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A Splicing Variant of the *RON* Transcript Induces Constitutive Tyrosine Kinase Activity and an Invasive Phenotype

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The Ron tyrosine kinase receptor shares with the members of its subfamily (Met and Sea) a unique functional feature: the control of cell dissociation, motility, and invasion of extracellular matrices (scattering). The mature Ron protein is a heterodimer of disulfide-linked α and β chains, originated by proteolytic cleavage of a single-chain precursor of 185 kDa. In a human gastric cancer cell line (KATO-III), we found abnormal accumulation of an uncleaved single-chain protein (Δ-Ron) of 165 kDa; 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. The deleted transcript originates by an alternatively spliced cassette exon of 147 bp, flanked by two short introns. The Δ-Ron tyrosine kinase is constitutively activated by disulfide-linked intracellular oligomerization because it contains an uneven number of cysteine residues. Oligomerization and constitutive tyrosine phosphorylation of the full-size Ron was obtained by site-directed mutagenesis of a single cysteine residue in the region encoded by the cassette exon, mimicking that occurring in the Δ-Ron isoform. Inhibition of thiol-mediated intermolecular disulfide bonding prevented Δ-Ron oligomerization. The intracellular activation of Ron is followed by acquisition of invasive properties in vitro. These data (i) provide a novel molecular mechanism for posttranscriptional activation of a tyrosine kinase receptor protein and (ii) suggest a role for the Ron receptor in progression toward malignancy.

One of the cellular mechanisms responsible for generating protein diversity is alternative RNA splicing, producing multiple isoforms from a single gene. Several examples have been described for transcripts encoding proteins involved in cellular signal transduction, including fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, nerve growth factor, and insulin receptors and the cytoplasmic kinases Src and Lyn (for a review, see references 3 and 6). Different isoforms generated by alternative splicing have also been described for Met, the hepatocyte growth factor receptor (HGF) (22, 35). Met is the prototype of a tyrosine kinase receptor family, including the product of avian *sea* (16) and Ron, the human receptor for the macrophage-stimulating protein (MSP) (13, 36).

Ron is a heterodimeric protein ($p185^{RON}$) composed of α and β subunits derived from proteolytic cleavage of a common precursor of 170 kDa (13), as previously described for the HGF receptor (14). $p185^{RON}$ is expressed in several epithelial tissues, in granulocytes, and in monocytes as the translation product of a 5.0-kb transcript. The extracellular domain of Ron binds the ligand; the membrane-spanning β subunit contains an active tyrosine kinase domain (13). Other important features common to the products of *RON* and *MET* are the major autophosphorylation sites, made of two neighboring tyrosine residues (23), and a multifunctional docking site located in the receptor tail that mediates signal transduction (32). Binding of MSP to $p185^{RON}$ stimulates the intrinsic tyrosine kinase activity and phosphorylation of the docking site recognized by multiple SH2 domain-containing transducer and adapter proteins. This activates the intracellular signaling cascade eliciting a distinctive biological response, shared by Ron and the other members of the *MET* gene family: cell dissociation, motility, and polarized growth (for a review, see reference 8).

In this paper, we report constitutive activation of the Ron receptor by intracellular oligomerization of an uncleaved protein isoform lacking 49 amino acids in the extracellular domain (Δ-Ron). This molecule originates from alternative splicing of one cassette exon of 147 bp, because of the presence of a noncanonical splice site consensus sequence. In a gastric carcinoma cell line (KATO-III), the Δ-*RON* mRNA is translated into an uncleaved precursor protein ($p165^{\Delta\text{-}RON}$) containing an uneven number of cysteine residues. Because of aberrant intermolecular disulfide bridge formation, the Δ-Ron tyrosine kinase oligomerizes in the intracellular compartment and is constitutively activated. This mechanism was mimicked by site-directed mutagenesis of one cysteine in the Ron extracellular domain. In the mirror experiment, inhibition of thiol-mediated intracellular oligomerization by addition of the reducing agent 2-mercaptoethanol prevented constitutive activation. Interestingly, transfection of Δ-*RON* cDNA was followed by the acquisition of an invasive phenotype by recipient cells.

MATERIALS AND METHODS

Cells lines and antibodies. GTL-16 is a clonal cell line derived from a poorly differentiated gastric carcinoma (15). The other cell lines used, KATO-III (stomach carcinoma), T47D (mammary carcinoma), PT45 (pancreatic carcinoma), MKN-1 (gastric carcinoma), SKOV3 (ovary adenocarcinoma), NIH:OVCAR3 (ovary adenocarcinoma), HOS (bone osteosarcoma), HeLa (cervix carcinoma), COS-1 (simian kidney), and U293 (human kidney), were from the American Type Culture Collection. Cells were grown in RPMI 1640 or in Dulbecco's modified Eagle medium containing 10% fetal calf serum and maintained at 37°C in a humidified atmosphere with 5% CO_2. Phosphotyrosine monoclonal antibodies were from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Ron antibodies were raised as previously described (13).

Immunoprecipitation and kinase assays. Treatment with reducing agents was performed by growing cells for 2 h in the presence of millimolar concentrations of 2-mercaptoethanol. Monolayers of subconfluent cell cultures were extracted for 20 min at 4°C with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholate, 100 μM Na_3VO_4) containing pro-

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tease inhibitors. For nonreducing analysis, 50 mM iodoacetamide was included in the lysis buffer. The cell lysates were cleared by centrifugation at 15,000 × *g* for 20 min at 4°C. Lysates were incubated with Ron antiserum (2.5 μl/ml of lysate) coupled to Sepharose-protein A (20 μl of packed beads per ml of lysate) for 2 h at 4°C with agitation. Sepharose-protein A-bound proteins were washed four times with RIPA buffer and incubated in 50 μl of kinase buffer (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM $MnCl_2$, 0.05% Triton X-100, 5 μM leupeptin), supplemented with 40 μM ATP and 10 μCi of [γ-^{32}P]ATP (specific activity, 5,000 Ci/mmol; Amersham, Little Chalfont, United Kingdom) for 10 min at room temperature. Proteins were separated on SDS–5 to 10% gradient polyacrylamide gels; gels were dried and exposed for autoradiography at −80°C, using intensifying screens. In vivo tyrosine phosphorylation was evaluated by Western blotting (immunoblotting) of the immunoprecipitates with phosphotyrosine antibodies. Subconfluent KATO-III and T47D cells were treated with conditioned medium from U293 cells, either control or MSP transfected, for 10 min at 37°C. Cells were lysed in RIPA buffer for immunoprecipitation.

