Altered Exon Usage in the Juxtamembrane Domain of Mouse and Human RON Regulates Receptor Activity and Signaling Specificity*

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Alternative splicing of signaling proteins can contribute to the complexity of signaling networks. We find that expression of mouse RON, but not human RON, results in constitutive receptor autophosphorylation, ligand-independent activation of the mitogen-activated protein kinase pathway, and association of the receptor with c-Src. Using chimeric receptors, we mapped the region for this difference in signaling capacity of mouse and human RON to the juxtamembrane domain. Expression of these receptors in primary erythroid progenitor cells also demonstrated a functional difference in the ability of mouse and human RON to support erythropoietin-independent colony formation that mapped to the juxtamembrane domain. Splicing of the mouse RON receptor tyrosine kinase transcript results in the constitutive deletion of an exon used by all other known RON orthologs that encodes part of the juxtamembrane domain of the receptor. Mutational analysis indicated that the two tyrosines present in this region in human RON, one of which has been previously shown to be a c-Cbl binding site, are not responsible for this difference. However, deletion of this region in the context of human RON enhanced receptor phosphorylation, activation of mitogen-activated protein kinase, and association of c-Src at levels comparable with those observed with mouse RON. These data provide direct evidence that the divergence of exon usage among different species can generate a protein with novel activity and subsequently add to the complexity of cellular signaling regulation.

The modular nature of signaling proteins greatly facilitates the rapid evolution of a large number of molecules with various combinations of functional domains, increasing the possibility for combinatorial regulation. Furthermore, individual exons frequently correspond to basic folding and functional modules of a given protein (1). Expectedly, alternative splicing constitutes an important mechanism for protein evolution and diversification. By simply including or excluding certain exons, genes could reorganize their functional motifs to achieve novel activities without taking the risk of deleteriously mutating the coding sequence. Receptor tyrosine kinases (RTKs) play a fundamental role in the regulation of animal development and pathogenesis. Intramolecular interactions, receptor dephosphorylation, and degradation form multiple layers of defense against uncontrolled kinase activity. The binding of growth factors enhances the enzymatic activity of the kinase domain by relieving structural constraints (2), resulting in tyrosine phosphorylation of the receptor that serves as an assembly platform for downstream signaling molecules (3). Alternative splicing, resulting in similar relief of these constraints, is one mechanism by which RTKs could become constitutively activated in tumor cells.

Recent studies have identified several transforming transcripts of the human RON receptor tyrosine kinase, generated by alternative splicing of exons 5, 6, and 11 in the extracellular domain in patients with colorectal carcinoma (4). RON belongs to a small vertebrate-specific family of receptor tyrosine kinases that includes the hepatocyte growth factor receptor (Met) and RON (also called Stk in mice and c-Sea in chickens) (5). The ligands for Met and RON, hepatocyte growth factor and macrophage stimulating protein (MSP), respectively, induce autophosphorylation of two highly conserved tyrosines in the C-terminal domain (6). These tyrosines subsequently mediate a number of biological functions, including epithelial cell migration, invasive growth, and protection from apoptosis. MSP, the ligand for RON, regulates macrophage activation in vitro (7, 8), and this regulation is dependent on the docking site tyrosines (9). Furthermore, a series of knock-out studies demonstrate that in vivo RON inactivation renders mice hypersusceptible to endotoxin challenge, indicating a critical role for RON in limiting the inflammatory response through regulation of macrophage activation (10, 11). However, MSP does not appear to be required for this response in vivo, suggesting the presence of alternative mechanisms of mouse RON regulation (12).

Recent knock-out studies have identified murine RON as a critical mediator of transformation in the progression of a murine model of breast carcinoma (13, 14). Furthermore, the murine RON gene encodes a truncated version of the receptor that is driven by an internal promoter within intron 10. This N-terminal truncated form of murine RON has been shown to confer susceptibility to Friend virus-induced erythroleukemia and mediate Epo-independent growth of primary erythroid progenitor cells upon interaction with the viral glycoprotein gp55 (15, 16). The intracellular domain of murine RON shares about 88% sequence similarity with its human counterpart (17). However, overexpression of wild type mouse RON, but not human RON, has been reported to induce the transformation of NIH 3T3 cells in vitro (18). We and others have observed that an exon encoding part of the juxtamembrane domain in major transcripts of all other known RON orthologs is missing in the murine RON transcript (19). Here we have shown that the resultant juxtamembrane domain in murine RON induces enhanced receptor phosphosaline; mRON, mouse RON; hRON, human RON; JM, juxtamembrane; SH, Src homology; VSV, vesicular stomatitis virus; Epo, erythropoietin.
phorylation, activation of the MAP kinase cascade, and association of RON with c-Src when compared with human RON. These studies have identified altered exon usage as a potential mechanism for the evolutionary diversification of mouse and human RON.

