Identification of a novel splicing product of the RON receptor tyrosine kinase in human colorectal carcinoma cells

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The RON receptor tyrosine kinase is a 180 kDa heterodimeric protein composed of a 40 kDa α chain and a 145 kDa β chain with intrinsic tyrosine kinase activity. Activation of RON causes cell dissociation, motility and invasion of extracellular matrices, suggesting that RON might be involved in tumor metastasis. We report here the cloning of a novel splice variant of RON in human colorectal carcinoma cell line HT-29. This RON variant is first produced as a single chain precursor with a molecular mass of 160 kDa. Proteolytic cleavage results in a 40 kDa α chain and a short form of the β chain with a molecular mass of 125 kDa. The altered receptor is synthesized from a transcript differing from the full-length RON mRNA by an in-frame deletion of 109 amino acids in the extracellular domain of the RON β chain. The consequence of the deletion is constitutive activation of the protein with autophosphorylation. Expression of the RON variant in colon epithelial CoTr cells results in increased cell migration and invasion of extracellular matrices. These data suggest that generation of the activated splice variant of RON may contribute to the invasive phenotype of human colorectal carcinomas in vivo.

Introduction

Receptor-type protein tyrosine kinases, such as those for EGF, FGF and NGF, are a group of cell surface proteins that play a critical role in cell growth, differentiation and movement (1,2). Constitutive activation of receptor tyrosine kinases has been implicated in the progression of certain types of human malignancies, such as tumors derived from breast and kidney tissues (3,4). It has been shown that receptor tyrosine kinases can be activated by a variety of mechanisms, including mutation, deletion, gene rearrangement and alternative mRNA splicing (5). A typical example is the MET proto-oncogene, which encodes the receptor for hepatocyte growth factor (6,7). Constitutive activation of the MET receptor (9,10) has been found by gene rearrangement, which generates a hybrid protein in which the MET kinase domain is fused with the sequence of the translocated promoter region derived from chromosome 1 (8). Mutational analysis also showed that point mutations in exons that encode the MET kinase domain cause constitutive activation of the MET receptor (9,10). Moreover, MET variants, generated through alternative splicing of the MET mRNA, also acquire constitutive tyrosine kinase activity (11,12). The abnormal accumulation and activation of these altered MET molecules have been implicated in MET-mediated cell transformation and invasive phenotypes of certain epithelial carcinomas (13,14).

The RON receptor tyrosine kinase is a member of the MET proto-oncogene family (15). The cDNA encoding human RON, also known as STK in mice (16), was originally cloned from a transformed human foreskin keratinocyte cell line (15). The ligand for RON was identified as macrophage stimulating protein (MSP) (17–19), also known as hepatocyte growth factor-like protein (20). The mature RON is a 180 kDa heterodimeric protein composed of a 40 kDa extracellular α chain and a 145 kDa transmembrane β chain (15,18). Both chains are derived from proteolytic conversion of a 180 kDa RON precursor (15). RON is expressed in several epithelial tissues (19,21), in certain types of tissue macrophages (22,23) and in some erythroleukemia cell lines (24). The roles of RON in cell transformation and tumor progression are largely unknown. Nevertheless, RON is overexpressed in many primary breast carcinomas (25). Point mutations in the tyrosine kinase domain are able to release the oncogenic potential of the RON receptor (26). Moreover, RON activation causes invasive growth and motility of certain epithelial tumor cells (27,28). Recently, a splicing variant of RON was identified in a human stomach cancer cell line KATO-III (29). This molecule is encoded by a transcript differing from the full-length RON mRNA by an in-frame deletion of 49 amino acids in the β chain extracellular domain (29). Expression of this RON variant promotes invasive growth of transfected epithelial cells (29). Considering these facts, it is suggested that RON might be involved in the progression of certain epithelial malignancies, particularly at the stage of tumor metastasis (25,28,29).

