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1 Introduction

The MET proto-oncogene, encoding the Hepatocyte Growth Factor receptor, is the prototype of a gene family encoding structurally homologous heterodimeric tyrosine kinase receptors, including human RON (19) and avian SEA (8). It can be converted into an oncogene by rearrangement of the kinase domain with a N-terminal unrelated sequence designated TPR (3). The kinase activity of the encoded hybrid protein (Tpr-Met) is deregulated, since two leucine-zipper motifs present in the Tpr moiety promote its constitutive dimerization (18). This conformation mimics receptor activation following ligand binding.

The product of RON has been identified as the receptor for MSP (Macrophage Stimulating Protein; 6, 24). MSP actually exerts a wide spectrum of biological activities, mainly on epithelial, neuro-endocrine and hemopoietic cells (7, 9). Furthermore, naturally-occurring transforming counterparts of RON have not been identified. On the contrary, both the human homologue of avian Sea and its ligand are still elusive. The oncogenic form of SEA (Sarcoma, Erythroblastosis, Anaemia) has been identified as the transforming component of the Avian Erythroblastosis S13 retrovirus, by fusion of extracellular and transmembrane regions of the viral envelope protein with the SEA tyrosine kinase (21).

It has been demonstrated that ligand-stimulation of Met, Ron and Sea induces cell growth, “scattering”, and tubulogenesis (13). These pleiotropic effects are elicited by receptor activation and phosphorylation of two critical carboxy-terminal tyrosine residues embedded in the sequence, which acts as docking site for multiple SH2-containing cytoplasmic effectors. The multifunctional docking site responsible for Met signalling is conserved in the evolutionary related receptors (9, 16).

2 Results and Discussion

2.1 Constitutive activation of Met, Ron and Sea tyrosine kinases

We used a recombinant approach to obtain constitutively active Ron and Sea tyrosine kinase designed according to the structure of Tpr-Met (Fig.1A). Tpr-Ron and Tpr-Sea cDNAs were stable expressed in NIH 3T3 fibroblasts. Level of expression and tyrosine phosphorylation of the recombinant chimaeras were analysed in stable transfectants by immunoprecipitation and Western blotting. Their enzymatic activity was examined by *in vitro* autokinase assays. All chimaeras were found to be expressed at comparable levels and highly phosphorylated on tyrosine both *in vivo* and *in vitro* (Table I).

Fig. 1. (A) Schematic representation of the chimaeric proteins containing Tpr and the intracellular domains of Met, Ron and Sea. The leucine zipper motifs (LZipA and LZipB) and the receptor intracellular subdomains are indicated at the top. (B) Schematic representations of the "swapped" chimaeras in which the tyrosine kinase subdomains are exchanged between Tpr-Met and Tpr-Ron. Acronyms on the right identifies the different constructs.

2.2 Transformation is linked to the functional features of the kinase domains

Given the strong correlation between the transforming ability and the tyrosine kinase activity of the *MET* oncogene product (3), we assayed the transforming ability of the constitutively activated Met, Ron and Sea kinases. Tpr-Met, Tpr-Ron and Tpr-Sea were tested in a focus forming assay following transfection in NIH3T3 fibroblasts. Cells transfected with Tpr-Sea yielded a two fold higher frequency of foci compared with Tpr-Met. The reason of this behaviour can be explained by the presence of a duplicated Grb-2 binding site in its C-terminal multifunctional docking site (Y^{1360}VNL-X_3 -Y^{1367}VNL). According to this hypothesis, it has been demonstrated that duplication of the Grb-2 binding site in Tpr-Met causes signalling reinforcement along the Ras pathway, and enhances transformation (17). Unexpectedly, Tpr-Ron was completely unable to induce foci of transformation (Table I).

To understand the differences observed in cell transformation we "swapped" the Ron tyrosine kinase subdomain with the corresponding Met region (Fig. 1B). The recombinant proteins were all expressed with the same efficiency, equally capable of autophosphorylation on tyrosine *in vivo*, and displayed comparable kinase activities *in vitro*. The transforming potential of "swapped" Tpr-chimaeras was analysed in focus forming assays and compared with that of Tpr-Met and Tpr-Ron. All Tpr-chimaeras bearing the Ron kinase domain (Tpr-Ron and Tpr-Met.K_R) did not induce foci of transformation. Conversely, the chimaeras containing the Met kinase (Tpr-Met and Tpr-Ron.K_M) were transforming.

