The C Terminus of RON Tyrosine Kinase Plays an Autoinhibitory Role*

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RON is a receptor tyrosine kinase in the MET family. We have expressed and purified active RON using the SF9/baculovirus system. The constructs used in this study comprise the kinase domain alone and the kinase domain plus the C-terminal region. The construct containing the kinase domain alone has a higher specific activity than the construct containing the kinase and C-terminal domains. Purified RON undergoes autophosphorylation, and the exogenous RON C terminus serves as a substrate. Peptides containing a dityrosine motif derived from the C-terminal tail inhibit RON in vitro or when delivered into intact cells, consistent with an autoinhibitory mechanism. Phenylalanine substitutions within these peptides increase the inhibitory potency. Moreover, introduction of these Phe residues into the dityrosine motif of the RON kinase leads to a decrease in kinase activity. Taken together, our data suggest a model in which the C-terminal tail of RON regulates kinase activity via an interaction with the kinase catalytic domain.

RON is a receptor tyrosine kinase that is involved in cell proliferation, survival, and motility (1–3). RON is one of the three members of the MET family of receptor tyrosine kinases (MET, RON, and SEA) (4, 5). The MET family members share a number of unique structural properties, most notably an αβ disulfide-linked heterodimeric structure. RON is composed of a 40-kDa extracellular α chain and a 150-kDa transmembrane β chain with intrinsic protein kinase activity (4, 6, 7).

RON is expressed in a variety of cells, including epithelial cells and macrophages (7, 8–11). The ligand for the RON receptor is macrophage-stimulating protein (MSP), also known as hepatocyte growth factor-like protein (6, 7). In normal cells, MSP binding leads to a transient increase in RON activity, whereas tumor cells often possess elevated levels of RON protein, expression of altered forms of RON, and increased RON kinase activity (12–14).

Upon activation of RON, the receptor becomes phosphorylated within the activation loop of the kinase catalytic domain, and the enzymatic activity of RON is enhanced. RON also possesses two tyrosine residues in the C-terminal tail in a motif (Y1353VQLPAT1360YMNL) that is conserved in all MET family members (15). Ligand stimulation of MET family members leads to phosphorylation of these two tyrosines (Ty1353 and Tyr1360). These phosphorylated tyrosine residues provide multifunctional docking sites for the p85 regulatory subunit of phosphatidylinositol 3-kinase (16), the Grb2-SOS complex (15, 17), STAT3 (18), and the adaptor protein Gab1 (19–21). Mutation of these two tyrosines (Y1353F/Y1360F) suppressed the transforming ability of activated forms of RON (15). A similar mutation caused a complete loss of transforming ability of the related SEA kinase (22). These results could be the result of the inability of the double mutant to engage SH2 domain-containing downstream signaling proteins. Another possibility, however, is that the intrinsic kinase activity of the Y1353F/Y1360F mutant is altered. Bardelli et al. (23) demonstrated that peptides containing C-terminal sequences inhibited MET kinase activity in vitro. A peptide derived from the C-terminal tail impaired MET-induced invasive growth in transformed epithelial cells. These studies suggested that the carboxy-terminal domain may act as an intramolecular modulator of MET receptor (23).

At present, the regulatory mechanism of MET family kinases is unclear, partly because no enzymatic studies have been carried out on a purified member of this family. We report the first purification and characterization of active RON from eukaryotic cells, using the SF9/baculovirus expression system. We present evidence that the C-terminal region of RON plays an autoinhibitory role.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibody against phosphorytrosine (4G10) and anti-MAPK antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-RON polyclonal antibody was obtained from BIOSOURCE (Camarillo, CA). Anti-phospho-MAPK antibody was purchased from BD Transduction Laboratories (San Diego). RON antibody (sc-322) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human RON was from R&D Systems (Minneapolis). pBAC-gus-9 transfer plasmid and Bacvector-3000 DNA transfection kits were from Novagen (Madison, WI). Protein A-agarose was from Sigma. Glutathione-agarose and glutathione S-transferase (GST) antibody were purchased from MolecProbes (Eugene, OR). Ni-NTA affinity resin was from Qiagen, and Affi-Gel-15 was from Bio-Rad. RON100K1300–1400 antibody was raised in rabbit. The Chariot peptide transfection reagent was purchased from Active Motif (Carlsbad, CA).

