SHORT REPORT

The Ron oncogenic activity induced by the MEN2B-like substitution overcomes the requirement for the multifunctional docking site

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Oncogenic activation of the Ron tyrosine kinase (Macrophage Stimulating Protein receptor) relies on substitutions of two highly conserved residues in the catalytic domain (D1232V and M1254T), which result in ligand-independent activation of the receptor, *in vivo* tumorigenesis and metastasis. We show here that the Y/F conversion of the Y^{1317} residue in the kinase domain impairs tumorigenic and metastatic properties of Ron activated by the MEN2B-like mutation (Ron^{M1254T}), but not by other two oncogenic substitutions. Furthermore, Ron^{M1254T} lacking the multifunctional docking site retains transforming and metastatic activity. These data reveal that the transforming activity of Ron^{M1254T} mutant is dependent on Y^{1317} phosphorylation, suggesting a shift in intramolecular substrate specificity. Consistently, a shift of Ron^{M1254T} kinase substrate specificity was observed by *in vitro* peptide phosphorylation assays and *in vivo* receptor autophosphorylation. The Y^{1317} phosphorylation elicits by itself activation of PI-3K/Akt and MAPK signalling pathways. Our data indicate that the accomplishment of the full oncogenic phenotype of Ron^{M1254T} requires the phosphorylation both of the canonical C-terminal docking site and of the unique Y^{1317} residue in the tyrosine kinase domain. *Oncogene* (2000) 19, 5208–5211.

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Ron is the receptor for MSP (Macrophage Stimulating Protein; Gaudino *et al.*, 1994; Wang *et al.*, 1994). Upon ligand stimulation, Ron signalling occurs through association to multiple effectors, as a consequence of the phosphorylation of a unique multifunctional docking site (Y^{1353}VQLPATY^{1360}MNL), conserved in the evolutionary related Met and Sea receptors (Ponzetto *et al.*, 1994; Iwama *et al.*, 1996; Wang *et al.*, 1996).

Receptor tyrosine kinases become oncogenic through a variety of mechanisms – including overexpression, point mutations, partial deletions and gene rearrangements – resulting in constitutive activation of the catalytic domain. Examples of activating mutations, directly affecting the structure of the TK domain are those found in the tyrosine kinases Kit, Met and Ret,

associated to human mast cell leukaemia and human mastocytosis (Longley *et al.*, 1996), to HPRC (Human Papillary Renal Carcinoma; Schmidt *et al.*, 1997) and to MEN2B (Multiple Endocrine Neoplasia type 2B; Carlson *et al.*, 1994). These consist of the substitution of a conserved aspartic acid residue in subdomain VII of the kinase with a neutral residue (in Kit and Met) and of a methionine residue in subdomain VIII of the kinase into threonine (in Met and Ret). Interestingly, these residues are highly conserved among the receptor tyrosine kinases, but have different identities in the cytosolic, non-receptor tyrosine kinases. Constitutive activation of a tyrosine kinase receptor can occur also by alteration of the sequence of the transmembrane domain. This is the case of p185^{neu*}, an oncogenic form of Neu, where a V→E substitution at position 664 induces constitutive dimerization, tyrosine kinase autophosphorylation and cell transformation (Weiner *et al.*, 1989; Sternberg and Gullick, 1989).

The Ron receptor acquires oncogenic potential by means of two single amino acid substitutions (Ron^{D1232V} and Ron^{M1254T}), targeted to the above mentioned conserved residues in the activation loop of the tyrosine kinase domain (Santoro *et al.*, 1998; Williams *et al.*, 1999).

In the present investigation we addressed the molecular mechanisms responsible for cell transformation by oncogenic Ron, abrogating potentially alternative phosphorylation sites in different Ron oncogenic mutants. These were the previously described receptors carrying D1232V and M1254T substitutions respectively (Santoro *et al.*, 1998) and the Ron^{Neu*} chimaera, where the transmembrane domain is replaced by the corresponding sequence of oncogenic p185^{neu*}. A similar strategy has been used to generate a constitutive active and transforming PDGFβ/Neu* receptor (Petti *et al.*, 1998).