Electroelution of proteins from SDS-gels. Kinase-assayed immunoprecipitates from KATO-III cells were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (5 to 10% gradient gel) under nonreducing conditions. The labeled high-molecular-weight bands of interest were located by autoradiography, excised from the dried gel, swollen in 20 μl of boiling water, mixed with an equal volume of 1× Laemmli buffer, and analyzed by SDS-PAGE in the presence of 2-mercaptoethanol.

Cell surface biotinylation. Cell surface biotinylation was performed by using an ECL (enhanced chemiluminescence) biotinylation kit (Amersham) as instructed by the supplier. After labeling, cells were extracted with ice-cold detergent-insoluble matrix buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 100 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% Triton X-100, protease inhibitors] for immunoprecipitation. Proteins were subjected to SDS-PAGE (7% gel) and Western blotting with streptavidin-horseradish peroxidase and an ECL kit (Amersham).

Northern (RNA) blotting. For Northern analysis, total RNA was extracted as described by Chomczynski and Sacchi (7). Formaldehyde-agarose gel electrophoresis and Northern transfer were performed as described by Wahl et al. (45). Hybridization was carried out at 65°C in Rapid Hyb Buffer (Amersham) in the presence of 10^6 cpm of a [α-^{32}P]dCTP oligo-random labeled probe (specific activity, $>10^8$ cpm/μg of DNA) per ml labeled with the Ready to Go system (Pharmacia, Uppsala, Sweden). The specific probes used were the cDNA encompassing the entire coding sequence of *RON* and a fragment of 147 bp, corresponding to the region of the receptor deleted in Δ-*RON*, obtained by PCR amplification. PCR-amplified fragments shown in Fig. 3B were subjected to 1% agarose gel electrophoresis, transferred to Hybond-N+ (Amersham) nylon membranes, and hybridized with the full-length *RON* cDNA probe by using a Direct nucleic acid labeling system kit (Amersham) as instructed by the supplier.

RT and PCR. Total RNA, extracted with guanidinium thiocyanate as described by Chomczynski and Sacchi (7), was used for reverse transcription (RT). The 40-μl reaction mixture contained the enzyme buffer as supplied by Bethesda Research Laboratories, (BRL), 2 μg of RNA, 1 U of RNAsin (Promega Biotec, Madison, Wis.) per μl, 50 pmol of the 3′ PCR primer (see below), 1 mM each deoxynucleotide triphosphate, and 10 U of Moloney murine leukemia virus reverse transcriptase (BRL) per μl. The reaction mixture was incubated at 37°C for 1 h, the enzyme was then denatured for 3 min at 95°C, and the products were kept at −20°C. PCR was carried out on the products of the RT reactions as follows. In a final volume of 100 μl, the reaction mixture contained 10 μl of the products of the RT reaction as source of a template, 50 mM Tris (pH 8.4), 50 mM KCl, 25 μg of bovine serum albumin (BRL) per ml, 2 μM each deoxynucleotide triphosphate, 35 pmol of the 3′ PCR primer, 35 pmol of the 5′ PCR primer, and a variable concentration of $MgCl_2$. Five units of *Taq* polymerase (Promega Biotec) was added, and 100 μl of mineral oil was overlaid on the reaction mixture. Thirty cycles of denaturation, annealing, and extension were then performed, using a Programmable Thermal Controller (M.J. Research, Inc.). Denaturation was at 92°C for 5 min for the first cycle and for 1 min for subsequent cycles. Annealing temperatures varied between 45 and 63°C according to the base composition of the primers; annealing time was 1 min. The extension temperature was 72°C. The extension time was calculated by assuming a rate of extension of 1,000 bases/min, according to the predicted length of the amplified product. The oligomers for PCR amplifications used to clone Δ-*RON* cDNA were designed on the basis of the *RON* cDNA sequence as follows: pair 1, sense oligomer corresponding to nucleotides −183 to −164 (5′-GAGGGCCGGGAA GGGATTTG-3′) and antisense oligomer corresponding to nucleotides 393 to 415 (5′-TGCTGGTGCTGGATCCCGCGCT-3′); pair 2, sense oligomer corresponding to nucleotides −38 to −12 (5′-CCGGTAGGGATCCTCTAGGGTC CC-3′) and antisense oligomer corresponding to nucleotides 2292 to 2315 (5′-T GCCCAGGTACCTGGTTCCTGGA-3′); pair 3, sense oligomer corresponding to nucleotides 2165 to 2184 (5′-ACCAGCCGGGCTGTGCTGGT-3′) and antisense oligomer corresponding to nucleotides 3650 to 3672 (5′-ACCTGGCTGC GCGGAACTGCAT-3′); and pair 4, sense oligomer corresponding to nucleotides 3235 to 3256 (5′-GTCAAGGATGTGCTGATTCCC-3′) and antisense oligomer corresponding to nucleotides 4366 to 4389 (5′-TCTGTGGAGTGAG GTACCTAATG-3′).

Sequences encompassing the region of divergence between the cDNA clones isolated from KATO-III cells were amplified on genomic DNA by using two primers flanking this region: sense oligomer corresponding to nucleotides 2619 to 2641 (5′-ATCCACCCAGTGCCAACCTAGTT-3′) and antisense oligomer corresponding to nucleotides 2873 to 2895 (5′-GGCCAGATGGGGTCCCACA GAG-3′). One hundred nanograms of genomic DNA was PCR amplified by 20 touchdown cycles (11) with denaturation at 95°C for 1 min, annealing temperature decreasing from 64 to 54°C for 30 s, extension at 72°C for 1 min, and 10 additional cycles at the lowest annealing temperature. The PCR products were subcloned into pUC18 vector and sequenced on both strands.

DNA sequencing. Double-stranded DNA sequencing was carried out by the dideoxynucleotide method (37), using Sequenase (U.S. Biochemicals) and the Sequenase protocol.

Site-directed mutagenesis. The C914A-Ron mutant was created by using a PCR cloning strategy. An antisense primer corresponding to nucleotides 2732 to 2777 (5′-GGCAGACAACCATGTCCCCCGGAACTCGTGCTGG**GC**GCTC TCACC-3′), including a *Drd*I restriction site for further cloning, was used to mutate Cys-914 to Ala (TGC to GCC), and a sense primer, corresponding to nucleotides 2246 to 2268 (5′-CCTGGGGCCACGGTGGCCAGTGT-3′), containing an *Sfi*I restriction site was used. The PCR product, double-strand sequenced, was inserted as an *Sfi*I-*Drd*I cassette in the pMT2*-*RON* vector. The pMT2*C914A/*RON* cDNA was used for transient expression in COS-1 cells.

