Uncoupling Ligand-dependent and -independent Mechanisms for Mitogen-activated Protein Kinase Activation by the Murine Ron Receptor Tyrosine Kinase

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Receptor tyrosine kinases (RTKs) activate downstream signaling through cognate growth factor receptor-induced dimerization and autophosphorylation. Overexpression of RTKs can lead to constitutive activation due to increased dimerization in the absence of ligand, and downstream signals are presumed to be the same as the ligand-induced signals. We have shown that the murine Ron (mRon) receptor tyrosine kinase exhibits constitutive activation of the MAP kinase pathway that is independent of the two docking site tyrosines, whereas activation of this pathway in response to ligand (macrophage-stimulating protein) is abolished in the absence of these tyrosines. Furthermore, we identified three tyrosines (Tyr-1175, Tyr-1265, and Tyr-1294) within the kinase domain that play critical but overlapping roles in controlling constitutive Erk activation by mRon. Phenylalanine mutations at these three tyrosines results in a receptor that fails to constitutively activate the Erk pathway but retains the ability to induce Erk phosphorylation in response to ligand stimulation. The ability of mRon to activate the MAP kinase pathway is dependent on c-Src activity, and we have shown that c-Src co-immunoprecipitates with mRon. c-Src fails to interact with mRon when the three tyrosines required for MAP kinase activation are mutated, whereas the presence of any one of these tyrosines alone restores Erk phosphorylation and recruitment of c-Src. Thus, the ligand-dependent and -independent activity of mRon can be uncoupled through the alteration of selective sets of tyrosines.

Polypeptide growth factors and their cognate receptor tyrosine kinases (RTKs) are essential for regulation of homeostasis and animal development. Deregelation of RTKs is involved in various pathogenic conditions, including tumorigenesis (1). RTK overexpression has been detected in several types of tumors in which high concentrations of the receptor on the cell surface allows homo-oligomerization of the receptor to occur in the absence of ligand. Alternatively, RTKs can be constitutively activated upon fusion with a dimerization domain (2), or mutations in the extracellular or juxtamembrane domain, all of which can confer constitutive kinase activation by promoting receptor oligomerization (3–6). In principle, these events and ligand stimulation activate RTKs through a common mechanism, receptor dimerization and concomitant tyrosine phosphorylation, leading to the enhancement of kinase activity and creation of a common assembly platform for downstream signaling partners. Alternatively, activating mutations in the kinase domain of RTKs, have been demonstrated to alter substrate specificity of the receptor, leading to induction of a distinct subset of signals culminating in transformation (7).

Overexpression of the Met/Ron family of RTKs has been implicated in the progression of a variety of tumors, mostly of epithelial origin. Met was originally isolated as a fusion partner with Tpr, resulting in transformation of a human osteogenic sarcoma cell line (8), and this fusion was demonstrated to result in constitutive tyrosine phosphorylation of the Met receptor (9). Furthermore, germ line point mutations in the kinase domain of Met resulting in constitutive activation of the receptor are primarily responsible for hereditary papillary renal carcinoma (10). Alternatively spliced forms in the extracellular domain of the related Ron receptor, resulting in constitutive activation of the receptor, have been isolated from patients with colon cancer (11), and through knock-out studies, murine Ron has been implicated in the progression of skin and lung epithelial malignancies (12, 13). In addition, murine and chicken Ron (also called STK and SEA, respectively), have been adapted by viruses to drive the progression of erythroleukemia (14). In the case of murine Ron, a naturally occurring truncated form of the receptor interacts in situ with the viral glycoprotein, gp55, resulting in stabilization and constitutive activation of the receptor (15). Alternatively, c-SEA is the cellular proto-oncogene of the avian erythroblastosis virus, S13, which causes erythroleukemia and sarcomas in chickens (16).