**Peptide Synthesis and Characterization**—The following synthetic peptides were used in this study: Abl substrate (EAIYAAPFAKKKG), Src substrate (AEEEITYGFEEAKKKK), EGFR substrate (AEELEYEELVAKKKK), insulin receptor substrate (KKEEEEYMMMMG), peptide Y1353/Y1360 (residues 1349–1367 of RON, LGDHYVQLPATYMNL), peptide Y1353 (1346–1359, SALLGDHYQVLAP), peptide Y1360 (1354–1367, VQLPATYMNLG), and peptide F1353/F1360 (1349–1367, LGDHFVQLPATFMNLG). Peptides were synthesized on an Applied Biosystems 431A automated peptide synthesizer using...
Autoinhibition by RON C Terminus

standard Fmoc (N-9-fluorenlymethoxycarbonyl) chemistry (24). The synthetic peptides were purified by preparative reversed phase high performance liquid chromatography and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Baculovirus Expression Vectors—The RON kinase C-terminal tail construct (RON-CT) was generated by PCR. This construct encoded amino acids AlaK392-Thr1605 of RON. The PCR 5′-primer had the sequence CGCGGATCCGCGCTCTTGCGTGAGGTCAAG, and the 3′-primer was GGAATTCGAGTGGGCGAGGCGTGTAGAG. These primers had 31 nucleotides (5′-primer) and 33 nucleotides (3′-primer) of complementarily with the template and encoded unique restriction sites (BamHI at the 5′-end and EcoRI at the 3′-end). The PCR product was ligated into plasmid pCR-BluntII-TOPO (Invitrogen). The resulting plasmid was digested with BamHI/EcoRI, and the RON insert was purified on an agarose gel. The RON fragment was subcloned into plasmid pBACgus-9 (N-terminal T7 tag, C-terminal CBD tag, and polyhistidine tag; Novagen), and expressed in Sf9 cells using the Baculo-Vector 3000 DH110 transfection kit (Novagen). The 2YF mutant form (RON-2YF) was generated by site-directed mutagenesis of the pBACgus-9 RON-CT construct using the QuikChange mutagenesis system (Stratagene). The mutation was confirmed by automated DNA sequencing.

To produce the isolated kinase catalytic domain of RON (RON-KIN), PCR was carried out with the 5′-primer 5′-CGCGGATCCGCGCTCTTGCGTGAGGTCAAG and the 3′-primer 3′-primer was GGAATTCGAGTGGGCGAGGCGTGTAGAG. These primers had 31 and 30 nucleotides of complementarily with the template, respectively, and encoded unique restriction sites (BamHI at the 5′-end and EcoRI at the 3′-end). The RON-KIN construct encodes amino acids AlaK367-Val1345 of RON-KIN was carried out using the methods described above for RON-CT. For production of RON-CT and RON-KIN proteins, 0.6 liter of Sf9 cells (1.8×10⁸ cells/ml) were infected with baculovirus at a multiplicity of infection of 7.5 and 9.0, respectively. We produced a RON construct containing the kinase domain plus the C-terminal tail (RON-CT) by infection of Sf9 cells with a recombinant baculovirus vector (Fig. 1A). We purified RON-CT to homogeneity by chromatography on Source-Q and Ni-NTA columns (Fig. 1B). Kinase activity was monitored during the purification by the phosphocellulose paper binding assay, using the Abl consensus peptide EAIYAAPFAKKKG as a substrate. Purified RON-CT migrates with the expected molecular mass (~55.4 kDa) (Fig. 1B). RON-CT was eluted with buffer containing 100 mM imidazole, 5 mM 2-mercaptoethanol, 2 mM Na3VO4, 10% glycerol, and 20 mM Tris-HCl (pH 8.0). RON kinase activity was measured using the phosphocellulose paper binding assay (25) with peptide EAIYAAAPFKKKKG as a substrate. Results were treated with anti-phosphotyrosine, anti-RON, anti-phospho-MAPK, and MAPK antibodies.

RESULTS

Phosphorylation of RON and Kinetics of Peptide Phosphorylation—We produced a RON construct containing the kinase domain plus the C-terminal tail (RON-CT) by infection of Sf9 cells with a recombinant baculovirus vector (Fig. 1A). We purified RON-CT to homogeneity by chromatography on Source-Q and Ni-NTA columns (Fig. 1B). Kinase activity was monitored during the purification by the phosphocellulose paper binding assay, using the Abl consensus peptide EAIYAAAPFKKKKG as a substrate. Purified RON-CT migrates with the expected molecular mass (~55.4 kDa) (Fig. 1B). RON-CT was eluted with buffer containing 100 mM imidazole, 5 mM 2-mercaptoethanol, 2 mM Na3VO4, 10% glycerol, and 20 mM Tris-HCl (pH 8.0). RON kinase activity was measured using the phosphocellulose paper binding assay (25) with peptide EAIYAAAPFKKKKG as a substrate. Results were treated with anti-phosphotyrosine, anti-RON, anti-phospho-MAPK, and MAPK antibodies.