**MATERIALS AND METHODS**

**Cell Culture, Antibodies, and Reagents**—Human embryonic kidney 293 cells, NIH3T3, and SYF (Src−/−, Yes−/−, Fyn−/−) cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Antibodies against phospho-Erk, Erk, phospho-Akt, Akt, and the HA epitope (262K) were purchased from Cell Signaling. Antibodies against phospho-tyrosine (4G10) were from Upstate Biotechnology and antibodies for c-Fos, hRON (c-20), c-Src (N-16), and GST (B-14) were from Santa Cruz. Antiserum against hRON was kindly provided by Dr. Massimo Santoro (University of Piemonte Orientale, Italy). Antiserum against mRON was raised against the last 15 amino acids of the carboxyl tail. The Akt in vitro kinase assay kit was purchased from Cell Signaling. Recombinant macrophage stimulating protein was from R&D Systems. The AP1 luciferase reporter and c-Src cDNA were kindly provided by Dr. Avery August (The Pennsylvania State University).

**Gene Construction and Mutagenesis**—The juxtamembrane fragment of human and mouse RON and the SH2 and SH3 domains of c-Src were amplified by PCR and cloned into the Smal site of pGEX3 (Amersham Biosciences). Human RON and mouse RON were expressed by the PCI-neo-mammalian expression vector or the MSCV retroviral vector. For C-terminal HA tagging, wild type and mutant forms of hRON, mRON, and their chimeras were PCR amplified and then cloned in-frame into the HindIII and NotI sites of pcDNA-c-2HA. All point mutations were created with the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Overlap extension PCR was performed to construct hRON/mRON chimeras and hRONJ. The N-terminal fragment of hRON-(1–1710) that is shared by all chimeras was excised from the PCI-hRON plasmid with XhoI and HindIII and cloned into the XhoI and HindIII sites of pBSKII. The resultant construct is named pBS-NR (pBS with N-terminal hRON). The remainder of hRON was amplified by PCR with a common external 5′ primer, 5′-CCACTGCCCACCAAGCTTAC-3′ (HindIII site is underlined), and unique internal 3′ primers 3′-CGACCTCGGTGTTGGGACGTACCTCGTAGG-5′ for hRONΔJ, 3′-CACCGCCTCCTCTCGTGACTCCAGTGAGGGAGAGGGTCC-5′ for hmRON1, 3′-GGGTTAC-TCCGCTCGACAGGGAGGTGACG-5′ for...
hmRON2, and 3'-CTCATGATCAACCTCCACACTTGCCATCCACGAGGTAGTGGCCTCACTGC 3' for hmRON3 (underlined sequences represent overlapping region). The mRON portion of the chimeric receptor was amplified by PCR with a common external 3' primer, 3'-GTCTGGTGGGAGCGGTTGAAGCATTAGCTATG-5', and unique internal 5' primers, 5'-GGTGCTACACTCCCTGCCCC-3' for hmRON1, 5'-ATCCATCTGACCAATGGTCATGGCC-3' for hmRON2, and 5'-AAGCGAGTTAGTGCCCTACCTGC-3' for hmRON3. The C-terminal fragment of hRON3 was amplified by PCR with internal 5' primer 5'-GCACAACCTCCATGGAACATCCTTCCCTCC-3' and external 3' primer 3'-GAGGAGGCCCGGTGAAACTAGATC-5'.

To generate chimeras, the PCR products were mixed for overlap extension PCR according to the following protocol: 95 °C 30 s, 55 °C 1 min, 72 °C 5 min for 1 cycle, and then two external primers were added and the reaction was continued for 30 more cycles. The hybrid segments were purified, phosphorylated by T4PNK, and cloned into the EcoRV site in pcDNA3.1. The overlap extension product for hRON3 was inserted into pcDNA3.1 in reverse orientation so that there are HindIII sites on both ends of the insert (one is carried by the insert and the other is provided by the vector). The resulting HindIII fragment was cloned into the HindIII site in pBS-NR. The full-length hRON provided by the vector. The resulting HindIII fragment was cloned into pcDNA3.1 in forward orientation, excised with HindIII and XbaI, and inserted into HindIII and XbaI sites in pBS-NR. All resultant chimeras were cut with XhoI and inserted into the XhoI site of MSCV.

