained with hematoxylin and eosin, and photographed. Sarcomas compress the normal alveolar tissue (d), are highly vascularized in appearance (e) and contain typical cell whorl structures (f). d and e,f were photographed at $\times 10$ and $\times 33$ respectively

latency of 3 weeks had been observed for Tpr–Met in similar experiments (Rong *et al.*, 1994).

The above mutations are more efficient in *in vivo* tumorigenesis when expressed in the constitutively active Tpr–Ron chimaera, rather than in the full-size Ron receptor, both in terms of onset of tumour mass and latency. This may be due to the signal-enhancing effect provided by the constitutive dimerization of the kinase domain. However, the oncogenic potential of the full-size receptor mutants is significant, since these may more accurately reflect possible naturally occurring mutations.

It has been demonstrated that transformation of NIH3T3 cells through the expression of activated oncogenes, like Ras, Rho or Met, is accompanied by the acquisition of metastatic properties in athymic mice (Muschel *et al.*, 1985; Rong *et al.*, 1994; Del Peso *et al.*, 1997). Here we show that oncogenic activation of Ron confers to NIH3T3 cells a metastatic phenotype. This means that mutated Ron can trigger all the events necessary to exit from vascular and/or lymphatic circulatory systems and to penetrate into the target organs, as well as cell proliferation and angiogenesis, required for colonization of the new site. Again, Tpr–Ron mutants exert their effect earlier than the corresponding Ron mutants.

Altogether these results demonstrate for the first time that Ron has the potential to transform cells and to elicit tumorigenic and metastatic programs.

RTKs mediate cell transformation when their activity is permanently stimulated. The occurrence of constitutively autophosphorylated receptor indicates

that the tyrosine kinase activity is blocked in a non-inhibitory condition, responsible for continuous downstream signalling to the nucleus (Hubbard *et al.*, 1994; Mohammadi *et al.*, 1996; Hubbard, 1997). Here we show that Ron receptor mutants are constitutively phosphorylated on tyrosine, as revealed by anti-phosphotyrosine antibodies. Furthermore, the difference observed in the level of tyrosine phosphorylation between mutants (being Ron M1254T more phosphorylated than Ron D1232V) correlates with their distinct transforming capacities.

Previous results obtained by kinetic analysis showed that the catalytic efficiency of the Ron kinase is inadequate to mediate transformation (Santoro *et al.*, 1996). The two mutations described here are very effective in shifting the kinetic parameters (V_{max} and K_M) of the Ron kinase, so that the catalytic efficiency can attain the threshold required for full transformation. Interestingly, the K_M of the kinase for the MBP substrate, widely used for measures of kinase activity, is shifted toward significantly lower values for the mutants, with respect to Tpr–Ron. This demonstrates that the oncogenic mutations alter the affinity of the Ron kinase, at least for the MBP exogenous substrate.

It has been demonstrated that the oncogenic forms of Kit and Ret kinases exert their function by altering the fidelity of signalling, by subversion from receptor-to cytosolic-specific substrates (Piao and Bernstein, 1996; Santoro *et al.*, 1995; Songyang and Cantley, 1995). Introduction of the Ret/MEN2B oncogenic mutation into the EGF receptor kinase enhanced its transforming ability and produced the same shift of

substrate specificity (Pandit *et al.*, 1996). Our data suggest that the D1232V and M1254T mutations alter substrate specificity also in the Ron receptor. The selective tyrosine phosphorylation of a co-immunoprecipitated 95 kDa protein and of the putative substrates of 80 and 100 kDa in cells expressing oncogenic Ron and Tpr-Ron, suggests that they may be positive effectors of cell transformation. Despite the efforts to characterize these putative substrates, their identity is still elusive. The attempts to these proteins are not recruited simply by functional (ligand-dependent) or constitutive (Tpr-Ron) activation of the kinase, but only upon structural alteration of the kinase by these oncogenic mutations.

Activation of the Ras/MAPK pathway has been shown to be required for transformation mediated by a number of tyrosine kinases (Stacey *et al.*, 1991). The two Ron oncogenic mutations must activate MAPK/Erk 2 activity above the threshold necessary for cell transformation. Interestingly the 'Ret-type' mutation, when inserted both in Ron and in Tpr-Ron, is less active on this pathway, than the 'Kit-type' mutation, despite its highest oncogenic potential. This suggests that other pathways may be involved in producing the biological effect.