Although signaling through this family of receptors is highly conserved, it remains to be determined whether activation of the Met/Ron receptor through various mechanisms results in the induction of the same signaling events. The potential diversity of RTK-mediated signaling is underscored by the ability of these receptors to phosphorylate a large repertoire of docking sites for downstream signaling proteins, various combinations of which could be exploited by the receptor to produce diverse biological outcomes. However, the Met/Ron family of RTKs generates signals sufficient to induce complicated biological outcomes through two phosphorylation sites on the carboxyl tail (17), supported by the observation that knock-in animals bearing mutations at these two sites have the same lethal phenotype as the complete null model (18). It was subsequently shown that the Met C-terminal tail mediates recruitment of the multifunctional scaffolding protein, Gab1 (19), through a unique Met binding domain in Gab1. Gab1 contains a number of potential phosphorylation sites, coupling Met to downstream signaling pathways through interaction with various adaptors, including Grb2, p85, Shp2, and CRKL, to mediate biological responses (20, 21). RTKs can also recruit the Gab family of scaffolding proteins through the unique C-terminal SH3 domain of the Grb2 adap-
tor protein (22), and the recruitment of Gab2 to murine Ron (STK) and chicken SEA is required for transformation by these receptors (23).3

Binding of Grb2 to the docking site tyrosines of the Met/Ron family of RTKs plays a critical role in the induction of cellular transformation by these receptors (24–26). Grb2-mediated activation of MAP kinase is a highly conserved mitogenic pathway in which the small GDP/GTP exchange factor, SOS, which is constitutively associated with the N-terminal Grb2 SH3 domain, is recruited to the vicinity of the cell membrane through interaction with the phosphorylated receptor. This results in the activation of its membrane target, the small GTPase, Ras, leading to the classic cascade initiated by Raf. Alternatively, recruitment of Gab1 to the Met receptor results in sustained activation of the MAP kinase pathway through recruitment of the tyrosine phosphatase, Shp2 (27). Although the mechanism by which Shp2 leads to MAP kinase activation is currently unclear, dephosphorylation of RasGAP binding sites in the receptor, as well as the dephosphorylation of the inhibitory tyrosine of Src family kinases, have been proposed (28). Here, we demonstrate that overexpression of the murine Ron receptor induces Erk-mediated signals through a mechanism distinct from the activation of this pathway following ligand stimulation. We show that, although ligand-induced Erk activation requires the two docking site tyrosines of mRon, ligand-independent activation of this same pathway does not require the docking site tyrosines or the C-terminal tail, but rather is dependent upon three tyrosines that reside in the kinase domain of mRon, and is associated with co-immunoprecipitation of the receptor with c-Src. These data indicate that the ligand-dependent and -independent signals emanating from mRon are initiated by distinct subsets of tyrosines in the receptor, and have broad implications for the study of RTK signaling.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Reagents—HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Antibodies against phospho-Erk, Erk, and HA epitope (262K) were purchased from Cell Signaling. Antibodies against Erk and phospho-tyrosine (4G10) were purchased from Upstate Biotechnology, and antibodies for c-Fos, Grb2 (c-23), and c-Src (N-16) were from Santa Cruz Biotechnology. Phospho-specific antibodies to the activation loop of Ron were kindly provided by Dr. Jiong Wu (Cell Signaling). Anti-serum against mRon was raised against the last 15 amino acids of the carboxyl tail. Recombinant macrophage stimulating protein (MSP) was from R&D Systems.

Gene Construction and Mutagenesis—A luciferase reporter plasmid containing an AP1 consensus binding site, wild-type c-Src CDNA, and the rat phospholipase Cγ1 gene was kindly provided by Dr. Avery August (The Pennsylvania State University). The c-Src dominant negative mutant (K296R/Y528F) was purchased from Upstate Biotechnology and subcloned into the HindIII site of pCDNA3. Mouse Ron is expressed in the PCI-neo mammalian expression vector or the MSCV retroviral vector. Point mutagenesis and C-terminal truncation were performed by PCR with the QuikChange site-directed mutagenesis kit (Stratagene). Primers for point mutagenesis: Y1074F, 5′-ctttggtgcttcagcgaatctac-3′; Y1078F, 5′-cttacacgaggaattacagcagc-3′; K1091M, 5′-ccatgtgcctcatcgtctgagg-3′; Y1140F, 5′-ctttggtgcttcagcgaatctac-3′; Y1175, 5′-ctttggtgcttcagcgaatctac-3′; Y1215F, 5′-ctctagacaaggtctctcgcctgtc-3′; Y1216F, 5′-ctctagacaaggtctctcgcctgtc-3′. These blunt fragments were terminally phosphorylated then cloned into the EcoRV site of pCDNA3.1. HindIII and NotI were used to excise the fragments that were then ligated into pCDNA3.1 containing the tandem HA sequence digested with HindIII and NotI. All point mutants and tagged constructs were sequenced to confirm the absence of additional mutations arising from PCR elongation.