Cell Transfection and Luciferase Assays—For the luciferase assay, 5 × 10⁴ human embryonic kidney 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 RON 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. 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 (Promega). For the immunoprecipitation assays, 4 × 10⁶ cells/well were seeded into 6-well plates. The next day cells were transfected with 0.5 μg of HA–RON or its derivatives and 0.5 μg of c-Src.

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, 25 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 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, incubated with protein A/G beads for 2–4 h, and washed with 1:1 mixture of cell lysis buffer and 1 M lithium chloride three times. Beads were resuspended in 20 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 + 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 reprobing, 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 Vitro Infection of Primary Bone Marrow Cells—Total bone marrow cells from RON+/– mice were harvested and incubated 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 VSV envelope protein-encoding constructs in the presence of 2.5 ng/ml interleukin-3, as previously described. Cells were then plated in methocult M3234 (StemCell Technologies) containing interleukin-3 (2.5 ng/ml) (Peprotech) in triplicate. Cultures were incubated for 2–8 days in 5% CO₂ at 37 °C. Epo-independent erythroid colonies, CFU-E and BFU-E, were visualized by acid-Benzidine staining as previously described (15) at days 2 and 8, respectively.

Primary Macrophage Isolation and Infection—Primary bone marrow macrophages were harvested and plated on 10-mm dishes. Macrophages were purified by adherence following an overnight culture. The primary macrophages were maintained in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum and 30% L929 supernatant for 3 days, followed by two consecutive viral infections for a total of 24 h in the presence of 8 μg/ml polybrene. Green fluorescent protein expression was used to monitor the infection efficiency. Resident peritoneal macrophages were harvested by lavage.
Immunofluorescence Staining—293 cells growing on coverslips overnight were co-transfected with c-Src and hRON or mRON as described. 24 h later the cells were fixed in methanol for 5 min at -10 °C. After rehydration with PBS, cells were blocked with 10% goat serum in PBS for 30 min and then incubated with anti-HA and anti-c-Src diluted in PBS containing 3% goat serum for 1 h. Following three washes with PBS, cells were stained with Alexa-488-and Alexa-568-conjugated goat anti-mouse or anti-rabbit secondary antibody (Molecular Probes) in PBS with 3% goat serum for 45 min. After extensive washing the cells were examined under a confocal fluorescence microscope with the appropriate filters.

GST Pulldown Assay—XL-1 Escherichia coli carrying pGEX3-SH2 or pGEX3-SH3 was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for GST fusion protein expression. Bacterial pellets were resuspended in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and lysed for 3 h in the presence of 500 μg/ml lysozyme (Sigma) and 50 μg/ml DNase I (Invitrogen). 60 μl of glutathione beads were added to 1.5 ml of cleared bacterial lysate. The beads were washed again with a 1:1 mixture of Western blot lysis buffer and 1 M LiCl three times, and 20 μl of glutathione beads (Santa Cruz) coated with GST-SH2 or SH3 were incubated with lysates from 293 cells transiently transfected with hRON, mRON, or their derivatives for 2 h. The beads were washed with a 1:1 mixture of Western blot lysis buffer and 1 M LiCl three times, and then resuspended in 20 μl of SDS loading buffer.

FIGURE 3. Constitutive activation of MAP kinase by murine RON maps to the juxtamembrane domain. A, schematic representation of chimeric receptors used in this experiment. ED, extracellular domain; TM, transmembrane domain; JM, juxtamembrane domain; KD, kinase domain; CT, C-terminal tail. B, expression of chimeric receptors in 293 cells over the time course of the experiment. C, 293 cells were co-transfected with the indicated wild type or chimeric receptors and an AP1 luciferase reporter. Cell lysates were immunoblotted and probed for phosphorylated Erk, total Erk, c-Fos, and RON receptor levels. D, AP1 luciferase activity in lysates from panel C. AP-1 transactivation induced by all indicated receptors was compared with empty vector control with Dunnett test. **, p <0.01.
resolved on an 8% SDS-PAGE gel, and Western blot analysis was performed.

Pulse-Chase—Transient transfection of 293 cells with hRON and hRONΔ was performed as described previously. After 36 h, cells were rinsed with PBS and starved in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium without serum for 1 h following the addition of 143 μCi of 35S-labeled methionine and cysteine (Amersham Biosciences). Cells were then washed with PBS once and cultured in complete Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. At the indicated times, cells were harvested in 600 μL of cell lysis buffer and subjected to immunoprecipitation with hRON antiserum and protein A beads. The immunocomplexes were resolved by 8% SDS-PAGE. The gel was then fixed in 50% methanol and 10% acetate for 30 min and dried under a vacuum for 2 h. The amount of 35S incorporated into the receptor was visualized using a Storm System (Amersham Biosciences).