It has been demonstrated recently that JNK activation is essential for transformation by the Tpr-Met oncogene (Rodrigues *et al.*, 1997). Our data show that non-transforming Tpr-Ron also activates the same pathway, to an extent even greater than MAPK. Surprisingly, the two oncogenic mutations seem not to influence JNK activity. This suggests that their effects may be mediated by alternative signalling due to the shift in substrate specificity, rather than to the potentiation of canonical pathways. To the best of our knowledge no other reports exist on the role played by these types of mutations on MAPK and JNK signalling.

This work reveals for the first time the tumorigenic and metastatic potential of Ron and suggests that activating point mutations in the *Ron* gene may play a role in the establishment of human cancers. The search for these mutations should be pursued in tumours derived from Ron expressing tissues.

Materials and methods

Molecular constructs

All the single point mutations were introduced by site-directed mutagenesis using the Alter Sites *In vitro* Mutagenesis System Kit (Promega Corp., Madison, WI). The cDNAs encoding for Ron (Gaudino *et al.*, 1994) and Tpr-Ron (Santoro *et al.*, 1996) were subcloned into the pAlter vector (Promega Corp., Madison, WI) and subjected to mutagenesis reaction. Mutations D1232V and M1254T were generated on *Ron* cDNA, by using the following oligonucleotides 5'-GCCCCGTCATCCTGGA-CAG-3' and 5'-TGAAGTGGACGGCGCTGG-3', respectively. Mutations were confirmed by DNA sequencing with T7 polymerase (Pharmacia, Uppsala, S) according to the dideoxynucleotide methods (Sanger *et al.*, 1977). All constructs were inserted into the pMT2 eukaryotic expression vector, under control of the Major Late Adenovirus promoter, and transiently expressed in COS-1 cells, as well as in G418-selected NIH3T3 mouse embryo fibroblasts.

Cell lines and experimental animals

NIH3T3 mouse fibroblasts, HeLa and COS-1 cells were purchased from ATCC (American Type Culture Collection). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD) and maintained at 37°C in a 5% CO₂-humidified atmosphere. NIH3T3 fibroblast stable cell lines expressing comparable levels of Ron/Tpr-Ron mutants were obtained by pools of cells isolated from *foci* of transformation. To establish cell lines expressing similar levels of the corresponding non-transforming wild type counterparts, G418-selection was used. Parallel cultures of the NIH3T3 cells stable transfected with the empty pSV2-neo vector were also established as control cell lines. The transfectants were obtained by co-transfection of each recombinant plasmid cDNA with pSV2-neo plasmid (ratio 15:1) using the calcium phosphate precipitation technique (CellPfect, Pharmacia, Uppsala, S). The following selection was performed in growth medium supplemented with 0.4 mg/ml of G418-sulfate (geneticin; GIBCO-BRL Life Technologies, Inc., Gaithersburg, MD). After 2 weeks, the neomycin resistant colonies were picked up and expanded into cell lines. Stable cell cultures expressing the respective recombinant proteins were selected by Western blot using specific Ron C-terminal antisera (Gaudino *et al.*, 1994). The mice utilized were 6 weeks old female athymic nudes (CD-1 nu BR) which were obtained from Charles River, Italy. Animal care was provided in accordance with the current national regulations regarding the protection of animals used for experiments and for other scientific purposes (Italian Ministry of Health, D.L.von. 116, 27/01/1992).