Transfection in COS-1 cells. The *RON* and Δ-*RON* cDNAs were inserted into the pMT2* eukaryotic expression vector, which contains the major late adenovirus promoter and the simian virus 40 origin of replication. Subconfluent COS-1 cells were transfected with 10 μg of the expression constructs by the DNA-calcium phosphate coprecipitation procedure (CellPhect Transfection kit; Pharmacia) (4). Three days after transfection, expression of the Δ-Ron and Ron proteins was analyzed by Western blotting.

Transwell migration and invasion assays. Transfected cells were starved for 24 h in serum-free medium before motility and invasion assays were performed, 100 h after transfection. Stimulation of cell invasiveness was determined by using a modification of the Matrigel method (21). Polycarbonate membranes (8-μm pore size) on the bottom of the upper compartment of the Transwells (6.5 mm; Costar Corporation, Cambridge, Mass.) were coated with 1.2 mg of Matrigel (Collaborative Research Incorporated, Waltham, Mass.) per ml. A total of 10^5 transfected cells resuspended in 200 μl of serum-free medium were placed on the Matrigel-coated polycarbonate membrane in the upper compartment. One milliliter of medium, either serum free or containing 20 ng of the stimulating factor, was added to the lower compartment. The plates were incubated at 37°C in a 5% CO_2 atmosphere saturated with H_2O for 24 h. At the end of incubation, the cells and Matrigel at the upper side of the polycarbonate filters were mechanically removed. Cells that had invaded the Matrigel and migrated to the lower side of the filter were fixed with 11% glutaraldehyde for 15 min at room temperature, washed three times with distilled water, and stained with 0.1% crystal violet–20% methanol for 20 min at room temperature. After three washes with water and complete drying at room temperature, the crystal violet was solubilized by immersing the filters in 100 μl of 10% acetic acid (5 min at room temperature). The concentration of the solubilized crystal violet was evaluated as A_{595}.

RESULTS

Detection of a tyrosine-phosphorylated 165-kDa Ron isoform (Δ-Ron) in a gastric carcinoma cell line. Carboxy-terminus-specific antibodies against the Ron receptor ($p185^{RON}$), after immunoprecipitation and Western blotting under reducing conditions identify one single band, with an apparent molecular mass of 150 kDa, corresponding to the β chain. In cells expressing high levels of Ron, the antibodies also identify the $pr170^{RON}$ precursor as a split double band due to a different extent of glycosylation (13). In a screening of tumor cells, the Ron antibodies identified a protein with apparent molecular mass of about 165 kDa (Δ-Ron) (Fig. 1). This abnormal molecule was expressed in the gastric cancer KATO-III cell line and coexisted with the regular 150-kDa Ron β chain, albeit expressed in low quantities.

After specific immunoprecipitation, Western blot experiments with phosphotyrosine antibodies showed that the Δ-Ron protein was phosphorylated on tyrosine either in the absence or in the presence of the ligand (Fig. 2). As described before (13), tyrosine phosphorylation of the Ron β chain is observed only upon MSP stimulation. The Ron β chain was found phosphorylated in KATO-III cells also in the absence of ligand, probably through Δ-Ron transphosphorylation.

The Δ-Ron protein originates from an alternatively spliced mRNA. Rearrangements causing aberrant migration and de-

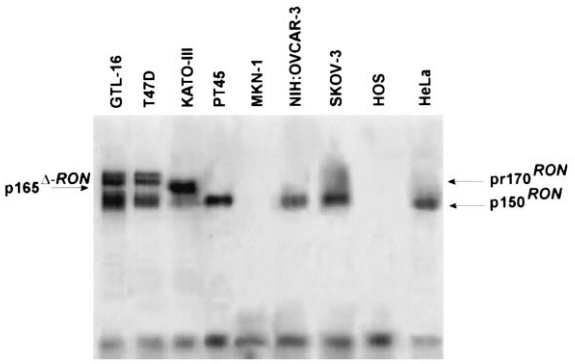
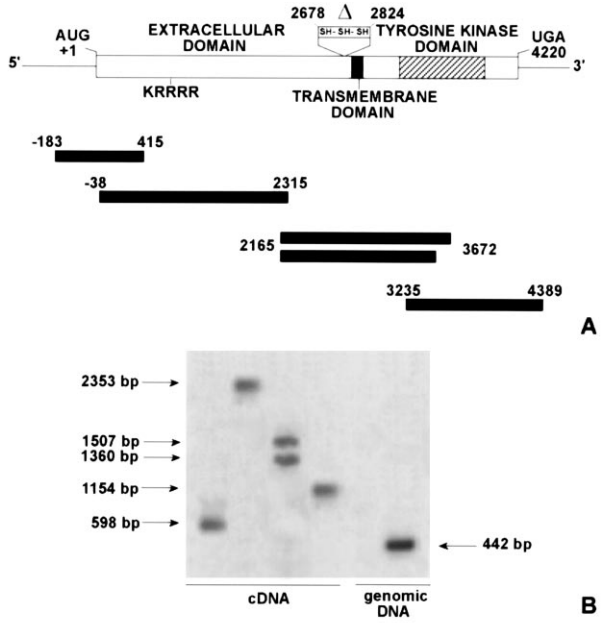


FIG. 1. Ron receptor expression. Detergent-solubilized proteins from tumor cell lines were immunoprecipitated with antibodies directed against the C terminus of the Ron β chain, separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose paper, and probed with the same antibodies. The specific antibodies identified one single band, with an apparent molecular mass of 150 kDa, corresponding to the Ron β chain. When expressed at detectable levels, the pr170^{RON} precursor was also evident as a tightly compressed double band. In the gastric cancer KATO-III cell line, the Ron antibodies identified a protein with an apparent molecular mass of about 165 kDa (Δ-Ron) which coexists with the 150-kDa Ron β chain, albeit expressed in low amount. Arrows indicate the uncleaved precursor (pr170^{RON}), the mature β chain (p150^{RON}), and the 165-kDa protein (p165$^{\Delta\text{-}RON}$).