RESULTS

Murine RON, but Not Human RON, Transmits Mitogenic Signals in a Ligand-independent Manner—Previous studies have shown that overexpression of even a constitutively dimerized human RON receptor tyrosine kinase (hRON) fails to transform NIH3T3 cells (20), whereas the wild type mouse RON (mRON) receptor is reported to induce cell transformation in this assay (18). To determine whether there are differences in the activation of mitogenic signaling induced by these receptors, we transiently transfected human embryonic kidney 293 cells with hRON or mRON in the presence or absence of MSP and tested the activation of the MAP kinase and phosphatidylinositol 3-kinase signaling pathways, both of which are required for cellular transformation by this family of receptors (21). Interestingly, although both hRON and mRON expression resulted in only mild activation of the phosphatidylinositol 3-kinase pathway as measured by Akt phosphorylation (Fig. 1B) and in vitro kinase assay (data not shown), there was a striking difference in the ability of these orthologous receptors to induce MAP kinase signaling. mRON expression strongly induced p44/42 Erk phosphorylation, immediate early gene expression as measured by c-Fos induction (Fig. 1A), and an AP1 transcriptional response (Fig. 1C) in the absence of ligand. However, hRON expression resulted in activation of this pathway only in the presence of MSP stimulation. hRON and mRON expression in these cells was confirmed by Western blot analysis with anti-hRON and anti-mRON.

Antibodies against hRON and mRON do not cross-react because the epitopes for antibody recognition reside on the C terminus of the receptor, a region with high sequence divergence between the human and murine RON receptors. To facilitate direct comparison of the expression levels of these two receptors, both hRON and mRON were tagged with a tandem HA epitope on the C terminus of the receptor. Comparable levels of receptor expression over the time course of the experiment are shown in Fig. 1D, ruling out the possibility that the difference in signaling capacity of hRON and mRON is caused by different expression levels. To determine whether the kinase activity of mRON is required for the induction of MAP kinase, we transiently transfected 293 cells with wild type or kinase-dead mRON, in which the essential lysine in the ATP binding site is mutated to methionine (K1091M), and compared the levels of Erk phosphorylation (Fig. 1E). The data from these studies demonstrate an absolute requirement for mRON kinase activity in the constitutive induction of the MAP kinase cascade. To examine receptor phosphorylation levels, HA-tagged hRON and mRON were expressed in 293 cells, and cell lysates were immunoprecipitated with anti-HA and blotted with anti-pTyr (Fig. 1F). mRON, but not hRON, is highly phosphorylated in these cells, and this phosphorylation is dependent on the kinase activity of the receptor.

To determine whether the constitutive phosphorylation of mRON is due solely to this overexpression system, we sought to compare levels of receptor phosphorylation in a known target cell of RON signaling. Previous studies have shown that the MSP/RON signaling pathway regulates the inflammatory activation of primary peritoneal macrophages from mice. The deregulation of macrophage activation in the absence of RON results in enhanced susceptibility to septic shock (10). Whereas murine peritoneal macrophages express endogenous mRON, bone marrow-derived macrophages do not express the receptor. Therefore, we infected primary bone marrow-derived macrophages with HA-tagged hRON or mRON in the context of the MSCV retroviral vector. In vitro infection of primary bone marrow macrophages with MSCV encoding GFP resulted in roughly 40% infection efficiency (Fig. 2A). Western blot analysis revealed that mRON was expressed at levels similar to those observed in the primary peritoneal macrophages (Fig. 2B), of which ~60% express endogenous RON (10). hRON and mRON were immunoprecipitated from these cells with anti-HA, and phosphorylation of these receptors was compared using anti-pTyr. Whereas HA-tagged mRON is clearly phosphorylated in these cells, hRON exhibits little or no constitutive phosphorylation in the absence of ligand. Similar levels of the two receptors in the precipitate were confirmed using anti-HA (Fig. 2C).