Cell Transfection and Luciferase Assays—For the luciferase assay, 5 × 104 HEK 293 cells/well were seeded into 24-well plates. The next day, a mixture of 0.2 μg of wild-type or mutant forms of mRon and 2 ng of AP-1 luciferase reporter plasmid was used for transient transfection with the FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s protocol. For experiments using wild-type or dominant negative c-Src, combinations of 0.15 μg of c-Src or control vector, 50 ng of mRon or its derivatives, and 0.5 ng of AP-1 were used for transfection. Cells were stimulated with 200 ng/ml MSP immediately after the DNA complex was added to the culture medium. 24–48 h later, luciferase assays were performed according to the manufacturer’s instructions (Promega). For the immunoprecipitation assays, 4 × 105 cells/well were seeded into 6-well plates. The next day cells were transfected with 0.5 μg of c-Src or its mutant as described above.

Immunoprecipitation and Western Blot Analysis—40 h following transfection, cells in 6-well plates were suspended in 600 μl of cell lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycero-phosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 100 mM NaF, 4 μg/ml aprotinin, and 2 μg/ml leupeptin. Cell lysates were centrifuged at 15,000 rpm for 15 min, and 300 μl of supernatant was used for immunoprecipitation. Briefly, cell lysates were incubated with the appropriate primary antibody at 4 °C overnight, then 20 μl of protein A or protein G (Santa Cruz Biotechnology) plus beads were added to the resultant immunocomplex for another 2–4 h. Beads were rigorously washed at 4 °C with a 1:1 solution of cell lysis buffer and 1 M lithium chloride three times. Beads were resuspended in 10 μl of SDS sample buffer, and denatured proteins were resolved on an SDS-PAGE gel and then transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in TBST buffer (TBS plus 0.1% Tween 20) for 1 h and probed with the appropriate primary antibody at 4 °C overnight. Membranes were washed with TBST three times and incubated with goat horseradish peroxidase-conjugated secondary antibody against mouse or rabbit IgG (Sigma). Blots were visualized using the enhanced chemiluminescence system (Pierce or Amersham Biosciences). For re-probing, membranes were stripped with Tris-HCl 62.5 mM, pH 6.8, SDS 2%, and β-mercaptoethanol 0.7% at 50 °C for 30 min.

Retrovirus Preparation and In Vivo Infection of Primary Bone Marrow Cells—Total bone marrow cells from mRon−/− mice were harvested and infected with viral supernatant from 293 cells transiently co-transfected with MSCV-based retroviral vectors expressing the indicated receptors under the transcriptional regulation of the viral long terminal repeat, along with pECO and VSVG envelope protein-encoding constructs in the presence of 2.5 ng/ml interleukin-3, as previously described (25). Cells were then plated in the Methocult M3234 (Stem Cell Technologies) containing interleukin-3 (2.5 ng/ml, Peprotech) in

3 H. E. Teal, J. Xu, S. Ni, L. D. Finkelstein, A. C. Cheng, R. F. Paulson, G. S. Ferg, and P. H. Correll, submitted for publication.
triplicate. Cultures were incubated for 2–8 days in 5% CO₂ at 37 °C. Epo-independent erythroid colonies (BFU-E) were visualized by acid-benzidine staining as previously described at day 8.

RESULTS

Murine Ron Induces Activation of the MAP Kinase Signaling Pathway in a Kinase-dependent, Ligand-independent Manner—Constitutive activity of RTKs has frequently been observed in a variety of tumors as well as in overexpression systems that are often utilized to study receptor signaling. To study signaling through the Ron receptor tyrosine kinase, we expressed murine Ron in 293 cells and examined the ability of the receptor to activate downstream signaling events. We found that transfection of 293 cells with mRon resulted in a robust activation of the MAP kinase pathway as determined by phosphorylation of Erk, up-regulation of c-Fos expression, and induction of AP1 activity and that this constitutive activity absolutely required the kinase activity of the receptor as determined by transfecting the cells with the kinase-dead mutant, mRonK1091M (Fig. 1, A and B). Interestingly, we failed to detect a similar up-regulation of the phosphatidylinositol 3-kinase pathway under these conditions as measured by Akt activity in an in vitro kinase assay (data not shown). We also observed similar activation of AP-1 in murine NIH3T3 cells transfected with mRon (data not shown). These data indicate that expression of mRon in the absence of ligand results in the selective up-regulation of the MAP kinase signaling pathway.