As an initial test of the substrate specificity of RON kinase, we carried out experiments with four peptides containing recognition motifs for different subfamilies of tyrosine kinases. The kinase recognition motifs included were for Src, Abl, EGFR, and insulin receptor. RON-CT preferred the EGFR substrate from this group of peptides (Table I). We carried out initial rate kinetic measurements with saturating concentrations of ATP and varying concentrations of peptides. These experiments yielded a Kₘ for the EGFR peptide of 520 μM and a kcat of 19.72 min⁻¹ (kcat/Km = 26.2×10⁻³ min⁻¹ μM⁻¹) (Table I). The next best substrate for RON, Abl peptide, was phosphorylated with a kcat/Km = 11×10⁻³ min⁻¹ μM⁻¹, 2.4 times lower than the EGFR substrate (Table I). Phosphorylation of the Src and insulin receptor-specific peptides was barely detectable. Thus, our kinetic experiments show that RON phosphorylates the EGFR peptide with a kcat/Km, value > 17-fold higher than that seen with a preferred substrate for another RTK, insulin receptor kinase. Previous experiments with immunoprecipitated RON also showed that RON preferred the EGFR peptide sequence (29). In those studies, however, a Src substrate sequence was phosphorylated roughly equally to the EGFR sequence, whereas in our studies (Table I) we found a difference of ~24-fold in terms of kcat/Km.
Autoinhibition by RON C Terminus

**Autoinhibition of RON**—Many tyrosine kinases are regulated by autophosphorylation within the activation loop, a segment that lies between the N- and C-terminal lobes of the catalytic domain. MET family receptors contain a pair of tyrosines in the activation loop (tyrosines 1238 and 1239 in RON). For the MET receptor itself, autophosphorylation has been mapped to the residues corresponding to Tyr1238 and Tyr1239, and phosphorylation at these sites activates MET (30, 31). By Western blotting with an antibody that recognizes RON that is doubly phosphorylated at Tyr1238/Tyr1239, we detected phosphorylation of RON-CT after expression in Sf9 cells (Fig. 1B). We investigated whether purified RON-CT can undergo autophosphorylation. Purified RON-CT was incubated with [γ-32P]ATP in kinase buffer, and the reaction mixtures were analyzed by SDS-PAGE and autoradiography. RON-CT activity was measured toward 0.5 mM peptide Abl substrate in the presence of various concentrations of ATP (data not shown). RON-CT alone was not a substrate for RON. GST-RON65 in particular contains no other tyrosines besides Tyr1353 and Tyr1360; thus, these results show that RON has the capacity to phosphorylate these sites, at least in the context of an exogenous fusion protein.

**Peptides Derived from the C-terminal Tail Inhibit RON Kinase Activity**—It has been demonstrated previously that a peptide corresponding to the C-terminal tail of the MET receptor inhibits MET kinase activity (23). To investigate whether the RON C-terminal tail regulates kinase activity, we tested peptides Y1353, Y1360, and Y1353/Y1360 as potential inhibitors. We also synthesized a peptide in which the two tyrosines in the C-terminal sequence (residues 1349–1367) were replaced with phenylalanines (peptide F1353/F1360). In these experiments, we first removed the CBD and His tags from RON-CT by treatment with thrombin to avoid any interference by the tags in our assays. RON-CT activity was measured toward 0.5 mM Abl peptide substrate in the presence of various concentrations of the C-terminal peptides. As shown in Fig. 4A, peptide F1353/F1360 dramatically inhibited RON-CT activity. Peptide Y1353 also showed inhibition, whereas peptides Y1360 and Y1353/Y1360 had very little effect (Fig. 4A). Consistent with the results shown in Fig. 4A, phosphorylation of GST-RON65 and

**Fig. 1. Purification of RON.** A, schematic representation of the RON constructs used in this study. B, proteins from each purification step were visualized with Coomassie Brilliant Blue (left panel). First lane, Sf9 cell lysate; second lane, flow-through fractions from Source Q chromatography; third lane, pooled fractions after Ni-NTA column. Center and right panels, purified RON was analyzed by Western blotting with anti-phospho-RON (center panel) or anti-RON antibodies (right panel).

**Fig. 2. Autophosphorylation of RON.** Purified RON-CT was incubated with 0.25 mM [γ-32P]ATP in kinase buffer for the indicated times. The reaction was terminated by the addition of SDS-sample buffer and analyzed by SDS-PAGE and autoradiography. In the lane marked +, RON was preincubated with 0.25 mM ATP for 40 min followed by an incubation with [γ-32P]ATP for 40 min as described above.