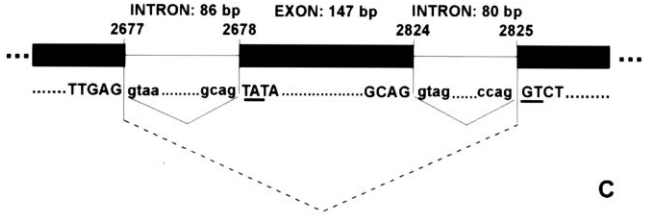


FIG. 3. The Δ-Ron protein originates from an alternatively spliced mRNA. (A) Schematic representation of the full-length Δ-*RON* cDNA cloned from KATO-III cells. RT-PCR was performed to amplify partially overlapping sequences, spanning the entire *RON* cDNA from position −183 in the 5′ untranslated region to position 4389 in the 3′ untranslated region. Two distinct cDNA clones were obtained during the amplification between nucleotides 2165 and 3525. All cDNA clones were sequenced on both strands. The consensus sequence for the precursor cleavage (KRRRR), the transmembrane and kinase domains, and the 147-bp (Δ) region deleted in Δ-*RON* are indicated. The three critical cysteine residues missing in Δ-*RON* cDNA are also shown. (B) The PCR amplification fragments were resolved by electrophoresis in 1% agarose gel, transferred to a nylon filter, and analyzed by hybridization with the entire *RON* cDNA as a probe. The difference in size of the cDNA clones between nucleotides 2165 and 3525 corresponded precisely to the length of the deletion (147 bp). One PCR product was obtained from the corresponding region on genomic DNA extracted from KATO-III cells. (C) The genomic PCR amplification product was sequenced, and exon-intron junctions were identified. The drawing represents the partial exon-intron structure surrounding the region of divergence between the *RON* and Δ-*RON* cDNAs. The alternative splicing event that generates the Δ-*RON* transcript is indicated by the dashed line. Uppercase letters designate coding sequences; nucleotides in the introns are in lowercase letters. The alternative acceptor sites are underlined. The alternative splicing of the 147-bp region occurs through the use of an acceptor site for the 86-bp 5′ intron. Its donor site, located at the 3′ end of the preceding exon, can be spliced at bp 2678, generating the *RON* mRNA, or at bp 2825, generating the Δ-*RON* transcript.

regulated activity have been reported for the *MET* gene (30), a *RON* homolog. To investigate if the Δ-Ron protein results from a possible genetic damage, the full-length cDNA was cloned from KATO-III cells. RT-PCR was performed to amplify partially overlapping sequences, spanning the entire *RON* cDNA from position −183 in the 5′ untranslated region to position 4242 in the 3′ untranslated region, using four oligonucleotide primer pairs designed on the basis of the previously reported *RON* sequence (36). Several cDNA clones obtained by amplification were sequenced on both strands (Fig. 3A). The only mutation detected was a single G-A transversion, resulting in a Gln-Arg substitution in the extracellular domain at amino acid position 322. (To exclude the possibility that this point mutation was responsible for the changes in properties of the p165 isoform [see below], the Gln→Arg substitution at position 322 was corrected in the Δ-*RON* cDNA by site-directed mutagenesis. No changes in biochemical or biological properties were observed [data not shown].) However, when the region between nucleotides 2165 and 3525 was amplified, two distinct fragments were obtained. The specificity of the fragments was ensured by hybridization analysis with a specific *RON* probe. The difference in size between the two bands corresponded to a sequence of 147 bp, i.e., 49 amino acids, matching the size reduction measured in Δ-Ron. The presence

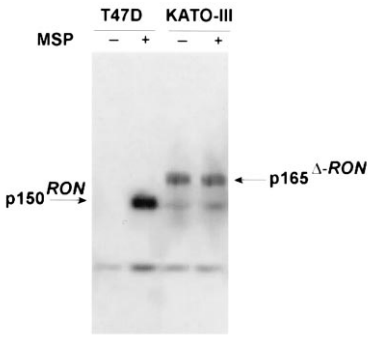


FIG. 2. Δ-Ron is constitutively tyrosine phosphorylated in vivo. Detergent-solubilized proteins from cells treated with conditioned medium from U293 cells, either control (−) or MSP transfected (+), were immunoprecipitated with Ron antibodies and Western blotted with phosphotyrosine antibodies. MSP-induced phosphorylation of the 150-kDa Ron β chain was detectable both in KATO-III and T47D cells, whereas the 165-kDa protein was tyrosine phosphorylated either in the presence (+) or in the absence (−) of the ligand. In KATO-III cells, the Ron β chain was tyrosine phosphorylated also in the absence of ligand, likely as consequence of transphosphorylation via the constitutively tyrosine phosphorylated Δ-Ron.

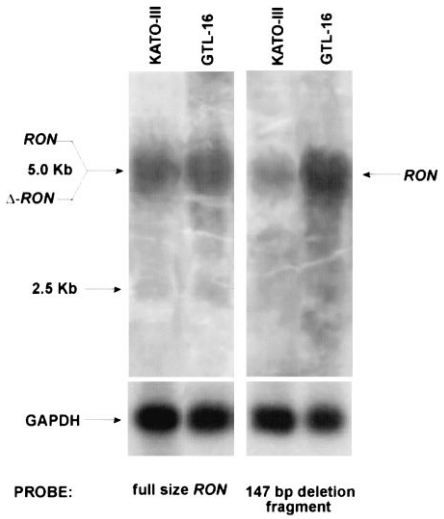


FIG. 4. Δ-*RON* is the most abundant mRNA in KATO-III cells. Ten micrograms of total RNA extracted from KATO-III and GTL-16 cells was subjected to differential hybridization with the full-size *RON* probe and with a probe containing only the 147-bp deletion fragment. The full-size probe hybridized with *RON* mRNA extracted from both cell lines. Conversely, the 147-bp deletion probe barely detected the KATO-III mRNA, indicating that the Δ-*RON* transcript was by far the most abundant. A probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to evaluate the amount of mRNA transferred to filters. The Northern blot was exposed for 96 h.

of two alleles (encoding a long and a short form of the protein) was ruled out since a single PCR product, corresponding to a size of 442 bp, was obtained from the genomic DNA (Fig. 3B). No mutations altering the normal splicing pattern were found in this DNA region; the genomic nucleotide sequence was identical to that of cell lines expressing the wild-type Ron receptor protein (T47D and GTL-16 [data not shown]).

These results can be explained only in terms of alternative splicing of a single *RON* primary transcript. Indeed, a minor cDNA variant lacking 49 amino acids has been described (13). Two other occurrences of alternative splicing in KATO-III cells have been reported (K-*sam* and E-cadherin transcripts [19, 29]).

The relative amounts of Δ-*RON* and *RON* transcripts were then quantitated in Northern analysis by differential hybridization with a full-size *RON* probe or with a probe corresponding to the 147-bp deleted fragment. The Δ-*RON* mRNA was by far the most abundant transcript in KATO-III cells, while the full-size *RON* transcript was predominant in control cell lines (Fig. 4). These data show that the gastric cancer cell line studied accumulates the Δ-*RON* mRNA that in other cells is a minor component.

The Δ-*RON* transcript is missing a 147-bp cassette exon. These data suggest that the two *RON* transcripts differ in the short 147-bp sequence as a result of a differential maturation of the primary transcript. To verify whether an alternative splicing can occur, the KATO-III genomic PCR amplification product was subcloned in the pUC18 vector and sequenced. Exon-intron junctions were identified by comparing genomic and cDNA sequences. This analysis showed that the 147-bp coding sequence is flanked at both 5′ and 3′ ends by two short introns of 86 and 80 bp, respectively.