To determine whether this constitutive activity is maintained at lower levels of receptor expression, we generated a C-terminally HA-tagged mRon and titrated out the amount of plasmid transfected into the 293 cells. Ron expression levels as detected by anti-HA were compared with the ability of the receptor to activate downstream signaling events. We found that even at levels of receptor expression barely detectable by Western blot analysis, Erk phosphorylation and c-Fos expression were induced (Fig. 1C). To determine whether mRon was constitutively phosphorylated in these cells, we immunoprecipitated cell lysates with anti-HA and blotted with anti-phospho-tyrosine, stripped, and reprobed with anti-HA to examine the phosphorylation status of the receptor.

FIGURE 1. Constitutive activation of the MAP kinase pathway by mRon is dependent on kinase activity and independent of the docking site tyrosines. A, HEK 293 cells were transiently co-transfected with WT or mutant forms of murine Ron and an AP1 luciferase reporter construct. Cell lysates were immunoblotted for phosphorylated Erk, total Erk, c-fos, and mRon. B, AP1 luciferase activity in the lysates from A. C, 293 cells were transiently transfected with a C-terminally HA-tagged mRon at the indicated concentrations. Cell lysates were immunoblotted for HA (mRon), phosphorylated Erk, total Erk, and c-fos. D, lysates from C were immunoprecipitated with anti-HA and blotted with anti-phospho-tyrosine, stripped, and reprobed with anti-HA to examine the phosphorylation status of the receptor.
The Docking Site Tyrosines (Tyr-1330/1337) of Murine Ron Are Required for MSP-dependent MAP Kinase Activation but Dispensable for Ligand-independent Activation of This Pathway—A signature motif of the Met/Ron family of RTKs is the presence of two conserved docking site tyrosines separated by six residues on the carboxyl tail. To determine the effect of these tyrosines on the activation of the MAP kinase pathway by mRon, we mutated Tyr-1330 and Tyr-1337 individually and in combination and co-transfected the mutant receptors and an AP1 luciferase reporter into HEK293 cells. We found that the ligand-independent phosphorylation of Erk and up-regulation of c-Fos is unaffected by mutation of the docking site tyrosines, both singly and in combination (Fig. 1A). This activity is reflected in the ability of the mutant receptors to up-regulate AP1 induced transcription as measured by luciferase activity (Fig. 1B).

A recent study demonstrated that the Ron carboxyl tail also provides phosphorylated serines as docking sites for 14–3-3 proteins, and this pathway is implicated in Ron-integrin cross-talk during epidermal wound healing (29). Therefore, we also mutated the two putative 14–3-3 binding sites, Ser-1366 and Ser-1372 to alanine, and results from expression of these mutants in 293 cells indicated that these serines are not responsible for the ligand-independent activation of the MAP kinase pathway by mRon (data not shown). In addition, we generated a series of C-terminal deletion constructs in which 16, 36, 42, 49, and 60 amino acids were progressively deleted. All of these mutants retained the ability to activate the MAP kinase pathway in the absence of ligand except for the final deletion, which extends into the kinase domain (data not shown). The same experiment was performed with mRon and the combined docking site mutant mRonY1330F/Y1337F in the presence or absence of MSP. While MSP stimulation resulted in enhanced Erk phosphorylation and c-Fos expression from the wild-type receptor, mRonY1330F/Y1337F failed to respond to MSP by further up-regulating this pathway (Fig. 2A). The inability of the docking site mutant to stimulate Erk phosphorylation in response to MSP was reflected in the absence of further up-regulation of AP1 activity under these conditions (Fig. 2B). Taken together, these data indicate that an intact kinase domain, but not the docking site tyrosines or other motifs in the C-terminal tail, is required for the constitutive activation of MAP kinase by mRon. However, the docking site tyrosines are essential for the activation of this pathway by mRon in response to ligand stimulation.