Ligand-independent Activation of the Ras-MAP Kinase Pathway by Murine RON Maps to the Juxtamembrane Domain—The cytoplasmic domain of RTKs that dictates signaling specificity can be divided into three regions: the juxtamembrane domain, the kinase domain, and the non-catalytic carboxyl tail. To determine which of these subdomains, if
FIGURE 5. Mouse RON transcripts are missing exon 13, included in all other RON orthologs. A, schematic representation of the genomic structure of mRON and hRON. Of the 20 exons encoding hRON, 19 are conserved in mRON. Exon 13 encoding for part of the juxtamembrane domain in hRON is missing in mature mRON transcripts. B, genomic sequence encoding the juxtamembrane domain of the RON receptor from human, feline, rat, and mouse (exon, red; intron, black). Splice donor and acceptor sites are boxed. *, premature stop codon in pseudo-exon 13 in mRON.
any, accounts for the difference in mitogenic signaling induced by hRON and mRON, we constructed fusion proteins of human and mouse RON at the relative junctions of each of the three subdomains (Fig. 3A). All chimeric receptors were expressed to similar levels in 293 cells over the course of the experiment (Fig. 3B). Although substitution of the mRON C-terminal tail in the context of hRON (hmRON3) had no effect on the inability of the hRON receptor to activate this pathway, inclusion of the mRON juxtamembrane domain (hmRON1) resulted in the induction of ligand-independent Erk activation by the chimeric receptor to levels similar to those induced by wild type mRON (Fig. 3C). Interestingly, we found that substitution of the mRON kinase domain in hRON (hmRON2) consistently induces slightly higher Erk activation than hRON and hmRON3, indicating a potential difference in the kinase domain of hRON and mRON. However, only hmRON1 and mRON are sufficient to induce a nuclear response as indicated by c-Fos induction and AP1 transactivation (Fig. 3, C and D). These results suggest that the juxtamembrane domain of RON plays a primary role in regulating the ability of the receptor to transduce mitogenic signals in the absence of ligand.

Previously we have shown that a naturally occurring N-terminal-truncated form of the mRON (Stk) receptor, Sf-Stk, mediates the transformation of primary erythroid progenitor cells in response to Friend erythroleukemia virus through activation of Sf-Stk by the viral glycoprotein gp55 (15, 16). Furthermore, a chimeric human RON receptor can substitute for Epo to induce the growth of erythroid progenitor cells upon ligand-induced activation (22). Here, we have demonstrated that expression of mRON, but not hRON, in the absence of ligand induces the formation of erythroid colonies (CFU-E and BFU-E) from primary bone marrow cells (Fig. 4, A and B). The kinase activity of the receptor is essential for the capability of mRON to promote progenitor cell proliferation, as demonstrated by the failure of kinase-inactive mRON (K1091M) to promote this response. Further, we showed that this activity maps to the juxtamembrane domain of mRON using the chimeric receptors described in Fig. 3. These experiments support the hypothesis that alterations in the juxtamembrane domain of mRON and the resulting changes in signaling capacity correlate with a functional response induced by this receptor.

FIGURE 6. Partial deletion of the juxtamembrane region in hRON results in receptor phosphorylation and activation of the MAP kinase pathway. A, 293 cells were transiently transfected with hRON and hRON with a deletion in the juxtamembrane domain (hmRONJ). Cell lysates were immunoblotted for phosphorylated Erk, total Erk, c-Fos, and receptor levels. B, expression of hRON and hmRONJ in 293 cells during the time course of the transfection. C, 293 cells were transiently transfected with HA-tagged hRON and hmRONJ. Lysates were immunoprecipitated with anti-HA and blotted with anti-pTyr. Blots were stripped and reprobed with anti-HA. D, pulse-chase analysis of hRON and hmRONJ turnover in 293 cells.
Amino acid sequence alignment of hRON and mRON revealed that mRON has a 27-amino acid deletion in the relatively diverse juxtamembrane region when compared with mRON (Figs. 5 and 7A) caused by exclusion of a potential exon in the mRON cDNA sequence, which has previously been reported by other investigators (19). Genomic sequence comparison showed that the sequence of the juxtamembrane domain-encoding exon exists in the major transcripts of all other known RON orthologs including human, rat, feline, and chicken, and no alternative splicing events in this region have been reported thus far. In addition, this exon appears in all hRON transcripts, but not mature mRON transcripts, found in the Expressed Sequence Tag data base. It is notable that all RON gene orthologs, including mouse, share a similar 5′-splicing donor and 3′-acceptor sequence surrounding this exon. However, we identified a premature stop codon arising from a 1-base pair frameshift in this pseudo-exon that, if included, would result in the translation of a severely truncated protein. This potential stop codon is conserved in two independent mouse strains, 129/SvJ and C57BL6 (23).