The alternative splicing of the 147-bp sequence occurs through the use of an alternative acceptor site for the 5′ intron. Its donor site, located at 3′ end of the preceding exon, can be spliced to two acceptor sites, (i) at bp 2678 and (ii) at bp 2825, located at the origin of the 147-bp sequence and at the origin of the following exon, respectively. In the first case, the spliced intron is of 86 bp and the mature RNA retains the 147-bp miniexon. A donor site at its 3′ end can be spliced to the acceptor site at position 2825, generating the second 80-bp intron. In the second case, the first donor site is spliced to the alternative acceptor site at bp 2825, consequently generating a long intron that contains the 147-bp cassette exon together with its flanking noncoding sequences (Fig. 3C). The predicted proteins encoded by the two mRNAs differ by 49 amino acids. Interestingly, among them are three cysteine residues. Thus, the short protein will have an uneven number of free thiol groups, a condition favoring interchain disulfide bond formation and stable oligomerization.

The Δ-Ron kinase forms oligomers in the intracellular compartment. To characterize the biochemical properties of the protein encoded by Δ-*RON*, the corresponding cDNA was expressed in COS-1 cells. As control, cells were also transfected with the *RON* cDNA. After lysis, proteins were analyzed by specific immunoprecipitation and Western blotting with Ron antibodies. The pattern of migration in reducing conditions of the recombinant Δ-Ron was found identical to that of the native form identified in the KATO-III cell line (Fig. 5).

The heterodimeric structure of the wild-type Ron protein was previously deduced from the different migration patterns in reducing and nonreducing conditions (13). As expected, in an in vitro kinase assay experiment, the shift was observed in the immunoprecipitates obtained from cells expressing the wild-type *RON* receptor. On the other hand, the $p165^{\Delta\text{-}RON}$ protein migrated to the same extent in reducing and nonreducing conditions. This finding showed that $p165^{\Delta\text{-}RON}$ is a single-chain molecule, with the properties of the $pr170^{RON}$ precursor, prior to its cleavage (Fig. 6A). Notably, $p165^{\Delta\text{-}RON}$, immunoprecipitated either from KATO-III cells or from transfected cells (not shown), was found in oligomers migrating as discrete bands of high molecular weight in nonreducing conditions. The oligomers, excised, reduced, and run again in the presence of 2-mercaptoethanol, were resolved in specific 165-kDa monomers (Fig. 6B), as confirmed by Western blotting with Ron antibodies (not shown), showing that the complexes

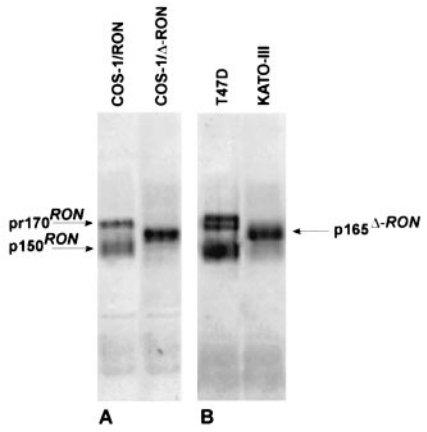


FIG. 5. Δ-*RON* cDNA encodes a 165-kDa protein. COS-1 cells were transfected with *RON* or Δ-*RON* cDNA, and proteins were analyzed as described for Fig. 1. The migration patterns of recombinant proteins immunoprecipitated from *RON*- and Δ-*RON*-transfected COS-1 cells (A) corresponded to those of native proteins extracted from T47D and KATO-III cells (B), respectively. Arrows indicate the uncleaved precursor ($pr170^{RON}$), the mature β chain ($p150^{RON}$), and the 165-kDa protein ($p165^{\Delta\text{-}RON}$). The $pr170^{RON}$ expressed by T47D cells migrates as a doublet as consequence of differential glycosylation.

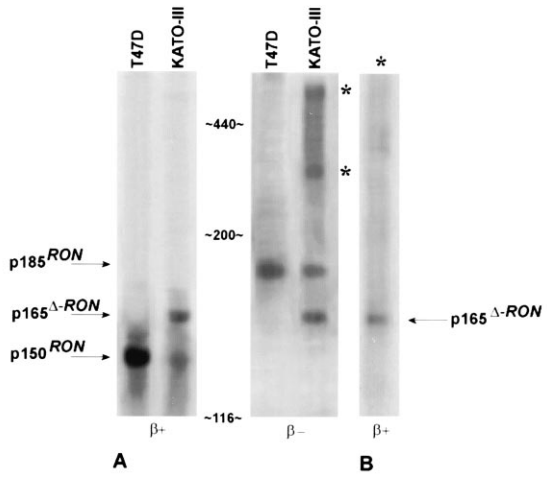


FIG. 6. The Δ-Ron protein is an oligomerized, single-chain, active tyrosine kinase. (A) Proteins solubilized from control (T47D) cells and from KATO-III cells were immunoprecipitated with Ron antibodies. A kinase assay was performed on immunocomplexes as described in Materials and Methods. Radiolabeled proteins were separated by SDS-PAGE (5 to 10% gradient gel) under reducing (β+) or nonreducing (β−) conditions. Gels were alkali treated, dried, and exposed for autoradiography with intensifying screens. A major tyrosine-phosphorylated band of 150 kDa, corresponding to the Ron β chain, was evident. As expected, under nonreducing conditions, the 150-kDa protein, immunoprecipitated from T47D and KATO-III cells, shifted from 150 to 185 kDa because of its association with the α chain. In contrast, the $p165^{\Delta\text{-}RON}$ protein migrated at the same extent both in reducing and nonreducing conditions. The immunocomplexes from KATO-III cells, analyzed by SDS-PAGE in the absence of 2-mercaptoethanol, contained high-molecular-weight oligomers, indicated by the asterisks. The high-molecular-weight bands, excised from the dried gel and reanalyzed under reducing conditions, were resolved in 165-kDa Δ-Ron monomers (B). Arrows indicate the mature αβ heterodimer ($p185^{RON}$), the 150-kDa β chain, and the uncleaved $p165^{\Delta\text{-}RON}$ protein. Molecular weights are indicated in thousands.

are formed by Δ-Ron proteins stabilized by intermolecular disulfide bonds. In principle, it is not possible to exclude that heterodimerization with other proteins could also occur. However, this possibility is reduced to a minimum by the fact that the high-molecular-weight complexes are roughly multiples (in weight) of Δ-Ron monomer (330 and 660 kDa).

The possibility that intermolecular disulfide bonds, hampering Δ-Ron gel mobility under nonreducing conditions, were postlysis artifacts was ruled out by adding an alkylating agent (iodoacetamide, 50 mM) to the extraction buffer.