Identification of Tyrosines within the Kinase Domain Critical for the Constitutive Activation of the MAP Kinase Pathway by mRon—Our data thus far have shown that the ability of mRon to activate the MAP kinase pathway in a ligand-independent manner is independent of the carboxyl tail, but requires the integrity of the kinase domain. There are ten tyrosines in the kinase domain of mRon in addition to the two docking site tyrosines in the C-terminal tail. Although specificity of SH2 domain recruitment is largely dictated by the composition of the three to five residues on the carboxyl terminus of the phospho-tyrosine, hydrophobicity at the pY+3 positions seems to be ubiquitously essential for module recognition. Tyr-1265 and Tyr-1294 in the C lobe of the kinase domain, as well as the docking site tyrosines, match the minimum requirement for SH2 binding. (Indicated by boxes in Fig. 3A.) However, we found that mutation of all four of these tyrosines (referred to as mRon Y4F) has no significant effect on the phosphorylation of Erk by mRon (Fig. 3B, lanes 1–4). Therefore, we addressed the role of the other tyrosines by individually mutating them to phenylalanine in the context of mRon Y4F. As expected, mutation of the activation loop tyrosine (Y1215F/Y1216F) completely abrogated receptor-mediated Erk activation by inactivating catalytic activity, as demonstrated by immunoprecipitation with mRon antiserum followed by immunoblot with anti-pTyr (data not shown).

Of the remaining six tyrosines, mutation of Tyr-1078 and Tyr-1140 in the context of mRon Y4F resulted in mild reduction of receptor-mediated Erk phosphorylation, whereas mutation of Tyr-1175 severely impaired activation of this pathway (Fig. 3B). However, only mutation of Tyr-1175 in the context of mRon4F, but not Tyr-1078 or Tyr-1440, was determined to be a critical residue for the activation of the AP1 transcriptional response (Fig. 3C). Tyr-1175 lies in the conserved αE region of the C lobe, raising the possibility that mutation of this tyrosine abolishes receptor-mediated Erk activation by inhibiting catalytic activity. Therefore, we compared the autophosphorylation of HA-tagged mRon4F alone or in the presence of T1078F, T1440F, or T1175F by immunoprecipitation with anti-HA and Western blot analysis with anti-pTyr. Although mutation of Tyr-1175 in the context of mRon4F resulted in reduced receptor phosphorylation, phosphorylation of mRon was not abrogated (Fig. 3D). In addition, mutation of T1175F in the context of the wild-type receptor did not inhibit the ability of the receptor to induce Erk phosphorylation (Fig. 3E). Therefore, regulation of kinase activity is not likely to be the sole mechanism by which Tyr-1175 regulates receptor-mediated MAP kinase signaling.

The Ligand-dependent and -independent Activities of the mRon Receptor Can Be Uncoupled Both Biochemically and Functionally—We have shown thus far that mRonY4F plus Tyr-1175F (Now called Y5F) fails to induce constitutive activation of the MAP kinase pathway. Here we set out to determine the role, if any, of the docking site tyrosines in the context of this mutant. Therefore, we added back Tyr-1330/1337 in the context of Y5F, resulting in a receptor containing the three kinase

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**FIGURE 2.** The docking site tyrosines are required for the induction of the MAP kinase pathway by mRon in response to ligand stimulation. A, 293 cells were transiently co-transfected with mRon or mRon Y1330F/Y1337F and an AP1 luciferase reporter construct in the presence or absence of MSP. Lysates were immunoblot with anti-phosphorylated Erk, total Erk, c-Fos, and mRon. B, AP1 luciferase activity in lysates from A.
mutations Y1265F/Y1294F/Y1175F. Transfection of 293 cells demonstrated that this mutant still lacked the ability to induce ligand-independent phosphorylation of Erk and up-regulation of c-Fos expression. However, upon ligand stimulation, this receptor was fully capable of inducing activation of this pathway (Fig. 4A). In addition, MSP stimulation of these cells resulted in increased phosphorylation of the receptor as determined by immunoprecipitation with anti-HA and blotting with anti-pTyr or phospho-specific antibody to the tyrosines in the activation loop (Fig. 4B). Thus, we are able to distinguish the tyrosines responsible for ligand-dependent activation of MAP kinase (Tyr-1330/1337) from the tyrosines involved in the constitutive induction of this pathway by mRon (Tyr-1265/1294/1275).

To determine if this uncoupling translates to a functional response by the receptor, we generated retroviruses expressing the wild-type and
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FIGURE 4. Tyrosines 1175, 1265, and 1294 are not required for ligand-induced activation of the MAP kinase pathway. A, 293 cells were transiently transfected with mRon containing tyrosine to phenylalanine mutations at positions 1175, 1265, and 1294 in the presence or absence of MSP. Cell lysates were immunoblotted for phosphorylated Erk, total Erk, c-Fos, and mRon. B, 293 cells were transiently transfected with the receptor from A in the presence or absence of MSP, and phosphorylation of the receptor was assessed following immunoprecipitation for mRon and blotting with anti-phospho-tyrosine. The blot was stripped and reprobed for mRon.