**Fig. 3. RON phosphorylates tyrosine residues in the bidentate motif.** GST-RON65 or GST-RON100 was incubated with purified RON-CT plus 0.25 mM [γ-32P]ATP in kinase assay buffer for 40 min. The reaction was stopped by the addition of SDS-sample buffer then subjected to SDS-PAGE. Reactions were analyzed by autoradiography (top panel) and by anti-GST Western blotting (bottom panel).
Autoinhibition by RON C Terminus

**Table I**

| Peptide       | Sequence                        | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ |
|---------------|---------------------------------|--------------|----------|-------|
|               |                                 | (min$^{-1}$ μM$^{-1}$) | (min$^{-1}$) | (μM)  |
| Abl substrate | EAIYAAPFAKKKG                   | 11.0 $\times 10^{-3}$ | 6.2      | 510   |
| Src substrate | AEKIEGFEAKKKKG                  | 1.1 $\times 10^{-3}$ | 0.89     | 800   |
| EGFR substrate| AEKIEYFLAKKKKG                  | 26.2 $\times 10^{-3}$ | 13.7     | 520   |
| IRK* substrate| KKEEEYMMMG                      | 1.5 $\times 10^{-3}$ | 1.4      | 950   |

IRK, insulin receptor kinase.

GST-RON100 was also inhibited by peptide F1353/F1360 but not by peptide Y1353/Y1360 (Fig. 4B). We also analyzed the effects of the synthetic peptides on RON-CT autophosphorylation (Fig. 4C). The results of this experiment correlated well with the results presented in Fig. 4A; peptide F1353/F1360 was the most effective inhibitor of RON-CT autophosphorylation, and peptide Y1353 also gave inhibition at higher concentrations. Peptides Y1360 and Y1353/Y1360 were inactive in this assay (Fig. 4C).

We investigated whether these peptides are specific for RON kinase. We carried out inhibition experiments using the purified catalytic domain of another receptor tyrosine kinase, the insulin-like growth factor I receptor (IGF1R). We measured phosphorylation of a specific IGF1R peptide substrate by the triply phosphorylated, activated form of IGF1R. Even at concentrations as high as 1 mM, none of the RON C-terminal peptides gave any inhibition of IGF1R (data not shown).

**Deletion of the C-terminal Tail Enhances RON Kinase Activity**—To test further the idea that the RON C terminus is inhibitory, we produced a form of RON containing the kinase catalytic domain alone (RON-KIN). RON-KIN was expressed in Sf9 insect cells and purified to homogeneity using a strategy similar to that for RON-CT. RON-KIN migrates with the expected molecular mass (~50.9 kDa) and reacts with anti-RON antibody (Fig. 5A). RON-KIN undergoes autophosphorylation, demonstrating that sites exist within the catalytic domain itself (data not shown). We compared the specific activity of RON-CT and RON-KIN using 0.5 mM Abl peptide as substrate. Initial experiments confirmed that the peptide assay is linear over the time period examined (Fig. 5B). RON-KIN had a specific activity ~3-fold higher than that of RON-CT. We carried out kinetic measurements with saturating concentrations of ATP and varying concentrations of EGFR peptide (Fig. 5C). RON-KIN had a $K_m$ for the EGFR peptide of 745 μM and a $k_{cat}$ value of 77.82 min$^{-1}$ (Fig. 5D). Thus, the $k_{cat}/K_m$ for RON-KIN was about four times higher than for RON-CT ($k_{cat}/K_m = 26.2 \times 10^{-3}$ min$^{-1}$ μM$^{-1}$; Table I).

The higher activity of RON-KIN suggests that the C terminus may contain an autoinhibitory element. We tested whether RON-KIN would be susceptible to inhibition by the C-terminal peptides. As shown above for RON-CT (Fig. 4), we carried out RON-KIN kinase assays with varying concentrations of the C-terminal peptides, using the Abl peptide as substrate. Peptides Y1353, Y1353/Y1360, and F1353/F1360 all showed significant inhibition in this experiment (Fig. 6A). Peptide Y1360 did not inhibit RON-KIN. Inhibition of RON-KIN was observed at lower concentrations of peptides than was observed for RON-CT (compare Figs. 4A and 6A). These results are consistent with a model in which the peptides can more easily access the kinase domain in the absence of the C-terminal tail. The results on peptide phosphorylation were mirrored in studies of RON-KIN autophosphorylation (Fig. 6B). Peptides Y1353/Y1360 and F1353/F1360 showed complete inhibition of RON-KIN autophosphorylation at the lowest concentration tested (25 μM). Peptide Y1353 gave inhibition at a slightly higher concentration (125 μM). To gain a better understanding of the mechanism of inhibition, we carried out kinetic measurements with varying concentrations of peptide F1353/F1360. Inhibition of RON-KIN by peptide F1353/F1360 was found to be competitive with respect to peptide substrate (Fig. 6C).