Sequence analysis of the Ron intracellular region identified, among several potential alternative substrates, the sequence LY^{1317}QVM that: (i) contains a very conserved tyrosine residue among the members of the receptor tyrosine kinase family (Hanks and Quinn, 1991), (ii) outlines a putative docking site for SH2-containing proteins (Songyang *et al.*, 1993), (iii) features a phosphorylation consensus for tyrosine kinases of the cytosolic type (Songyang and Cantley, 1995). This tyrosine was converted to phenylalanine in all mutationally activated Ron receptors ('single F' mutation: Y/F^{1317}). Mutants suppressing the multifunctional docking site ('double F' mutation: Y/F^{1353},
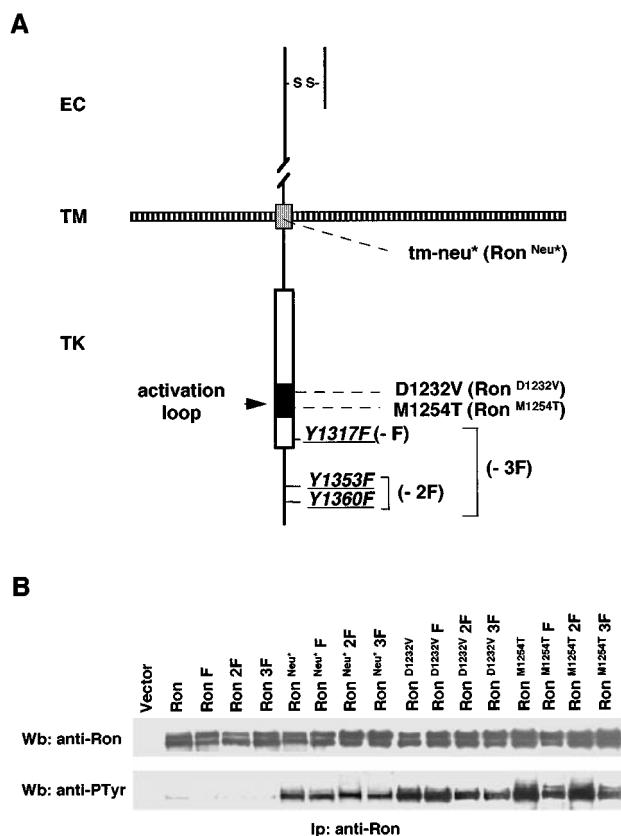
Figure 1 Schematic representation, expression and tyrosine auto-phosphorylation of the Ron mutants. (**a**) Schematic representation of the oncogenic mutations and tyrosine substitutions in the Ron receptor. In the mutant Ron^{Neu*} the native transmembrane domain was replaced by that of the oncogenic rat neu (tm-neu*). The Ron mutationally active Ron^{D1232V} and Ron^{M1254T} were generated by single point substitution in activation loop of the conserved residues D1232V and M1254T, respectively. The Y/F substitution of the critical tyrosine residues of the canonical multifunctional docking site (Y1353F and Y1360F) as well as of the putative alternative phosphorylation site (Y1317F) are indicated in underlined italic. The cDNA encoding for Ron, Ron^{D1232V} and Ron^{M1254T} have been previously described (Gaudino et al., 1994; Santoro et al., 1998). The chimeric Ron^{Neu*} receptor was generated by replacing the transmembrane (TM) domain of human Ron with that of rat p185^{neu*}, bearing the oncogenic Val/Glu^{664} substitution. PCR mutagenesis was employed to generate a unique SfiI site, used for deleting the fragment encoding the TM domain of Ron. Synthetic double-stranded oligonucleotides (sense 60 bp: 5′-TGTTCCTAATATTAGTG-GTGGT CGTTGGAATCCTAA TCCGGAGGAACAGCTAGT-TCTTC-3′; antisense 79 bp: 5′-ACCACTAATATTAGGAA-CAGCA GGACGCCCTCTACAGTTG CAATGA TGAATGTC-ACCGTGCTCTGGACCCCATCTG-3′) were inserted into the SfiI site, generating the Ron^{Neu*} chimaera. All single (F), double (2F) and triple (3F) tyrosine/phenylalanine (Y/F) point mutations were introduced by site-directed mutagenesis, using the pAlterSites in vitro Mutagenesis System (Promega). Mutations were confirmed by direct DNA cycle-sequencing (Amersham). (**b**) Expression and tyrosine phosphorylation of Ron mutants in NIH3T3 cells. All constructs were inserted in the pMT2 eukaryotic expression vector and co-transfected with pSVneo in NIH3T3 mouse embryo fibroblasts by the DNA-calcium phosphate procedure. The transfectants were selected in growth medium supplemented with 0.4 mg/ml of G418-sulphate. The panel shows Western analysis on anti-Ron immunoprecipitates from lysates of pooled NIH3T3 stable cell lines, expressing the indicated wild-type or mutated receptors, using anti-Ron antiserum (Gaudino et al., 1994) or anti-phosphotyrosine antibody (Upstate Biotechnology Inc.). Immunoprecipitation and Western blotting experiments were performed as previously described (Santoro et al., 1998)

Y/F^{1360}) were also generated as well as combined 'triple F' (Y/F^{1317}, Y/F^{1353}, Y/F^{1360}) mutants (Figure 1).