Biochemical assays

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with an ice-cold RIPA buffer containing 1 mM sodium orthovanadate and inhibitors of proteases (aprotinin, 10 µg/ml; pepstatin, 10 µg/ml; leupeptin, 50 µg/ml; phenylmethanesulfonyl fluoride, 1 mM). The cell lysates were cleared by centrifugation at 15 000 g for 15 min at 4°C; an equal amount (800 µg) of total proteins from each cell lines, determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA), were immunoprecipitated with stirring for 2 h at 4°C with the anti-Ron specific antisera absorbed to 40 µl of protein A-Sepharose 4B packed beads (Pharmacia, Uppsala, S). The immunocomplexes were washed twice with lysis buffer and proteins from immunoprecipitates were solubilized in boiling Laemmli buffer in reducing conditions. The proteins were separated on 8% SDS-PAGE and transferred to nitro-cellulose filters (Hybond, Amersham, UK). Filters were probed with the appropriate antibodies and specific binding was detected by the enhanced chemiluminescence system (ECL, Amersham, UK). Intracellular substrates tyrosine phosphorylation was evaluated by Western blotting as described above, on immunoprecipitates using phosphotyrosine monoclonal antibodies from UBI (Lake Placid, NY) or using anti-Ron specific antisera (Gaudino *et al.*, 1996). Quiescent NIH3T3 fibroblasts were pre-treated with 1 mM sodium vanadate prior to cell extraction, performed in two different conditions. For anti-phosphotyrosine immunoprecipitates, cells were lysed in a buffer containing 10 mM PIPES pH 6.8, 100 mM NaCl, 5 mM MgCl₂, 300 mM sucrose, 5 mM EGTA (DIM buffer), 1% Triton X-100, 100 µM sodium orthovanadate, 10 mM sodium pyrophosphate and inhibitors of proteases (aprotinin, 10 µg/ml; pepstatin, 10 µg/ml; leupeptin, 50 µg/ml; phenylmethanesulfonyl fluoride, 1 mM). For anti-Ron immunoprecipitates, cells were lysed in RIPA buffer, as described above.

Transforming assays

The *focus* forming assay was performed on NIH3T3 fibroblasts (5×10^5 cells) that were co-transfected with 10 μg of each recombinant plasmid and 0.8 μg of pSV2-neo, using the calcium phosphate precipitation technique (CellPfect, Pharmacia, Uppsala, S). Twenty-four hours after DNA transfection the cultures were split at low cell density into 100 mm dishes, and incubated in DMEM medium supplemented with 5% foetal calf serum. The cell cultures were maintained at confluence and screened for *foci* formation 10–18 days later. Spontaneous formation of *foci* was negligible. All the experiments were performed in triplicate. To verify the efficiency of transfection, a fraction of cells were selected in G418-containing medium. *Foci* derived cells and G418-resistant clones were established as stable lines, to verify chimaeric protein expression.

Analysis of kinetic parameters

An equal amount (10 μg) of *Tpr-Ron* and relative mutant cDNAs were transfected in COS-1 cells by the DNA-calcium phosphate co-precipitation procedure. After 60 h of transient transfection the confluent cell cultures were placed on ice, washed twice with cold PBS, and solubilized in 1 ml of ice-cold DIM buffer containing 1% Triton X-100, 100 mM sodium orthovanadate, and inhibitors of proteases (aprotinin, 1 $\mu\text{g}/\text{ml}$; pepstatin, 50 $\mu\text{g}/\text{ml}$; leupeptin, 500 $\mu\text{g}/\text{ml}$; soybean trypsin inhibitor, 500 $\mu\text{g}/\text{ml}$; phenylmethanesulfonyl fluoride, 1 mM). Cell lysates were cleared by centrifugation at 4°C for 15 min at 13 000 g. The supernatants were immunoprecipitated with Sepharose-protein A (70 ml of packed beads per ml of lysate) and with the relative Ron antibody for 3 h at 4°C with stirring. To quantify the relative amounts of protein immunoprecipitated by the same antibody from different samples, aliquots of immunocomplexes were analysed by Western blotting. After immunoprecipitation beads were washed two times with lysis buffer and two times with the kinase buffer (KB: 25 mM HEPES-NaOH pH 7.4, 5 mM MnCl_2 , 100 mM DTT). Washed immunocomplexes were divided into equal aliquots and used for the experiments. To measure the catalytic efficiency, the kinase reaction was performed in 30 μl of reaction buffer supplemented with increasing concentrations of MBP exogenous substrate (0.7, 1.4, 2.8, 5.6 μM) and of a constant concentration of ATP (100 μM). ATP concentration was adjusted to the specific activity (s.a.) of 0.5–0.6 mCi/mmol with [γ - ^{32}P]ATP (Amersham, UK). The range of ATP concentrations was determined by serial dilutions, maintaining the specific activity unchanged. The standard reaction time for exogenous substrate phosphorylation was 3 min at 4°C with continuous stirring, as determined by preliminary time-course experiments. The reactions were stopped by adding boiling Laemmli buffer, and the eluted proteins were separated on 15% SDS-PAGE followed by autoradiography at -70°C with intensifying screens. Phosphate incorporation was estimated by scintillation counting of the excised labelled bands in a Packard β -counter.