FIGURE 5. The docking site tyrosines are required for ligand-dependent induction of erythroid colony formation by mRon, whereas tyrosines 1175, 1265, and 1294 are required for ligand-independent colony growth. Bone marrow was harvested from the fibias and tibias of mRon knock-out mice and transduced with retroviral vectors harboring wild-type or mutant forms of mRon. The infected cells were plated in methylcellulose in the presence or absence of MSP, and erythroid colonies were counted on day 8 following benzidine staining for hemoglobin. *, p < 0.01.

Tyrrosines 1175, 1265, and 1294 Play Critical but Redundant Roles in the Activation of the MAP Kinase Pathway by mRon and Association of the Receptor with c-Src—Based on previous results indicating that mutation of Tyr-1175 alone did not abrogate constitutive MAP kinase activation by mRon, we set out to examine the individual contributions of tyrosines 1175, 1265, and 1294 to the induction of this pathway in the absence of ligand by mRon. Therefore, we mutated back each of these phenylalanines to tyrosine, both singly and in combination, in the context of the Y5F mutant that failed to induce constitutive Erk activity (Fig. 6A). Our data demonstrate that adding back Tyr-1175, Tyr-1265, or Tyr-1294 is able to partially restore the ability of the receptor to induce Erk phosphorylation, c-fos expression, and AP1 transactivation in the absence of ligand, compared with the adding back of Tyr-1330 and Tyr-1337, which we have shown has little effect on this pathway (Fig. 6, B and D). Adding back each of these tyrosines also enhanced receptor phosphorylation as demonstrated by immunoprecipitation with anti-mRon and blotting with anti-pTyr or phospho-specific antibody to the tyrosines in the activation loop (Fig. 6C).

To confirm that mutation of the two docking site tyrosines regulates Grb2 recruitment to the receptor, we co-transfected a FLAG-tagged Grb2 with wild-type and mutant mRon. Data from these studies show that ‘Tyr-1337’ is the primary Grb2 binding site and that mutation of both docking site tyrosines almost completely abrogates Grb2 binding (Fig. 7A). This suggests that Grb2 is not mediating the ligand-independent activation of the MAP kinase pathway by mRon. Multiple mechanisms other than those mediated by Grb2 have been implicated in the activation of MAP kinase by RTKs, including phospholipase Cγ/protein kinase C and 14-3-3 signaling pathways, which are utilized by the vascular endothelial growth factor receptor and insulin-like growth factor, respectively (31, 32). By using a lipase-dead mutant of phospholipase Cγ and a panel of protein kinase C inhibitors, as well as mutating potential 14-3-3 binding sites, we concluded that these pathways were not likely to be responsible for the ligand-independent activation of the MAP kinase pathway induced by mRon.

Src family kinases are an important class of signaling effectors that are used by multiple cell surface receptors to propagate proliferative signals. To explore the potential for mRon to recruit c-Src, we co-transfected 293 cells with c-Src and the indicated HA-tagged receptors and immunoprecipitated lysates with anti-c-Src. These data demonstrate that c-Src interacts with mRon in the absence of ligand and that this interaction requires the kinase activity of receptor, but not the docking site tyrosines (Fig. 7B). To examine the potential role of Src family kinases in the constitutive activation of the MAP kinase pathway by mRon, we co-transfected a dominant interfering c-Src (Y296R/Y528F), in which the Src activation loop tyrosine and the C-terminal tyrosine are mutated, with mRon and examined Erk phosphorylation. Co-expression of the dominant negative Src blocked the ability of mRon to constitutively activate the MAP kinase signaling pathway (Fig. 7C). Although the kinase activity of mRon is required for the interaction with c-Src, co-transfection of a kinase inactive c-Src with mRon demonstrates that this interaction does not require the kinase activity of c-Src (Fig. 7D). In addition, we show that receptor expression and mRon autophosphorylation were not altered by the overexpression of dominant negative c-Src (Fig. 7E). To determine whether mutation of the tyrosines responsible for the constitutive activation of MAP kinase by mRon also affects the ability of c-Src to interact with the receptor, we co-transfected wild-type and mutant mRon with c-Src, immunopre-
Tyrosines 1175, 1265, and 1294 play critical but overlapping roles in the induction of the MAP kinase pathway by mRon in the absence of ligand. A, schematic of mutant receptors used in this experiment. B, 293 cells were transiently co-transfected with mRon or the indicated mutant receptors and an AP1 luciferase reporter construct. Cell lysates were immunoblotted for phosphorylated Erk, total Erk, c-Fos, and mRon. C, cell lysates from B were immunoprecipitated with anti-mRon and blotted with anti-phosphotyrosine. Blots were also probed with phospho-specific antibody to the two tyrosines in the activation loop (anti-RonYY) as a measure of kinase activity. Blots were stripped and re-probed for mRon. D, AP1 luciferase activity in lysates from B.
precipitated with anti-c-Src and blotted with anti-mRon. As demonstrated previously, wild-type mRon interacts with c-Src, and this interaction is dependent on the kinase activity of mRon. However, mRonY5F failed to co-immunoprecipitate with c-Src (Fig. 7F). Interestingly, restoration of any one of the three tyrosines 1175, 1265, or 1294 restored the ability of mRon to interact with c-Src and blotted with anti-mRon. The blots were stripped and re-probed for c-Src. The expression of mRon in the samples was verified by Western blotting of whole cell lysates with anti-mRon.