Wild-type and mutant cDNAs were tested for focus forming activity in NIH3T3 fibroblasts. As previously shown (Santoro et al., 1998), wild-type Ron was totally ineffective on cell transformation, whereas Ron^{D1232V} and Ron^{M1254T} were highly transforming. Interestingly, the Ron^{Neu*} chimaera yielded the highest number of foci (957/10 μg transfected cDNA). The transforming properties of Ron^{D1232V} and Ron^{Neu*} were completely abolished by 'double F' and 'triple F' mutations. Surprisingly, the 'double F' suppression mutant, when generated in Ron^{M1254T}, preserved the transforming ability of this receptor, although half-reduced. Conversely, the 'single F' substitution of residue Y^{1317} did not alter the focus forming activity of Ron^{Neu*} and Ron^{D1232V}, whereas in the case of Ron^{M1244T} the number of foci was significantly reduced, being 50% less. Interestingly, 'triple F' mutations, suppressing both the multifunctional docking site and the Y^{1317} site, totally abolished transforming ability of Ron^{D1232V} and Ron^{Neu*}, while a residual number of foci were induced by this mutant generated in Ron^{M1254T} (Figure 2). These data demonstrate that the M1254T substitution forces Ron^{M1254T} to become partially independent from the phosphorylation of the canonical docking site.

We have previously demonstrated that NIH3T3 cells expressing Ron^{D1232V} and Ron^{N1254T} mutants mediate metastasis formation in nude mice (Santoro et al., 1998). Similar results were obtained for Met (Jeffers et

constructs	Focus-forming activity (foci/ 10 ug of cDNA)	No. of athymic mice with metastases/total number of mice[§]	Latency (weeks)
vector	≤ 1	n.d.	-
Ron	≤ 1	n.d.	-
Ron^{Neu*}	957 ± 41	4/4	6
Ron^{Neu*} F	986 ± 45	4/4	6
Ron^{Neu*} 2F	≤ 1	0/4	-
Ron^{Neu*} 3F	≤ 1	0/3	-
Ron^{M1254T}	797 ± 35	4/4	8
Ron^{M1254T} F	406 ± 17	1/4	8
Ron^{M1254T} 2F	355 ± 15	4/4	8
Ron^{M1254T} 3F	94 ± 4	1/3	10
Ron^{D1232V}	397 ± 16	4/4	8
Ron^{D1232V} F	331 ± 14	4/4	8
Ron^{D1232V} 2F	≤ 1	0/4	-
Ron^{D1232V} 3F	≤ 1	0/3	-

Figure 2 Oncogenic and metastatic properties of the Ron mutants. Transforming activity and in vivo experimental metastasis of NIH3T3 cells expressing the mutated Ron constructs. The focus forming assays were 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 (CellPhect, Pharmacia). Twenty-four hours after DNA transfection the cultures were split at low cell density into a 100 mm dishes, and incubated in DMEM medium supplemented with 5% foetal bovine serum. The cell cultures were maintained at confluence and screened for foci formation 15–21 days later. All experiments were performed in triplicates and values represent the average ($\pm$s.d). Spontaneous formation of foci was negligible. To verify the transfection efficiency, a fraction of cells were selected in G418-containing medium. Cells derived from foci were used only to test protein expression. To avoid clonal variability, in the Ron-mediated experimental lung colonization, 10^6 cells from pooled G418-selected NIH3T3 populations expressing each type of construct, were injected intravenously into the tail vein of athymic female nu/nu mice, as previously described (Santoro et al., 1998). The animals were subsequently observed for lung metastases for next 2 months. nd: not determined

al., 1998). Thus, we investigated whether the Y→F conversion of residue 1317 could also affect lung tissue colonization induced by Ron oncogenic forms. G418-resistant NIH3T3 cells, expressing equal levels of mutationally activated Ron, carrying or not the 'Y/F' substitutions, were injected intravenously into the tail vein of athymic *nu/nu* mice, subsequently examined for evidence of lung metastases. Six weeks after injection of cells expressing both Ron[Neu*] and its Y/F[1317] mutant, severe lung metastases were present in all sacrified animals. Metastasis formation was equally observed for Ron[D1232V] and for its Y/F[1317] mutant, both after a longer latency of 8 weeks. Surprisingly, the Y/F[1317] substitution totally abolished the strong metastatic phenotype induced by Ron[M1254T], whereas suppression of multifunctional docking site in Ron[M1254T] maintained the ability to induce transformation and metastasis (Figure 2). The importance of Y[1317] is highlighted by the fact that all animals injected with cells expressing the 'double F' mutant exhibited severe lung metastases, as in the case of non mutagenized Ron[M1254T] (data not shown). These results obtained *in vivo* reveal that the M1254T mutation completely subverts the canonical docking site required for Ron-induced lung tissue colonization.