We report here the cloning of a novel splice variant of RON (RONΔp160) from the human colorectal carcinoma cell line HT-29. The function of RONΔp160 in regulating cell migration and matrix invasion was also studied. We found that RONΔp160 is constitutively activated in HT-29 cells and in transfected CoTr colon epithelial cells. CoTr cells expressing RONΔp160 showed increased cell migration and matrix invasive activity. These data suggest that RONΔp160 might play an important role in vivo in promoting the invasive phenotype of colorectal carcinoma cells.

Materials and methods

Cells and reagents

Human colorectal epithelial cell line CoTr and carcinoma cell lines HT-29 and Colo-201 were from the ATCC (Rockville, MD). RE7 cells, a clone of Madin–Darby canine kidney cells transfected with a human RON cDNA, were as described (18). Human mature MSP was kindly provided by Dr E.J.Leonard (National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD). Mouse mAb (clone ID2) to the extracellular domain of human RON and rabbit IgG antibodies against the synthetic C-terminal peptide of the RON β chain were as described (18,27). The mouse mAb to phosphotyrosine (clone 4G10) was from Upstate Biotechnology (Lake Placid, NY). Enhanced chemiluminescence (ECL) detection reagents were
from Amersham (Arlington Heights, IL), Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were from Life Technologies (Gaithersburg, MD).

Immunoprecipitation and western blotting

Cells (3–5×10⁶ per sample) were lysed with 200 µl of lysis buffer (50 mM Tris, pH 7.4, 0.5% Triton X-100, 0.5% NP-40, 150 mM NaCl, 2 mM EDTA, 100 mM vanadate, 5 µg/ml leupeptin, 5 µg/ml aprotinin and 10 µg/ml soybean trypsin inhibitor). Cellular proteins were incubated overnight at 4°C with 1 µg of mAb ID2 coupled to protein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). Samples were separated on an 8% polyacrylamide gel under reducing or non-reducing conditions and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Western blotting was performed using rabbit IgG to RON or mAb 4G10 to phosphotyrosine, followed by goat anti-rabbit or rabbit IgG conjugated with horseradish peroxidase (Roche). The reaction was developed with ECL reagents. In some experiments the membrane was treated with SDS/2-mercaptoethanol erasure buffer (18) and re-probed with other antibodies.

Reverse transcription (RT)–PCR and DNA sequencing

Total RNAs were isolated from HT-29 or Colo-201 cells using TRIzol (Life Technologies). Reverse transcription was carried out using 2 µg of total RNAs with a SuperScript Preamplification Kit (Life Technologies). PCR was conducted on the products of RT reactions. The oligomers for PCR amplification used to clone the RONp160 cDNA were designed on the basis of the RON cDNA sequences (15): pair 1, sense oligomer, nt –183 to –164 (5′-GAGGGCCCGGGAGGGATTG-3′) and antisense oligomer, nt 2292 to 2315 (5′-TGCCAGATCTGCTGCTCCGAG-3′); pair 2, sense oligomer, nt 2165 to 2189 (5′-ACACCCTGGCCGGTGCTGCTCAAT-3′) and antisense oligomer, nt 3650 to 3672 (5′-ACCTGGTCGGCCGAACTGCAT-3′); pair 3, sense oligomer, nt 5122 to 5145 (5′-ACACCTGGTCATCGGACATCC-3′) and antisense oligomer, nt 4366 to 4389 (5′-CCATTAGCTCCCTACTCACAGA-3′). Three amplified fragments were designated fragments I, II and III. Double-stranded DNA sequencing was performed in the University of Colorado Cancer Center DNA Sequence Core facility.