A Deletion in the Juxtamembrane Domain of hRON Confers Ligand-independent Phosphorylation of the Receptor, Induction of MAP Kinase, and Association of the Receptor with c-Src—To assess whether we could reproduce the mitogenic signaling potential of mRON in the context of the hRON receptor, we deleted the 27 amino acids from the juxtamembrane domain of hRON that correspond to the region missing in mRON. RONΔJ behaved similarly to hmRON1, which contains the juxtamembrane domain of mRON, in terms of its ability to induce ligand-independent Erk phosphorylation and c-Fos expression (Fig. 6A). We demonstrated that this difference was not due to differences in receptor expression, by comparing hRON and hRONΔJ expression over the time course of the experiment (Fig. 6B). The structure of the juxtamembrane domain has been widely reported to impact the level of receptor phosphorylation. Therefore, we tagged hRONΔJ with HA and immunoprecipitated hRON-HA and hRONΔJ-HA from 293 cells using anti-HA antibody and performed Western blot analysis with antiphosphotyrosine. We found that, like mRON, hRONΔJ, but not hRON, is constitutively tyrosine phosphorylated in the absence of ligand (Fig. 6C). Altogether these experiments demonstrate an important role for the juxtamembrane domain in regulating the tyrosine phosphorylation and signaling potential of RON.

The juxtamembrane domain of hRON contains several potential regulatory sites that are missing in mRON (Fig. 7A). For instance, DY1017R, conserved in all Met/RON family members except mRON, has been identified as a c-Cbl TKB binding motif (24). Furthermore, the substi-
Regulation of RON Signaling by the JM Domain

In the current study, we present evidence that the JM domain of mRON, a murine receptor tyrosine kinase, largely diverges from its other mammalian orthologs. The difference is due to the absence of a single exon in this region, encoded by other family members, resulting in enhanced kinase activity, ligand-independent activation of the Ras-MAP kinase pathway, and association with c-Src. Based on these data, we propose a model in which the peptide encoded by exon 13 of human RON causes the receptor to adopt a conformation in which kinase activity is largely inhibited. Upon ligand binding, kinase activity of hRON is up-regulated and tyrosines in the C-terminal tail are phosphorylated, leading to the activation of the MAP kinase signaling cascade. Alternatively, the absence of this juxtamembrane domain fragment in mRON may lead to a structural change in the receptor, resulting in relief of the autoinhibitory constraints of the receptor, leading to constitutive kinase activation in the absence of ligand-induced dimerization that is permissive for the activation of the MAP kinase cascade and association with c-Src. This evolutionary divergence of mRON could create a novel function for mRON in the absence of ligand.

How this species-specific alteration in RON occurred is not clear. However, the presence of a frameshift-derived premature stop codon in the pseudo-exon 13 of mRON could be responsible. Frameshift and nonsense mutations can lead to production of a non-functional protein. Recent studies indicate that the intron-exon boundary sequences provide insufficient information for exon definition and that additional splicing signals are carried out by intrinsic exon sequences called exonic splicing enhancers that, if mutated, could lead to exclusion of the exon (29). The presence of exonic splicing enhancers could allow an organism to circumvent a nonsense mutation by skipping the lethal exon in order to maintain the correct reading frame at the expense of generating either a partial loss-of-function or gain-of-function allele. However, we failed to detect any critical splicing enhancer sequence in the exon that is impaired by the frameshift mutation as predicted by splicing enhancer prediction software (30). Interestingly, feline RON contains the same shift in reading frame at this site, but instead of skipping the entire exon carrying the nonsense mutation, feline RON uses an ectopic splice enhancer site, thus maintaining most of the coding sequence. We cannot, however, rule out the possibility that this premature stop codon is not causative but rather arose as a consequence of the absence of selective pressure following the loss of this exon.

Recent data have suggested that the juxtamembrane domain of RTKs represents an important autoinhibitory switch that sets the receptor in a closed conformation as a default state, requiring ligand binding and dimerization to release the structural constraints and activate the kinase (31). Moreover, the autophosphorylation of JM tyrosines in many RTK families plays a vital role in regulating the transition of the kinase domain from an inactive to active conformation. The addition of a charged bulky phosphate group in the JM region by tyrosine phosphorylation would destabilize the autoinhibitory folding of the activation loop and subsequently allow its access to peptide substrate and the ATP binding site (32). Our study provides a unique scenario in which the deletion of a JM sequence containing two tyrosines releases the kinase activity, whereas mutation of these tyrosines to phenylalanine has no significant effect on receptor-mediated signaling in this system.

Germ line JM point mutations, small deletions, and insertions constitute causative factors for various human hereditary cancers through releasing constitutive kinase activity, as revealed by a series of mutations in KIT found in gastrointestinal stromal tumors and an internal tandem duplication in the JM domain of Flt3 in acute myeloid leukemia (33). Several germ line point mutations in the Met juxtamembrane domain have also been identified in small cell lung cancer and gastric cancer (34, 35). The physiological relevance of juxtamembrane regulation of the Met/RON family of receptors has not been extensively studied to date. Alternative splicing of the Met receptor, resulting in deletion of exon 14 encoding part of the juxtamembrane domain, has been shown to produce an active isoform in a subset of mouse tissues (36). In addition, a search of the human Expressed Sequence Tag data base revealed a splicing variant of hRON missing JM exon 14 (BG289902) that is expressed in a bladder papilloma cell line. On the contrary, there is no evidence...
thus far indicating that human RON exon 13 undergoes alternative splicing in vivo.