MAPK and JNK kinase assays

Subconfluent HeLa cells were co-transfected with pcDNA3-HA-JNK (Coso *et al.*, 1995) or pcDNA-HA-MAPK/Erk 2 (Crespo *et al.*, 1994) and the various cDNAs of the Ron/*Tpr-Ron* mutants using the lipofectAMINE method (GIBCO-BRL Life Technologies, Inc., Gaithersburg, MD) according to the protocol of the manufacturer. Total amount of DNA was adjusted to 2.5 μg per 35 mm plates. Twenty-four hours after transfection the cells were starved 16 h in serum-free medium and UV/stress-free conditions. The cells were then left untreated. As control

cells were stimulated with 100 ng/ml EGF for 15 min or with 20 $\mu\text{g}/\text{ml}$ anisomycin for 20 min. Cells were washed with ice-cold PBS, and lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 20 mM β -glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4 , 1 mM phenylmethanesulfonyl fluoride, 1 mg/ml aprotinin, 50 $\mu\text{g}/\text{ml}$ pepstatin, 500 $\mu\text{g}/\text{ml}$ leupeptin. Lysates were cleared by centrifugation at 13 000 g for 15 min at 4°C and the protein concentration in the supernatant was determined using the micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The epitope-tagged MAPK/Erk 2 or JNK were immunoprecipitated from aliquots (800 μg) of cell protein by incubation with the anti-HA specific monoclonal antibody 12CA5 (BABC0) and protein G Sepharose (Pharmacia) for 2 h at 4°C with stirring. Immunocomplexes were washed three times with PBS containing 1% NP-40 and 2 mM Na_3VO_4 once with 100 mM Tris-HCl (pH 7.5), 0.5 M LiCl and once with kinase reaction buffer (12.5 mM MOPS pH 7.5, 12.5 mM β -glycerophosphate, 7.5 mM MgCl_2 , 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4). The MAPK or JNK activity was determined by resuspending the immunoprecipitates in 30 μl of kinase reaction buffer containing 20 μM of unlabelled ATP, 1 μCi of [γ - ^{32}P]ATP and, as substrate, 10 μg of myelin basic protein for the MAPK assay of 2 μg of GST-c-Jun (1–79) fusion protein for the JNK assay (Coso *et al.*, 1995). After 30 min at 30°C, the reactions were stopped by adding of Laemmli's buffer and boiled for 5 min under reducing conditions. The samples were analysed by SDS electrophoresis on 12% polyacrylamide gels. Phosphorylated substrates were detected by autoradiography and quantitated using a phosphoimager (Molecular Dynamics). The values of fold increase were reported relative to MAPK/Erk 2-HA or JNK-HA protein contents of the immunocomplexes, by Western blotting with the anti-HA specific monoclonal antibody 12CA5 and densitometer analysis.

In vivo tumorigenesis and lung colonization assays

To avoid clonal variations, in our analyses we used pooled populations stable transfected NIH3T3 cells described above, rather than isolated and specific clones for the different biological assays. All cells were trypsinized, neutralized with growth media and resuspended in 0.1 ml of serum-free medium (DMEM, GIBCO-BRL Life Technologies, Inc., Gaithersburg, MD) prior to injection. In the tumorigenic assay the cells were subcutaneously inoculated into the posterior flank of athymic nude mice. The tumour formation was monitored weekly. Latency was expressed as the period of time required by tumours to reach a diameter of 15–20 mm. The animals, in which tumours were not formed, were observed for 8 weeks. For the experimental lung metastasis assay cells were injected intravenously into the tail vein of athymic *nu/nu* mice. To examine the presence of lung tumour masses the animals were sacrificed when they appeared distressed or after 12 weeks from injection. For histological examinations the lungs of tested mice were immersion fixed in formalin, embedded in paraffin and sectioned at 5 μm . The sections were stained with hematoxylin/eosin and evaluated by light microscopy for identification of tumours.

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