Construction of the full-length RONp160 cDNA and its expression in CoTr cells

The 2.5 and 2.2 kb PCR fragments covering the nucleotide sequence from –183 to 2315 were amplified from RT products using oligomer pair 1 (–183 to –164 and 2292–2315). The fragments were then subcloned into the pGEM-T Easy vector (Promega). After confirming the deleted regions in the 2.2 kb fragment by DNA sequencing, the cDNA fragment was digested with restriction enzymes EcoRI and Nhel. The resulting 700 bp fragment, designated ΔRON cDNA, was isolated. This fragment is shorter than the regular RON PCR product derived from Colo201 cells and has an in-frame deletion of 327 nt. To construct the RONp160 cDNA, the mammalian expression vector pDR2 (Clonetech) containing a full-size wild-type RON cDNA (pDR2–RON) was digested with EcoRI and Nhel to eliminate a 1 kb fragment. The digested pDR2–RON fragment was then ligated with the 0.7 kb ΔRON cDNA fragment to create the full-length RONΔ160 cDNA (pDR2–ΔRON). Transfection of CoTr cells with the pDR2–ΔRON vector was performed using the DOTAP (Roche) transfection reagent as described (18). Cells were selected with 50 µg/ml hygromycin B. Cells expressing RONΔ160 were isolated by positive selection using Dynal beads (Dynal) coupled with mAb ID2. Expression of RONΔ160 was determined by western blotting with rabbit IgG to RON peptide.

Cell migration assay

The migration assay was performed as previously described (18). Briefly, bottom wells of a multiwell chemotaxis chamber were filled with 30 µl of DMEM containing different amounts of MSP in duplicate and then covered with a collagen IV-coated membrane. Upper wells were filled with 45 µl of cell suspension (4×10⁶ cells/ml in DMEM). The chamber was disassembled after a 5 h incubation at 37°C. The membranes were air dried and stained with Diff-Quik. The cells which had migrated were counted in three randomly selected areas. The results are expressed as the percentage of input cells that migrated.

Matrigel invasion assay

The assay was performed as described (30) with slight modification. Briefly, a polycarbonate membrane (10 µm pore size) was placed on the bottom of the upper well of the Transwells (Costar) and coated with 1.2 mg/ml Matrigel (Collaborative Research). Cells (1×10⁶ in 200 µl of serum-free medium) were then added to the coated membrane. The bottom wells were filled with medium or MSP (5 nM). The plate was incubated for 24 h at 37°C. Cells that invaded the Matrigel and adhered to the lower side of the membrane were fixed. The cells which had migrated were counted in three randomly selected areas. Results are expressed as the percentage of input cells that invaded.

Results

Detection of RONΔp160 in human colon carcinoma cell line HT-29

During the course of studying RON expression in human colorectal epithelial cells, we found that RON is highly expressed in carcinoma cell lines HT-29 and Colo-201. In contrast, SV-40-immortalized colon epithelial cells (CoTr) barely expressed RON. These results are shown in Figure 1. The mature RON β chain with a molecular mass of 140 kDa was present in both Colo-201 and HT-29 cells. Interestingly, a protein strongly reacting with anti-RON antibodies was also detected in HT-29 cells. Under reducing conditions this protein showed a molecular mass of 125 kDa (Figure 1A), which represents a short form of the RON β chain. However, under non-reducing conditions the protein has a molecular mass of 160 kDa (designated RONΔp160) (Figure 1B). RONΔp160 is composed of a regular α chain and the short form of the β chain (see details below). These results indicate that HT-29 cells express two forms of RON, a mature RON and an isoform of RON. Because the RON isoform could be immunoprecipitated with mAb ID2 to the extracellular domain of RON and detected by antibodies against the C-terminal peptide of RON, we reasoned that this short form of RON is a novel RON variant.

Constitutive activation of RONΔp160 in HT-29 carcinoma cells

To determine if RONΔp160 could be activated by MSP, HT-29 cells were serum starved overnight and then stimulated with or without 5 nM MSP. CoTr cells were used as a negative control. After immunoprecipitation of RON and RONΔp160 with mAb ID2, the phosphorylation assay was performed by western blotting using mAb 4G10 to phosphotyrosine. The results are shown in Figure 2. In non-stimulated HT-29 cells autophosphorylation of the 140 kDa RON β chain was observed. MSP stimulation did not further increase the phosphorylation status of RON. Interestingly, p125 was also autophosphorylated, even in the absence of MSP. MSP stimulation moderately increased p125 phosphorylation. These results suggest that RON and its variant form RONΔ160 are constitutively active in HT-29 cells.