**DISCUSSION**

The central role of the docking site tyrosines on the carboxyl tail of the Met receptor was underscored by the discovery that in vivo mutation of these tyrosines lead to an embryonic lethal phenotype.
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resembling that of a Met null mutant model (18). However, this Met mutant is not signaling-dead, as revealed by the observation that it is still capable of conveying a residual signal leading to activation of Ras and MAP kinase via Gab1 phosphorylation, although several important binding effectors are uncoupled with the mutant receptor (33).

We have shown that Ron plays a central role in the regulation of macrophase activation both \textit{in vitro} and \textit{in vivo} (34–36), and this function is dependent on the docking site tyrosines (37). In addition, the ability of the N-terminally truncated form of murine Ron (Sf-Stk) to transform erythroid progenitor cells in response to Friend erythroleukemia virus requires the second docking site tyrosine (25). However, an oncogenic point mutation in human Ron has been shown to elicit cellular transforming activity independent of the docking site tyrosines, indicating that additional oncogenic signals might emanate from a novel site on the receptor (38).

Oncogenic mutations in the kinase domain of RTKs can induce ligand-independent signaling downstream of the receptor by altering substrate fidelity and subsequently creating novel signaling pathways (7). In contrast, constitutive signaling induced by wild-type receptors in the absence of ligand stimulation is usually attributed to "leaky" activity of the kinase due to low levels of receptor homodimerization caused by overexpression. Therefore, ligand-independent and -dependent activity of wild-type RTKs is considered to be generically indistinguishable, differing only at the quantitative level. In this study, we present evidence demonstrating that the C-terminal docking site tyrosines in murine Ron are responsible for ligand-inducible MAP kinase activation, whereas constitutive activation of this pathway by mRon is independent of these tyrosines. Through systematic point mutagenesis, we were able to attribute the strong residual activity of mRon to three tyrosines in the C lobe of the kinase domain, which, when mutated together, dramatically reduce the constitutive receptor phosphorylation and MAP kinase activation. However, this mutant receptor retains the full response to MSP stimulation with regards to receptor phosphorylation and activation of the MAP kinase cascade.

Identifying the mechanism by which Tyr-1175, Tyr-1265, and Tyr-1294 influence receptor activity and downstream activation of the MAP kinase pathway presents a challenge. Here we show that mRon co-immunoprecipitates with c-Src and that the mutation of these three tyrosines abrogates this interaction. Interestingly, adding back any one of these three tyrosines restores the ability of mRon to co-immunoprecipitate with c-Src. Using a dominant negative c-Src, we demonstrate that Src activity is required for the ability of mRon to constitutively activate the MAP kinase pathway. However, the dominant negative Src does not influence receptor expression or phosphorylation. The requirement for mRon kinase activity in the activation of MAP kinase and recruitment of c-Src, suggests the possibility that these three tyrosines could serve as docking sites for c-Src upon receptor autophosphorylation. Sequence comparison indicates that these three tyrosines are highly conserved in the RTK superfamity of transmembrane receptors, suggesting the possibility of a shared role for these tyrosines in regulating kinase activity and/or receptor signaling. A previous study indeed showed that Tyr-1294 plays a critical role in oncogenic Ron receptor-mediated 3T3 cell transformation (38). Similarly, the equivalent tyrosine to Tyr-1294 (Tyr-730) on the fibroblast-like growth factor receptor has been reported to serve as an autophosphorylation site \textit{in vivo} (39), and recent studies have shown that the equivalent tyrosine to Tyr-1294 (Tyr-981) of the Ret receptor serves as a novel autophosphorylation site and binds to the Src SH2 domain (40). Together, these data indicate that it might be a common feature for these structurally conserved tyrosines in the catalytic domain to serve as a docking site.