We ruled out that Tpr-Ron signalling involved specific effectors different from those recruited by Tpr-Met, on the basis of the experiments performed with the C-terminal "swapped" chimaeras. Surprisingly, the Ron C-terminal tail was found to be

even better than the Met tail in inducing cell transformation. The Ron tail includes the conserved multifunctional docking site (Y^{1353}VQL-X_3-Y^{1360}MNL) that *in vitro* and *in vivo* binds the same set of SH2-containing signal transducers bound by Met (Y^{1349}VHV-X_3-Y^{1356}VNV; 16). Actually, the Ron tail can recruit the Grb-2/Sos complex through Y^{1360}MNL (9).

Altogether these experiments show that the transforming ability of Tpr-Ron and Tpr-Met is linked to their kinase domains.

2.3 Catalytic efficiency of Met and Ron kinases

There are several reports showing that the oncogenic potential of a tyrosine kinase is dramatically influenced by differences in the catalytic efficiency; as in the case of the EGF receptor (15), of $pp60^{c\text{-}src}$ (10) and the proto-oncogene Neu (11). To evaluate the catalytic efficiency of Tpr-Met and Tpr-Ron we determined the kinetic parameters for tyrosine autophosphorylation and for the exogenous substrate MBP (Myelin Basic Protein) phosphorylation.

The apparent Michaelis-Menten constant [K_M (app)] of Tpr-Met and Tpr-Ron for MBP was in the same order of magnitude (1.76 ± 0.5 μM and 1.79 ± 0.3 μM, respectively). On the contrary, there is a strong difference in V_{max} between Met and Ron kinases (1.15 ± 0.07 μM and 0.24 ± 0.01 μM, respectively).

The reported data show that the catalytic efficiency of Tpr-Ron - expressed as a ratio between the V_{max} and the K_M (MBP) - is five times lower than the V_{max} of Tpr-Met (0.13 vs. 0.65 pmol/min, respectively). This suggests that catalytic efficiency is the parameter that discriminates the oncogenic potential of the two kinases.

2.4 Invasive phenotype evoked by the Tpr-chimaeras

We next investigated cell motility, and invasiveness. NIH3T3 fibroblasts expressing Tpr-Ron - despite the low efficiency of its kinase - migrated through polycarbonate filters and displayed invasive migration through the artificial basement membrane. Cell migration and matrix invasion induced by Tpr-Ron were comparable to those induced by transfection of Tpr-Met (Table I). Tpr-Ron, in spite of its weak kinase, fulfils the requirements for activating cell migration and matrix invasion, and provides a naturally occurring example of dissociation between the two arms of the biological response triggered by the Met family of receptors.

In contrast with the above, cells expressing Tpr-Sea, that displays an higher transforming ability than Tpr-Met, did not elicit a fully invasive phenotype and displayed only a modest increase in cell motility as well as in matrix invasion. Tpr-Sea docking site has two identical Y*VNL sequences, both binding Grb-2 at high affinity (22). This may prevent recruitment of the necessary amount of PI 3-Kinase for promoting motility and invasion, as demonstrated by a Tpr-Met mutant which binds two Grb-2 molecules but is lacking for binding to PI 3-Kinase. This mutant transformed host cells with higher efficiency, but was unable to trigger matrix invasion and metastasis, indicating that concomitant activation of the two pathways is necessary for the fully malignant phenotype (Giordano *et al*., 1996, submitted for publication).

2.5 Cell polarisation induced by Tpr-Chimaeras

MDCK epithelial cell line is a sensitive target for signals controlling polarised growth. These cells, when seeded in 3D collagen gels and stimulated with HGF, migrate, proliferate, and polarise into collagen matrices. This complex regulation results in the formation of branched tubular structures (14). MDCK cells expressing recombinant Tpr-Ron formed cysts developing few spikes that evolved into long and unbranching tubules. On the other hand, uncontrolled activation of Tpr-Met in these epithelial cells boosts cell proliferation, as shown by the formation of larger spherical cysts, but fails to activate the differentiative program. Also the clones expressing recombinant Tpr-Sea grew as larger spherical cysts, which never formed tubular structures (Table I). Tpr-Ron appears to be able to drive part of the morphogenetic program, inducing linear tubulogenesis but not branching, as occurred in the case of HGF stimulation in presence of TGF-β and vitronectin (20). This suggests that number and morphology of the tubule structures are influenced by the combination of both tyrosine kinase signalling and ECM receptors (2).