Molecular cloning of the cDNA fragment encoding the RON variant

To study RONΔp160 in more detail, RT–PCR was performed to determine the deleted region and to clone the cDNA encoding RONΔp160 expressed in HT-29 cells. Figure 3 illustrates the procedures used in cloning and constructing the
A RON variant in colon cancer cells

Fig. 2. Spontaneous phosphorylation of RON and its variant in HT-29 cells. Cells (3 × 10^6/ml) were stimulated with or without 5 nM MSP at 37°C for 10 min. Immunoprecipitation was performed as described. The mAb 4G10 was used to detect phosphorylated protein. The reaction was developed with ECL reagents. Results shown here are from one of three experiments with similar results.

Fig. 4. Amplification of RON cDNA fragments from HT-29 and Colo-201 cells. RT–PCR was performed using a pair of primers covering nucleotides –183 to 2315. Two prominent bands with sizes of 2.5 and 2.2 kb were identified in HT-29 cells. The 2.5 kb fragment was also found in Colo-201 cells. These three fragments were isolated for DNA sequence analysis. The 2.8 kb fragment from Colo-201 cells is a non-specifically amplified cDNA unrelated to RON.

was ligated into the pDR2 mammalian expression vector (Clontech) and then transfected into CoTr cells. RON ∆p160 expression was determined by western blotting. The results are shown in Figure 6A. In RON ∆p160 cDNA-transfected cells two bands with molecular masses of 160 and 125 kDa, respectively, were observed. The 160 kDa band is the uncleaved pro-RON ∆p160 protein. The 125 kDa band is the RON ∆β chain. These data suggest that RON ∆p160 is first synthesized as a single chain precursor and then proteolytically cleaved into the two chain form. The α chain of RON ∆p160 was not seen because our antibodies did not recognize it.

The data in Figure 6B show the results from tyrosine phosphorylation experiments. RON ∆p160 is constitutively activated in CoTr cells even without ligand stimulation. MSP stimulation slightly increased phosphorylation of the RON ∆β chain. These results are consistent with those shown in Figure 2, indicating that deletion of 109 amino acid residues in the full-length RON ∆p160 cDNA-transfected cells was independent of MSP.

Expression and activation of RONΔcDNA in CoTr cells

The full-length cDNA of RONΔp160 was constructed by replacement of the Eco47III–NheI fragment in the wild-type RON cDNA with the cloned fragment containing the deleted 327 nt (Figure 3). The resultant RONΔp160 cDNA was ligated into the pDR2 mammalian expression vector (Clontech) and then transfected into CoTr cells. RONΔp160 expression was determined by western blotting. The results are shown in Figure 6A. In RONΔp160 cDNA-transfected cells two bands with molecular masses of 160 and 125 kDa, respectively, were observed. The 160 kDa band is the uncleaved pro-RONΔp160 protein. The 125 kDa band is the RONΔ β chain. These data suggest that RONΔp160 is first synthesized as a single chain precursor and then proteolytically cleaved into the two chain form. The α chain of RONΔp160 was not seen because our antibodies did not recognize it.

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Induction of cell migration and matrix invasion by RONΔp160

To study the function of RONΔp160, we established CoTr cell lines expressing this RON variant. These cells, together with CoTr cells transfected with wild-type RON, were used in a cell migration assay. The results are shown in Figure 7A. CoTr cells transfected with the pDR2 vector alone did not migrate even in the presence of MSP. These data are consistent with the results shown in Figures 1 and 2, which indicate that CoTr cells do not express RON. In contrast, CoTr cells expressing wild-type RON responded to MSP. Cells migrated in a MSP concentration-dependent manner. Maximal cell migration was observed when 2.5 nM MSP was added. Interestingly, expression of RONΔp160 induced increased motility of transfected cells. The number of cells that migrated was comparable with CoTr cells expressing wild-type RON, however, migration of RONΔp160 cDNA-transfected cells was independent of MSP. This effect was probably caused by constitutive activation of RONΔp160 in transfected cells.
Fig. 5. Partial nucleotide sequence of the RON cDNA fragment II obtained from HT-29 cells. RT–PCR and DNA sequencing were performed as described in Materials and methods. The deleted 109 amino acids in RONΔp160 are underlined with a solid line. The cysteine residues in the sequences are boxed.