Molecular modeling of the three-dimensional structure of mRon, based on that of the Met kinase domain, which shares 63% sequence identity with mRon, demonstrates that Tyr-1265 and Tyr-1294, along with the C-terminal docking site tyrosines, reside on the surface of the kinase domain. In the resting conformation, both Tyr-1265 and Tyr-1330 have over 40% surface area exposed to the environment, making them good candidates for incoming protein binding. However, Tyr-1175 originates from the αC helix and directly points to the hydrophobic pocket on the base of the αC helix in the N lobe. We found that 13 out of 18 RTKs examined contain a tyrosine residue at this position, whereas the remaining receptors harbor a phenylalanine. This suggests that the presence of an aromatic ring at this site is likely to be important for the regulation of kinase activity. Our data indicate that restoration of Tyr-1265 and Tyr-1294, as well as Tyr-1175, partially rescues receptor kinase activity as measured by autophosphorylation of the receptor. However, unlike Tyr-1175, the location of Tyr-1265 and 1294 does not evidently link them to regulation of the kinase activity.

Conversely, it is also difficult to postulate how Tyr-1175 could become phosphorylated and potentially serve as a binding site for the c-Src SH2 domain based on the inactive structure. A recent study indicated that Tyr-1194, the equivalent site to Tyr-1175 in Met, is very likely to serve as an autophosphorylation site (41). Likewise, the JM tyrosines in the EphR and Fk3 are deeply immersed in the inter-domain pocket between the JM region and the kinase N lobe, but nevertheless can be rapidly exposed at the exterior of the protein core by phosphorylation induced conformational change (42). Therefore, the possibility that Tyr-1175 is phosphorylated and serves as a docking site for downstream signaling proteins such as c-Src cannot be discounted. The important role of Src family kinases downstream of RTK signaling can be generalized to other families. For example, the restoration of an Src family kinase docking site, but not sites for phosphatidylinositol 3-kinase, Grb2, or phospholipase Cγ, to a functionally inert c-Kit receptor, rescues most of the downstream signaling and cellular responses promoted by c-Kit (44). Furthermore, the embryonic lethal phenotype of the Met docking site mutation "knock-in" model can be partially rescued by introduction of an optimal c-Src recruitment motif to the C terminus of the mutant Met receptor (33).

In this study, we have demonstrated that ligand-independent signaling from an RTK may not necessarily reflect the signals generated by that receptor in response to ligand, underscoring the importance of distinguishing between these events when studying RTK signaling under normal physiological conditions and those induced by overexpression, a common feature in transformed cells. Interestingly, although we showed that expression of full-length mRon in primary erythroid cells induced Epo-independent colonies in the absence of ligand, expression of a chimeric human Ron receptor in these cells required ligand stimulation to induce Epo-independent growth of erythroblasts (30). In addition, transformation of primary erythroid progenitor cells by Sf-Stk (truncated mRon) in response to Friend virus infection requires the interaction of Sf-Stk with the viral glycoprotein, gp55 (14). It is possible that the complex formed by Sf-Stk and gp55 could adopt a conformation similar to that of full-length mRon in the presence of ligand. However, the role of the three tyrosines in the kinase domain in the transformation of cells by Sf-Stk/gp55 has not yet been evaluated.

Perhaps most importantly, the phenotype of the mRon knock-out animals (increased susceptibility to septic shock) (34), was not recapitulated in the MSP knock-out animals (45). The presence of an alternative ligand has been evoked to explain this difference. However, based on our studies here, it is possible that signaling through mRon \textit{in vivo} is not strictly ligand-dependent and that the signals generated by mRon in
the presence and absence of MSP in the regulation of macrophage activation and susceptibility to septic shock may be distinct.

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**Uncoupling Mechanisms for MAP Kinase Activation**