It has been suggested that the M/T substitutions in the activation loop of Ret (Songyang *et al*., 1995; Santoro *et al*., 1995), Kit (Piao *et al*., 1996) and Met (Bardelli *et al*., 1998), can induce a subversion of substrate specificity of the kinase. To investigate whether M1254T mutation could alter the Ron substrate fidelity, we performed *in vitro* phosphorylation assays on optimal peptide substrates for EGF receptor (EGFR) and for the Abl and Src cytosolic kinases, using Ron and Ron[M1254T] recombinant kinases expressed in insect cells. A marked alteration in substrate specificity was observed for the Ron[M1254T] kinase, which phosphorylated efficiently the peptides optimal substrate both for EGFR and for the cytosolic tyrosine kinase Abl. These results reveal that the M1254T substitution uniquely confers substrate specificity of the cytosolic-type to the Ron kinase, in addition to its original unaltered specificity for substrates of the receptor-type (Figure 3a). Conversely, the other two oncogenic mutations did not affect Ron substrate specificity (data not shown). This might explain why Ron[M1254T] phosphorylates both the tyrosine residues of the multifunctional docking site and Y[1317] residue, because the latter is embedded in a consensus sequence typical of cytosolic-type tyrosine kinases (Songyang and Cantley, 1995). Consistently, the level of *in vivo* tyrosine phosphorylation (receptor trans-autophosphorylation) was markedly reduced only in cells expressing Ron[M1254T] F, but not Ron[M1254T] 2F (Figure 1b). This suggests for the Y[1317] residue the role of an alternative optimal tyrosine phosphorylation site for the kinases bearing the M1254T substitution.

It has been demonstrated that the Ron signalling to cell interior requires at least activation both of the PI3-kinase/Akt and of Ras/MAPK pathways (Li *et al*., 1995; Wang *et al*., 1996; Santoro *et al*., 1996). This prompted us to investigate how mutationally activated Ron[M1254T] affects these signalling pathways in cells expressing Ron[M1254T] and its Y/F mutants. By using antibodies recognizing the phosphorylated (activated) form of Akt and MAPK, we found that
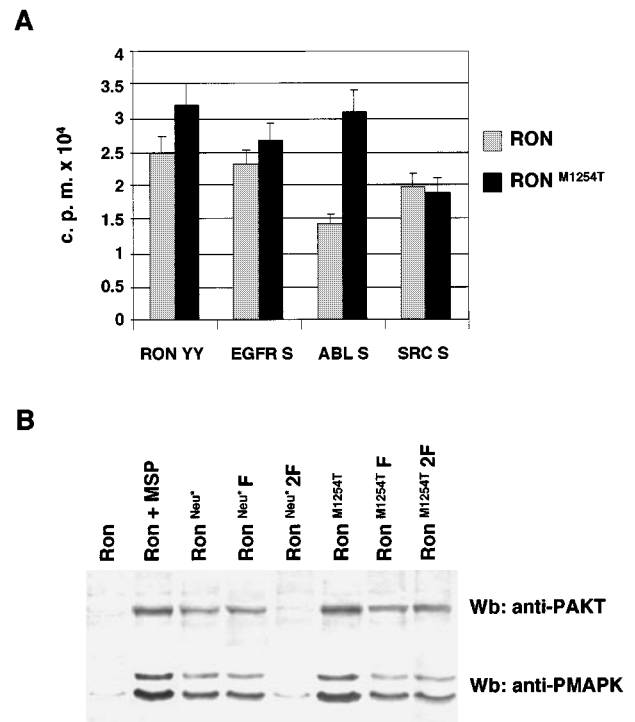
A