Fig. 6. Expression and activation of RONΔp160 in CoTr cells. Transfection, immunoprecipitation and detection of RONΔp160 were performed as described. To determine whether RON is phosphorylated, cells were stimulated with 5 nM MSP as described in Figure 2. One of three experiments with similar results is presented.

The matrix invasiveness of CoTr cells expressing wild-type RON or RONΔp160 was also studied in an invasion assay. The results are shown in Figure 7B. Activation of wild-type RON by MSP caused CoTr cells to invade the Matrigel. CoTr cells expressing RONΔp160 also showed increased invasive activity. The addition of MSP further increased the invasiveness of RONΔp160-expressing cells. These results are consistent with the data shown in Figure 7A, indicating the effect of constitutive activation of RONΔp160 in transfected cells. Taken together, these results suggest that RONΔp160 expression is characterized by the acquisition of a motile–invasive phenotype by transfected colon epithelial cells.

Discussion

We report here the identification and cloning of a novel splice variant of RON, namely RONΔp160, in human colorectal carcinoma cell line HT-29. The functional properties of RONΔp160 are particularly interesting with regard to the role...
of RON in tumorigenesis of colorectal carcinoma cells for several reasons. First, RON\(\Delta p160\) is constitutively active in HT-29 cells. Expression of RON\(\Delta p160\) in CoTr cells also results in autophosphorylation of the protein, suggesting that deletion of the extracellular domain of the RON \(\beta\) chain causes structural changes that lead to spontaneous tyrosine phosphorylation. Second, expression of RON\(\Delta p160\) in CoTr cells results in cells with increased motility and invasiveness. These data indicate that RON\(\Delta p160\) has the ability to transduce signals that activate the motile machinery of the cells. In other words, the motile–invasive activities of RON\(\Delta p160\) could play an important role in promoting the invasive phenotype of colorectal carcinoma cells in vivo. Finally, generation of an altered form of the receptor tyrosine kinase by the RON gene may represent a unique mechanism for RON in the progression of colorectal carcinomas.

RON\(\Delta p160\) is the product of a transcript differing from the full-length RON mRNA. As described for the RT–PCR experiments, two PCR fragments of 2.5 and 2.2 kb could be amplified from mRNAs derived from HT-29 cells, using primers covering nt –183 to 2315. DNA sequence analysis confirmed that a deletion of 327 nt occurred in the 2.2 kb fragment. Thus, the predicted RON variant has an in-frame deletion of 109 amino acids in the extracellular domain of the RON \(\beta\) chain, which will have a molecular mass of 125 kDa. The results of our western blot analysis confirmed this prediction. The \(\beta\) chain of RON\(\Delta p160\) displayed a molecular mass of 125 kDa, which matches the predicted size of the protein.

Deletion of large numbers of amino acids within a protein is usually the result of mRNA splicing (31). The existence of splicing variants has been found in a variety of oncoproteins, such as HER2, Smad2 and MET (11,32,33). These splicing products display different biological properties in comparison with the non-spliced proteins (11,32,33). The available information on the genomic structures of the human RON gene (34) prompted us to determine whether production of the RON\(\Delta p160\) protein is due to deletion of certain exons in the RON gene. The human RON gene consists of 20 exons and 19 introns (34). Comparing genomic sequences that encode the extracellular domain of RON, we found that the 109 amino acids from Phe574 to Arg627 represent two exons of the RON gene, exons 5 and 6. Exon 5 contains 161 nt and encodes 54 amino acid residues from Phe574 to Arg627. Exon 6 has 166 nt and encodes 55 amino acid residues from Pro628 to Met682. Deletion of these 109 amino acids results in the direct connection of Glu573, the last amino acid residue of exon 4, with Glu683, the first amino acid residue of exon 7. Currently, the mechanism(s) that governs the splicing process of RON mRNA in HT-29 cells is unknown. However, because selection of a splicing site depends not only on its own sequence but also on the context around that sequence (31), it is reasonable to think that the elimination of exons 5 and 6 is not a random splicing process occurring in HT-29 cells. Our recent data also indicate that deletion of these two exons is not related to a genomic abnormality in the boundary sequences between introns and exons, because no mutations were found in the deleted region and the surrounding sequences (our unpublished data).