The mature, cleaved Ron protein is exposed at the cell surface, as demonstrated by domain-specific surface biotinylation of T47D cells or of cells transfected with the wild-type *RON* cDNA. In contrast, no biotin-labeled $p165^{\Delta\text{-}RON}$ proteins were immunoprecipitated from KATO-III cells or from COS-1 cells transiently transfected with Δ-*RON* cDNA (Fig. 7). These data show that the deleted Δ-Ron is retained in the intracellular compartment, possibly in the pre-Golgi vesicular system, as indicated by endoglycosidase-H digestion susceptibility (data not shown).

Δ-Ron oligomers originate by intermolecular disulfide bonding. To confirm that intracellular oligomerization of Δ-Ron is due to the uneven number of cysteine residues, we generated the mutant C914A-Ron. One of the highly conserved cysteine residues (position 914, included in the 49-amino-acid deletion fragment) was converted to alanine by site-directed mutagenesis. Expression in COS-1 cells of the mutated cDNA was followed by accumulation of the Ron single-chain precursor ($pr170^{RON}$) and retention in the intracellular compartment. The mutant with uneven cysteine residues was constitutively phosphorylated on tyrosine (Fig. 8A), mimicking that occurring in the Δ-Ron isoform.

Covalent disulfide bonding is known to be reversible in the presence of reducing agents. Therefore, we added exogenous 2-mercaptoethanol to cells naturally expressing Δ-Ron (KATO-III) at concentrations not affecting cell viability, terminal glycosylation, or membrane exposure of proteins (1). Mercaptoethanol allowed maturation of Δ-Ron, and the correctly processed αβ heterodimer was exposed to the cell surface (Fig. 8B). The effect of 2-mercaptoethanol was dose dependent, ranging from 2 to 32 mM (maximal activity). Under these conditions, the high-molecular-weight Δ-Ron aggregates disappeared (data not shown), confirming the central role played by free thiol groups.

Δ-Ron induces cell motility and invasiveness. Under physiological conditions, the Ron receptor transduces mitogenic, motogenic, and invasive signals when stimulated by the growth factor MSP (25). The ligand-independent tyrosine phosphorylation of Δ-Ron prompted us to verify if constitutive activation of Ron could be associated with transformation and/or acquisition of an invasive phenotype.

Surprisingly, NIH 3T3 cells transfected by Δ-*RON* and expressing the constitutively activated kinase did not form foci of transformation, nor did they grow in soft agar.

A motility assay was then performed to measure the chemotactic and chemokinetic properties of a panel of cells expressing the wild-type Ron or Δ-Ron. As a control, cells not expressing Ron were studied. Figure 9 shows that the latter were inactive, either in the absence or in the presence of MSP; as expected, the cells expressing wild-type Ron migrated only in the presence of MSP. Interestingly, the cells expressing Δ-Ron displayed a constitutive motile phenotype. This phenotype was driven either by the endogenous Δ-Ron, the recombinant Δ-Ron (cloned from KATO-III cells), or the mutant C914A-Ron, bearing an uneven number of cysteine residues. Notably, the motile-invasive phenotype was expressed constitutively also by NIH 3T3 cells stably transfected with Δ-Ron and expressing a normal level of protein.

An invasion assay was also performed to assess the ability of Δ-Ron to drive migration through an artificial basement membrane, made of collagen IV, laminin, and glycosaminoglycans.

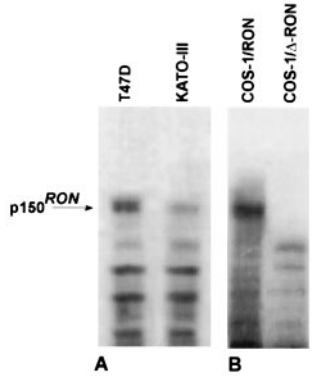


FIG. 7. Δ-Ron is not exposed at the cell surface. COS-1 cells transfected with both *RON* and Δ-*RON* cDNAs, T47D cells expressing endogenous Ron, and KATO-III cells expressing endogenous Δ-Ron were subjected to domain-selective cell surface biotinylation. Proteins from cell lysates were immunoprecipitated with Ron antibodies, separated by SDS-PAGE under reducing conditions, and transferred to nitrocellulose. Biotinylated proteins were visualized by using streptavidin-horseradish peroxidase and ECL. The mature 150-kDa Ron protein from T47D cells and KATO-III cells was exposed at the cell surface (A) as well as the recombinant Ron (B). Δ-Ron, both endogenous and recombinant, was retained in the intracellular compartment.