B

Figure 3 Peptide substrate selectivity of Ron and Ron[M1254T] kinases and Ron mutant-induced AKT/PKB and MAPK activation. (**a**) Phosphorylation assay on peptide substrates to determine the catalytic specificity of Ron and Ron[M1254T] kinases. Ron and Ron[M1254T] intracellular domain were cloned in the Baculovirus Transfer Vector pBlueBacHis2 (Invitrogen) and expressed in recombinant form using Sf9 baculovirus cells. The insect cells were lysed 72 h after infection in lysis buffer containing 1% Triton X-100, 2 mM sodium orthovanadate and inhibitors of proteases (aprotinin, 1 μg/ml; leupeptin, 500 μg/ml; phenilmethanelsulphonyl fluoride, 1 mM). The cellular extracts were immunoprecipitated with Sepharose-protein A (Amersham-Pharmacia) and with Ron C-terminal antisera, and used to phosphorylate the optimal peptide substrates for epidermal growth factor receptor (EGFRs) AEEEEYFELVAKKKK (100 μM), Src (SRCs) AEEEIYGEFEAKKK (100 μM), Abl (ABLs) EAIYAAPFAKKK (100 μM) and Ron (RONs) RDIL-DREYYSVQQHR (100 μM). The reaction was performed in the kinase buffer (25 mM HEPES, pH 7.5, 5 mM MnCl₂, 2 mM DTT) in the presence of [γ-³²P]ATP for 15 min at 4°C (Songyang *et al*., 1994). The amount of radioactivity incorporated was determined using the phosphocellulose assay (Pierce). The error bars indicate the standard deviation between two independent experiments. (**b**) Effect of Y/F substitution on oncogenic Ron-mediated activation of AKT and MAPK signalling pathways. Total cell lysate (20 μg) from NIH3T3 cells expressing the indicate mutants were analysed by SDS–PAGE and Western blotting with anti active-Akt (anti-phospho S473 Akt, New England Biolabs) or anti active-MAPK (anti-diphosphorylated MAPK, Sigma) antibodies, respectively. The cells were serum-starved for 16 h before the lysis

phosphorylation of residue Y[1317] in the Ron[M1254T] receptor devoid of a functional canonical docking site (Ron[M1254T] 2F) led to activation of Akt and MAPK effectors. On the contrary, the corresponding mutants of Ron[Neu*] and Ron[D1232V] (Ron[Neu*] 2F and Ron[D1232V] 2F) were totally inactive on these pathways (Figure 3b and data not shown). Moreover, Ron[M1254T] delivered a stronger signal that Ron[Neu*] and Ron[D1232V], possibly because of the synergy between the two phosphotyrosine motifs (Y[1317] and YY[1353,1360]) occurring only in the receptor bearing the M1254T mutation. This assumption is strengthened by the fact that abrogation of Y[1317] in Ron[M1254T] F) reduces by half the levels of Akt

and MAPK activities, in contrast to what occurs in the case of the corresponding Ron[Neu*] mutant (Ron[Neu*] F), that maintain unchanged these activities (Figure 3b).

These results show that the Ron[M1254T] F and Ron[M1254T] 2F mutants, when independently expressed, do not abrogate completely the signalling, which is maintained at appreciable levels. This is explained by a specific effect of the M1254T mutation, leading residue Y[1317] and multifunctional docking site to vicariate each other in transducing the signal triggered by Ron[M1254T] kinase. On the other hand, the M1254T mutation can enhance signalling because of the specific engagement of residue Y[1317] in addition of the conventional C-terminal docking site.

The pattern is completely different for the other two mutationally active Ron receptors, Ron[Neu*] and Ron[D1232V]. Here the abrogation of the multifunctional docking site totally suppressed Ron signalling and consequently transformation and metastasis. Moreover, in these oncogenic Ron receptors, the expression of the Y/F[1317] substitution did not affect signalling and biological activity at all.

Recently, similar results were obtained for the mutationally activated Met receptor, bearing L1213V or M1268T substitutions, that overcomes the require-ment for the phosphorylation of the multifunctional docking site. This suggests that the activating mutations stabilize an active conformation of the Met kinase, signalling through mechanisms distinct from those of wild-type Met (Jeffers and Vande Woude, 1999).

In conclusion, our results demonstrate that the Ron receptor carrying the M1254T mutation requires for the attainment of its full oncogenic program the phosphorylation of the unique Y[1317] residue as well as of the canonical C-terminal docking site. This is the first demonstration by *in vivo* assays that the MEN2B-like amino acid substitution influences the biological properties of a tyrosine kinase receptor, by altering its substrate specificity.

It will be interesting to investigate whether a similar behaviour occurs in Ret and Met receptors, that are targets of the same M/T mutation in MEN2B and HPRC neoplasia, respectively.

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