The mature two chain form of RON is derived from a single chain precursor, which is cleaved at the Lys305-Arg-Arg-Arg sequence, generating \(\alpha\) and \(\beta\) chains linked by a disulfide bond (15). At present it is not known whether cleavage of pro-RON occurs at the cell surface or in the cytoplasm, however, this conversion is critical for exposure of the RON receptor on the cell surface because unprocessed pro-RON remains in the cytoplasm (29). Using cDNA transfection techniques, we confirmed that RON\(\Delta p160\) expressed in CoTr cells follows the same proteolytic mechanism as wild-type RON. The generated proteins are composed of an intact \(\alpha\) chain and the short \(\beta\) chain. More importantly, the proteolytic process allows RON\(\Delta p160\) to reside on the cell surface and interact with MSP. These results suggest that deletion of 109 amino acids in the extracellular domain of the RON \(\beta\) chain does not affect cellular protease(s) access to the cleavage site. This information is of interest, because a previous report showed that deletion of 49 amino acids encoded by exon 11 in the extracellular domain of the RON \(\beta\) chain affected conversion of the RON precursor and its transportation (29). The protein did not undergo proteolytic conversion and remained in the cytoplasm (29). The reason(s) that this particular RON variant was not cleaved is unknown. One possible explanation is that deletion of these 49 amino acids fundamentally changes three-dimensional structures of the pro-RON variant, which results in ‘masking’ of the cleavage site, however, in our case the cleavage site is still exposed on the surface of the pro-RON\(\Delta p160\) molecule despite deletion of a large number of amino acids. Furthermore, RON\(\Delta p160\) is capable of interacting with the ligand MSP, as is evident from its further phosphorylation upon MSP stimulation.

As observed, RON\(\Delta p160\) is constitutively active in TH-29 cells and in transfected CoTr cells. Activation could be further increased by MSP, as is evident from the tyrosine phosphorylation of RON\(\Delta p160\) in transfected CoTr cells. Although we did not study the mechanisms of how the deletion caused receptor activation, it is worth pointing out that there are three cysteines in the deleted region. Lack of these critical amino acids creates an uneven number of cysteine residues in the extracellular domain of the RON\(\Delta p160\) \(\beta\) chain. It has been shown that the unbalanced number of cysteine residues in the extracellular domain of the RON variant results in abnormal formation of intermolecular disulfide bonds (29). The consequence of such abnormal bond formation is oligomerization of the altered receptor and autophosphorylation (29). We think that the same mechanism may apply to the spontaneous activation of RON\(\Delta p160\) in HT-29 cells, however, further studies using DNA mutation techniques are needed to reveal whether our hypothesis is valid.

It is clear from our current findings that deletion of 109 amino acid residues located in the extracellular domain of the RON \(\beta\) chain has a significant impact on receptor function. Similar results have also been demonstrated in a RON variant reported in a previously published paper (29). With spontaneous activation of RON\(\Delta p160\) the downstream motile–invasive program is induced, and further increased by ligand stimulation. Moreover, transfected CoTr cells acquire a phenotype characterized by the ability to migrate through a membrane and penetrate an artificial basement membrane in vitro. This property correlates with the metastatic phenotype displayed in vivo by malignant tumors (35,36). Because of the unique biological properties of RON\(\Delta p160\), it will be of interest in the future to determine such functions of RON\(\Delta p160\) as cell transforming potential and to study its role in the progression of human colorectal carcinoma cells in vivo.
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References

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