The Tpr-Ron.K_M chimaera did not induce tubules in MDCK cells, but formed large cysts as well, whereas the counterpart construct, Tpr-Met.K_R, led to unbranching morphogenesis as did Tpr-Ron. A potential explanation for the behaviour of the Met kinase-based constructs could be that the high level of signalling conveyed by the Met kinase, optimal to induce unrestrained proliferation, interferes with the accomplishment of the morphogenic program. On the other hand, the low signalling threshold attained by the Ron kinase seems permissive and adequate to activate at least part of the morphogenic program. This can be explained by differential activation of critical genes due to a lower dosage of transcriptional activators induced by Ron (1).

These data demonstrate that constitutive activation of Ron and Met kinases differentially induces the morphogenic program, independently from the nature of the transducing multifunctional docking site.

Table I: Biochemical and biological characterisation of the Tpr-chimaeras.

Chimaera	Kinase activity*	Density arrested growth	Transforming ability	Migration and invasion	Cell polarisation
Tpr-Met	++	-	+	+	-
Tpr-Ron	++	+	-	+	+
Tpr-Sea	++	-	++	+/-	-
Tpr-Met.K_R	++	n.d.	-	+	+
Tpr-Ron.K_M	++	n.d.	++	++	-

* Measured by *in vivo* tyrosine phosphorylation and *in vitro* autophosphorylation

2.6 MAP kinase activation by Tpr-Met, Tpr-Ron and Tpr-Sea

Cell transformation requires a strong mitogenic signal for which MAP kinase phosphorylation is a mandatory step (4). MAP kinase activation in pooled stable NIH 3T3 transfectants was analysed by phosphorylation of MBP exogenous substrate after specific immunoprecipitation. In cells expressing Tpr-Sea and Tpr-Met, the MAP kinase was activated seven and six fold over the background respectively, while expression of Tpr-Ron resulted only in a modest increase of MAP kinase activity (Fig. 2).

Fig. 2. MAP kinase activation by Tpr-Sea, Tpr-Ron and Tpr-Met. The assay was performed by measuring the amount of ^{32}P transferred to myelin basic protein (MBP) by MAP kinase immunoprecipitated with anti-$p42^{ERK2}$ antibodies. MAP kinase activation is expressed as fold increase over the background, in triplicate determinations (bars = S.D.).

MAP kinase stimulation, however, was significant and correlated with induction of cell proliferation. Cells expressing Tpr-Met or Tpr-Sea acquired a transformed behaviour characterised by unrestrained proliferation. Cells expressing Tpr-Ron were able to grow in low serum, but their growth was arrested when saturation density was reached (contact-arrested condition)(Table I).

Then we conclude that the Ron-dependent activation of the MAP kinase pathway does not lead to cell transformation, as occurs in the case of Tpr-Met and Tpr-Sea. A possible explanation is that Met and Sea kinases activate the MAP kinase pathway above a given threshold, that is not reached by the weak intensity of the Ron kinase signal, due to the relative low catalytic efficiency of its kinase. It has been demonstrated that a quantitative difference in MAP kinase activation is translated into a qualitative difference in transcription factors activation, leading to specific gene expression (12). The proliferative *vs* differentiative/morphogenetic responses are modulated by the intensity and duration of the MAP kinase activation, that results in the phosphorylation of transcription factors at various levels (5, 23). According to this interpretation, the higher threshold of MAP kinase activity induced by Tpr-Met and Tpr-Sea causes a higher nuclear concentration of transcription factors that can induce the expression of specific genes associated to cell transformation. On the contrary, the lower threshold of MAP kinase activity attained by Tpr-Ron signalling maintains a lower nuclear level of transcriptional activators, leading to expression of other critical different genes, associated to cell invasiveness and morphogenesis but not to cell transformation.

3 References

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