A constitutive active mutant of hRON (M1254T), mimicking the point mutation in the activation loop of Met (M1268T) associated with hereditary papillary renal carcinoma type I, behaves similarly to mRON with regard to its strong autophosphorylation, ligand-independent activation of MAP kinase, and ability to recruit c-Src (37, 38). It is interesting to speculate that these distinct mutations in the Met/RON family of receptors, ranging from the JM domain to the activation loop, could induce signaling through a common mechanism. However, the constitutive activity of mRON could be detrimental if not properly controlled, suggesting the presence of other layers of negative regulation in vivo. RON has been shown to associate with a number of cell surface receptors, including integrins and adhesion molecules, the yc chain shared by a number of cytokines, the EpoR, and other RTKs, including Met and epidermal growth factor receptor (39). Recent studies have demonstrated that the interaction of RON with the hyaluronidase 2 receptor negatively influences its activity and that binding of the envelope protein from jaagsieepie virus to Hyal2 frees RON, resulting in its activation (40). Therefore, the activity of RON could be regulated under physiological conditions by its interaction with other signaling molecules at the cell surface.

Studies from our laboratory and others’ have demonstrated a defective inflammatory response in mice with a targeted deletion in the RON receptor (10, 11), whereas the phenotype of MSP knock-out animals appears to be normal (12). The possible existence of an alternative ligand for RON has been evoked to explain this difference. However, we hypothesize that the constitutive activity of mRON could largely compensate for the loss of ligand. This is consistent with our results demonstrating the constitutive phosphorylation of mRON when expressed at near physiological levels in primary macrophages. In addition, NFκB-dependent transcription of the human immunodeficiency virus long
terminal repeat is enhanced in RON / macrophages in the absence of MSP, indicating a potential role for mRON in setting thresholds for classical macrophage activation (41). However, because we have been unable to immunoprecipitate the endogenous receptor from peritoneal macrophages with the reagents currently available, the phosphorylation status of endogenous mRON in these cells remains speculative.

Previous studies have shown that a chimeric RON receptor can compensate for the lack of Epo-induced signaling in erythroid progenitor cells in response to ligand stimulation (22). Here, we have demonstrated that the juxtamembrane domain of RON regulates cytokine-independent growth of primary hematopoietic cells, resulting in the ability of mRON, but not hRON, to support Epo-independent colony formation in the absence of ligand. It is therefore possible that expression of mRON in primary erythroid progenitors could enhance the erythropoietic response, especially under conditions of stress. The studies described here underline the importance of considering the potential for species-specific difference in signaling when interpreting biological outcomes. Future comparison of the human and mouse RON receptors will likely shed light on the requirements for this receptor in a normal physiological context as well as in cellular transformation.