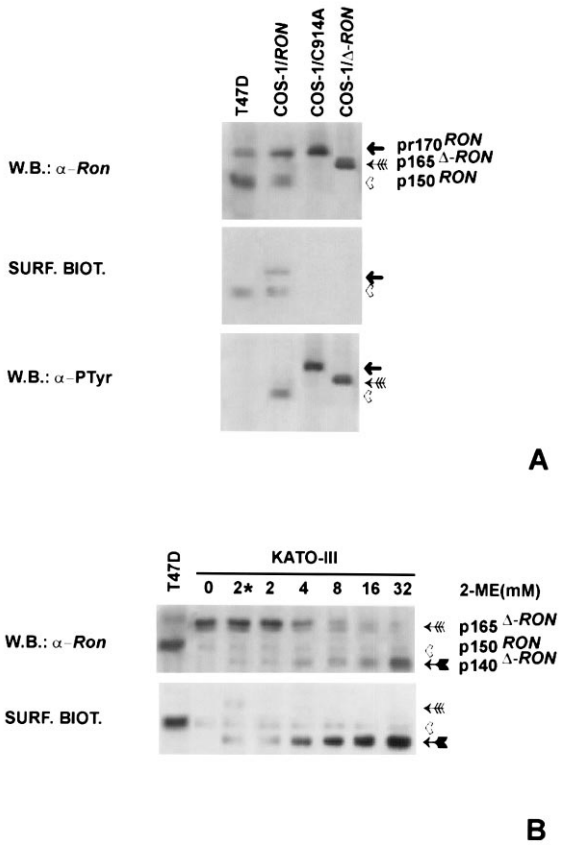


FIG. 8. Δ-Ron oligomers formation is thiol mediated. (A) Expression in COS-1 cells of the C914A-Ron mutant (bearing an uneven number of cysteines) is followed by accumulation in the intracellular compartment of the single-chain precursor (pr170^{RON}) and by constitutive tyrosine phosphorylation, mimicking what occurs for the Δ-Ron isoform. Detergent-solubilized proteins from surface-biotinylated (SURF. BIOT.) cells were immunoprecipitated with Ron antibodies (α-*Ron*), separated by SDS-PAGE under reducing conditions, and Western blotted (W.B.) with the indicated antibodies. Biotinylated proteins were visualized by using streptavidin-horseradish peroxidase and ECL. Arrows indicate the uncleaved precursor (pr170^{RON}), the Δ-Ron protein (p165$^{\Delta\text{-}RON}$), and the mature Ron β chain (p150^{RON}). α-PTyr, antiphosphotyrosine. (B) Covalent disulfide bonds are reversible in the presence of reducing agents. KATO-III cells, treated with millimolar concentrations of 2-mercaptoethanol, were subjected to domain-selective cell surface biotinylation. Detergent-solubilized proteins were immunoprecipitated and blotted with Ron antibodies. Biotinylated proteins were visualized as described above. Arrows indicate the Δ-Ron protein (p165$^{\Delta\text{-}RON}$), the 150-kDa Ron protein (p150^{RON}), and the mature 140-kDa Δ-Ron β chain (p140$^{\Delta\text{-}RON}$). The lane labeled with an asterisk contains Δ-Ron immunoprecipitates obtained from cells permeabilized to allow biotin labeling of intracellular proteins.

Again, the cells expressing the full-size Ron, either endogenous or transfected, were found to cross the basement membrane only after stimulation with MSP. On the other hand, the cells expressing Δ-Ron or the C914A-Ron mutant constitutively displayed invasive properties (Fig. 10).

DISCUSSION

Structural alterations of oncogene-coded proteins can deregulate critical cellular functions. Several examples have been reported for tyrosine kinase receptors, such as the activating rearrangements of *TRK*, *RET*, and *MET* (9, 24, 41). Moreover 5′ or 3′ deletions activate v-*erbB* (12), v-*kit* (33), v-*fms* (47), and *HER2* (5). The data presented here demonstrate the existence of a structurally altered form of the tyrosine kinase receptor encoded by the *RON* gene. This isoform (Δ-Ron) is generated by alternative splicing, segregates in the intracellular compartment, oligomerizes by stable intermolecular disulfide bonds, and displays constitutive tyrosine phosphorylation. The cells expressing Δ-Ron acquire a migratory and invasive phenotype.

Δ-Ron differs from the full-length sequence by lacking 49 amino acids in the extracellular domain. The two transcripts, encoding full-size Ron and the Δ-Ron isoform, are generated by alternative splicing of a true cassette exon of 147 bp encoding a short segment of the extracellular domain. Cassette exons are nucleotide sequences that may or may not be incorporated into mRNA, but their immediate flanking introns are invariably removed during the splicing process (6). In the case of the *RON* mRNA, when the introns flanking the cassette exon are individually removed, the 147 bp are included in the mature transcript; when the flanking introns are removed together, the embodied cassette exon is spliced out and the Δ-*RON* mRNA is generated. The second event is due to the existence, at the boundary between the 5′ intron and the 147-bp cassette exon, of a noncanonical 3′ splice site consensus sequence (-TA instead of -GT) which can be randomly ignored by the splicing machinery. In general, it has been shown that the selection of a splice site depends not only on its own sequence but also on the context around that sequence (28). Regarding this point, exons that are intrinsically poor splicing substrates, because of small size or weak flanking splice sites, have been proposed to

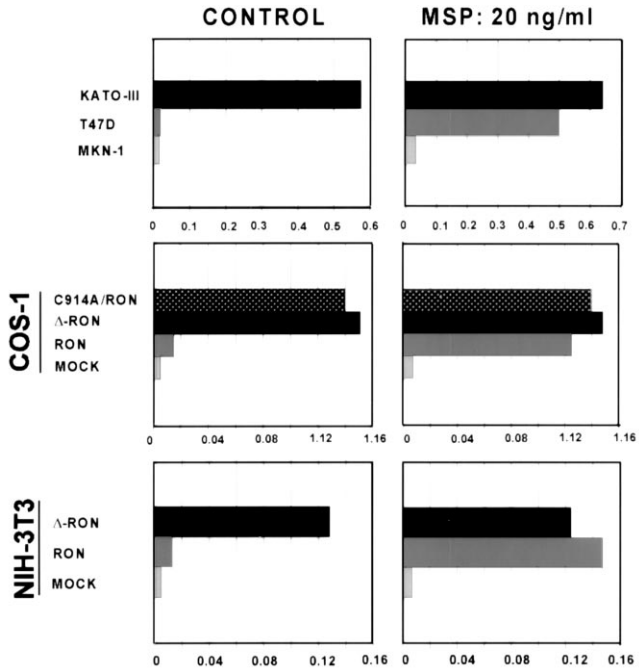


FIG. 9. Δ-Ron induces a motile phenotype in vitro. A motility assay was performed to measure chemotactic and chemokinetic properties of a panel of cells expressing Ron and Δ-Ron, either endogenous or transfected. As control, cells not expressing Ron (MKN-1 cells) were studied. Cells were plated on the upper side of an 8-μm-pore-size Millipore filter in a Transwell chamber and incubated for 24 h in serum-free medium, either in the absence or in the presence of 20 ng of MSP per ml in the lower compartment. The reported values (averages of triplicate determinations) represent the A_{595} of cells that migrated to the lower side of the Transwell and were fixed and stained with crystal violet. The cells expressing endogenous or recombinant Ron (T47D, COS-1, and NIH 3T3 cells transfected with *RON* cDNA) migrated into the lower compartment of the chamber only in the presence of MSP. Cells expressing endogenous or recombinant Δ-Ron (KATO-III, COS-1, and NIH 3T3 cells transfected with Δ-*RON*) displayed a motile phenotype also in the absence of MSP. Constitutive migration was observed also in cells expressing the C914A-Ron mutant.