REFERENCES

1. Go, M. (1985) Adv. Biophys. 19, 91–131
2. Blume-Jensen, P., and Hunter, T. (2001) Nature 411, 355–365
3. Pawson, T., and Scott, J. (1997) Science 278, 2075–2080
4. Wang, M., Wang, D., and Chen, Y. (2003) Carcinogenesis 24, 1291–1300
5. Furge, K., Zhang, Y., and Vande Woude, G. (2000) Oncogene 19, 5582–5589
6. Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santoro, M., Gallo, K., Godowski, P., and Comoglio, P. (1994) EMBO J. 13, 3524–3532
7. Morrison, A., and Correll, P. (2002) J. Immunol. 168, 853–860
8. Morrison, A., Wilson, C., Ray, M., and Correll, P. (2004) J. Immunol. 172, 1825–1832
9. Liu, Q., Fruit, K., Ward, J., and Correll, P. (1999) J. Immunol. 163, 6606–6613
10. Correll, P., Iwama, A., Tondat, S., Mayrhofer, G., Suda, T., and Bernstein, A. (1997) Genes Funct. 1, 69–83
11. Waltz, S., Eaton, L., Hess, K., Peace, B., Tonti, J., Wang, M., Kaestner, K., and Degen, S. (2001) J. Clin. Invest. 108, 567–576
12. Bezerra, J., Carrick, T., Degen, J., Witte, D., and Degen, S. (1999) J. Clin. Invest. 101, 1175–1183
13. Chan, E., Peace, B., Collins, M., and Waltz, S. (2005) Oncogene 24, 479–488
14. Peace, B., Toney-Earley, K., Collins, M., and Waltz, S. (2005) Cancer Res. 65, 1285–1293
15. Finkelstein, L., Ney, P., Liu, Q., Paulson, R., and Correll, P. (2002) Oncogene 21, 3562–3570
16. Persons, D., Paulson, R., Loyd, M., Herley, M., Bodner, S., Bernstein, A., Correll, P., and Ney, P. (1999) Nat. Genet. 23, 159–165
17. Iwama, A., Okano, K., Sudo, T., Matsuda, Y., and Suda, T. (1994) Blood 83, 3160–3169
18. Peace, B., Hughes, M., Degen, S., and Waltz, S. (2001) Oncogene 20, 6142–6151
19. De Maria, R., Magni, P., Bosisio, B., Prat, M., Comoglio, P., Castaggini, M., and Di Renzo, M. (2003) Oncogene 22, 1785–1790
20. Santoro, M., Collesi, C., Grisendi, S., Gaudino, G., and Comoglio, P. (1996) Mol. Cell. Biol. 16, 7072–7083
21. Agazie, Y., Iwama, A., and Hayman, M. (2002) Oncogene 21, 697–707
22. van den Akker, E., van Dijk, T., Parren-van Amelsvoort, M., Grossman, K., Schaepfer, U., Toney-Earley, K., Waltz, W., Lowenberg, B., and von Linder, M. (2004) Blood 103, 4457–4465
23. Waltz, S., Toms, C., McDowell, S., Clay, L., Muraoka, R., Air, E., Sun, W., Thomas, M., and Degen, S. (1998) Oncogene 16, 27–42
24. Peschard, P., Ishiyama, N., Lin, T., Lipkowitz, S., and Park, M. (2004) J. Biol. Chem. 279, 29565–29571
25. Peschard, P., Fournier, T., Lamorte, L., Naujaks, M., Band, H., Langdon, W., and Park, M. (2001) Mol. Cell 8, 995–1004
26. Hashigasaki, A., Machide, M., Nakamura, T., Matsunuma, K., and Nakamura, T. (2004) J. Biol. Chem. 279, 26445–26452
27. Danilkovitch, A., and EJ, L. (1999) J. Leukocyte Biol. 65, 345–348
28. Wei, X., Ni, S., and Correll, P. (August 2005) J. Biol. Chem. 10.1074/jbc.M050377200
29. Cartegni, L., Chew, S., and Krainer, A. (2002) Nat. Rev. Genet. 3, 285–298
30. Cartegni, L., Wang, J., Zhu, Z., Zhang, M., and Krainer, A. (2002) Nucleic Acids Res. 31, 3658–3571
31. Hubbard, S. (2004) Nat. Rev. Mol. Cell. Biol. 5, 464–471
32. Griffith, J., Black, J., Faerman, C., Swenson, L., Wynn, M., Lu, F., Lippe, J., and Sxzena, K. (2004) Mol. Cell. Biol. 13, 169–178
33. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., Kawano, K., Hanada, M., Kurata, A., Takeda, M., Muhammad Tunio, G., Matsuzawa, Y., Kanakura, Y., Shimomura, Y., and Kitamura, Y. (1998) Science 279, 577–580
34. Lee, J., Han, S., Cho, H., Jennings, B., Gerrard, B., Dean, M., Schmidt, L., Zbar, B., and vande Woude, G. (2000) Oncogene 19, 4947–4953
35. Ma, P., Kijima, T., Maulik, G., Fox, E., Sattler, M., Griffin, J., Johnson, B., and Salgia, R. (2003) Cancer Res. 63, 6272–6281
36. Lee, C., and Yamada, K. (1995) J. Biol. Chem. 270, 507–510
37. Santoro, M., Peseng, L., Orechov, S., Cilli, M., and Gaudino, G. (2000) Oncogene 19, 5208–5211
38. Nakaigawa, N., Weirich, G., Schmidt, I., and Zbar, B. (2000) Oncogene 19, 2996–3002
39. Danilkovitch-Miagkova, A., and Leonard, E. (2001) Histol. Histopathol. 16, 623–631
40. Danilkovitch-Miagkova, A., Duh, F., Kuzmin, I., Angeloni, D., Liu, S., Miller, A., and Lerman, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 4580–4585
41. Lee, S., Kelantari, P., Tsutsui, S., Kla, A., Holden, J., Correll, P., Power, C., and Henderson, A. (2005) J. Immunol. 173, 6864–6872