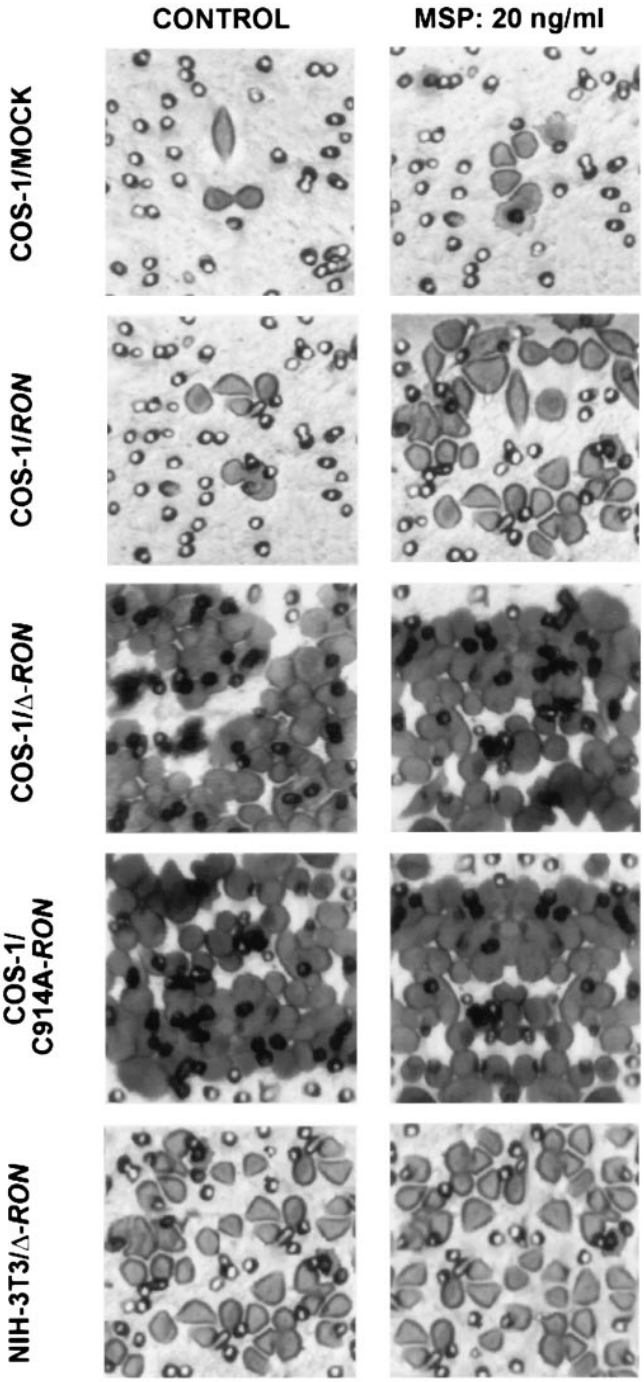


FIG. 10. Δ-Ron induces an invasive phenotype in vitro. An invasion assay was performed to monitor the ability of cells to migrate through an 8-μm-pore-size Millipore filter coated with an artificial basement membrane made of polymerized collagen IV, laminin, and glycosaminoglycans (Matrigel). The cells expressing recombinant full-size Ron required stimulation by exogenous MSP to cross the Matrigel barrier. Cells expressing recombinant Δ-Ron or the C914A-Ron mutant displayed the invasive phenotype constitutively.

contain positive-acting splicing elements, referred to as exon-splicing enhancers (17, 42, 48). Canonical exon-splicing enhancer sequences were, however, not easily identifiable in the 147-bp cassette-exon region.

As noted above, most of the known structural defects leading to oncogene activation are due to genetic alterations such as mutations, deletions, or rearrangements. The mechanism leading to the generation of the constitutively tyrosine phosphorylated Δ-Ron in the cell line studied is posttranscriptional. Posttranscriptional activation has also been observed in the case of the homologous Met/HGF receptor. In a colon carcinoma, accumulation of the uncleaved precursor was responsible for constitutive activation of the intrinsic tyrosine kinase. In this case, however, the precursor accumulation was due to lack of a specific proteolytic enzyme of the *trans*-Golgi network rather than to an intrinsic structural defect of the protein (26).

The in-frame deletion induces a critical change in the Δ-Ron structure. Among the missing 49 amino acids are three cysteine residues normally involved in intramolecular disulfide bonds. Therefore, the unbalance of the cysteine pairs renders the uneven cysteines available for intermolecular disulfide bonding with other Δ-Ron partners, creating oligomers. The existence of high-molecular-weight Δ-Ron complexes sensitive to reducing agents strongly supports this hypothesis. This situation has already been shown to occur in the case of epidermal growth factor and erythropoietin receptor mutants (40, 46), and a similar mechanism has been proposed for constitutive activation of the epidermal growth factor, Neu, and fibroblast growth factor receptors (27, 39). Another example is the germ line mutation, occurring in the multiple endocrine neoplasia 2A (MEN2A) syndrome, that activates *RET* by replacement of Cys-634; the receptor, containing an uneven number of thiol groups, undergoes steady-state dimerization and constitutive tyrosine kinase activation (38). Moreover, in the case of the *TRK* oncogene, a 51-amino-acid deletion in the extracellular domain, removing one cysteine residue, generates a conformation usually achieved by ligand binding (10). We reproduced biochemical and biological properties of Δ-Ron by substitution of a single cysteine residue in the extracellular domain. COS-1 cells expressing the C914A-Ron mutant, bearing an uneven number of cysteines, were endowed with deregulated motile-invasive properties, mimicking the phenotype elicited by Δ-Ron.

The Δ-Ron molecules oligomerized by interchain cysteine oxidation never reach the cell surface, nor are they correctly processed by proteolytic cleavage. In the Met/HGF receptor, a molecule highly homologous to Ron, a 51-amino-acid deletion variant (with an uneven number of cysteine residues) is not correctly processed and is retained intracellularly (35). This is somewhat expected, since as a rule, only proteins that have attained the proper three-dimensional structure are secreted or expressed at the cell surface. Folding and assembly intermediates are retained intracellularly. This quality control of newly synthesized molecules takes place notably in the endoplasmic reticulum (18, 20, 31). For example, it has been shown that the transport of immunoglobulin λ light chains with exposed thiols is hindered by the formation of reversible disulfide bonds with the protein matrix of the endoplasmic reticulum (34). Such interactions may be prevented by altering the intracellular redox potential or by site-directed mutagenesis of one relevant cysteine residue, as demonstrated in the case of secretion of immunoglobulin M assembly intermediates (1). Similarly, we show here that maturation and cell surface exposure of Δ-Ron can be restored by addition of 2-mercaptoethanol. This observation indicates that disulfide-interchange reactions are responsible for the intracellular retention of Δ-Ron in the form of partially assembled intermediates.

Accumulation of the misfolded immature Δ-Ron protein in the intracellular vesicular compartment and its oligomerization due to aberrant intermolecular disulfide bonds have important consequences on receptor functions. The receptor kinase is

constitutively activated, and the downstream motile-invasive program is induced in the absence of exogenous signaling. As observed, the affected cells acquire a phenotype characterized by the ability to migrate through basement membranes in vitro. This property correlates with the metastatic phenotype displayed in vivo by malignant cells (2, 43, 44). Interestingly, although cells expressing a constitutively activated Ron tyrosine kinase are invasive, they do not form foci of transformation in vitro, nor do they grow in soft agar. This observation strengthens the prominent biological features of the tyrosine kinase receptor subfamily to which Ron belongs, i.e., the control of cell dissociation, motility, and invasion, rather than mere growth.

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