**Tyr to Phe Substitutions in RON-CT Decrease Kinase Activity**—Because peptide F1353/F1360 was a more effective inhibitor than peptide Y1353/Y1360 (Figs. 4 and 6), we produced a mutant form of RON (designated RON-2YF) containing Tyr to Phe substitutions at positions 1353 and 1360 in the tail. We expressed RON-2YF in Sf9 cells and purified the enzyme to homogeneity using procedures similar to those described above for RON-CT. The C-terminal mutations severely impaired RON kinase activity (Fig. 5D). The $k_{cat}/K_m$ for RON-2YF (using EGFR peptide as substrate) was 0.6 $\times 10^{-3}$ min$^{-1}$ μM$^{-1}$, ~43 times lower than for RON-CT. Our interpretation (see “Discussion”) is that the C-terminal tail of the 2YF mutant form of RON is engaged in a strengthened interaction with the kinase domain (relative to the wild-type RON-CT).

**RON Inhibition in Intact Cells**—To test the ability of the RON C-terminal peptides to inhibit full-length RON receptor, we introduced peptides Y1353/Y1360 and F1353/F1360 into NIH3T3 cells using Chariot, a commercial kit for protein transduction. As a control, we used the Src peptide substrate, the weakest of the RON kinase substrates (Table I). MSP treatment led to an increase in tyrosine phosphorylation of RON in these cells (Fig. 7A). Although the control peptide had no effect on RON activation at the two concentrations tested (9 and 90 μM), peptide Y1353/Y1360 gave some inhibition at the higher concentration. Peptide F1353/F1360 showed inhibition at both concentrations, with a substantial reduction in phosphorylation at 90 μM (Fig. 7A). The blot was reprobed with RON antibody to confirm that peptide treatment did not affect RON expression (Fig. 7A). We carried out similar experiments to examine MAPK activation in these cells. Treatment of the NIH3T3 cells with MSP led to a stimulation of MAPK, as detected by phospho-MAPK Western blotting (Fig. 7B). In these experiments, peptides Y1353/Y1360 and F1353/F1360 both gave a significant reduction in MAPK activation at 90 μM. These experiments show that the RON C terminus has the capacity to inhibit kinase activity in the context of the intact, ligand-responsive receptor.

**Discussion**

An emerging theme in the regulation of receptor tyrosine kinases is that domains outside the core catalytic domain play important roles in regulating kinase activity. The crystal structures of more than a dozen RTKs have been reported, typically of the kinase domains alone. These studies have highlighted the importance of the conformation of the activation loop, which regulates substrate access and the proper positioning of catalytic residues (32–34). In many cases, however, there may be aspects of the RTK regulatory mechanisms which are not present in these structures; biochemical data for several RTKs show a more complex mechanism of activation. The juxtamembrane region (JM) of some RTKs clearly plays a negative role. Tyrosine residues in the JM region of several RTKs function as direct inhibitors of catalytic function and also serve as recruitment sites for various downstream signaling proteins. These
Autoinhibition by RON C Terminus

they demonstrate the structural basis for the negative regulatory role of the JM region in RTKs. The JM regions of these RTKs prevent the activation loop from adopting an active conformation, and phosphorylation within the JM region relieves autoinhibition. In the case of c-Kit, addition of a peptide corresponding to the JM region inhibited kinase activity, providing biochemical evidence for the intramolecular regulation (39).

Biochemical experiments have implicated the C-terminal portion of the cytoplasmic domain of several RTKs in kinase regulation. Substitution of the C-terminal residues of c-Fms with residues of v-Fms enhanced receptor autophosphorylation and transforming activity (40). The deletion of the C-terminal residues of ErbB2 increased receptor kinase activity as well as transforming ability (41). A peptide containing the C terminus of platelet-derived growth factor β receptor inhibited its kinase activity (42). The crystal structure of the Tie2 RTK has been solved with an intact C-terminal tail. The Tie2 C-terminal tail is present in an extended conformation. The tail interacts with the C-lobe of the kinase catalytic domain and ends near the substrate binding site. The activation loop of Tie2 is in an “active-like” conformation (43), and the end of the C-terminal tail might prevent access of substrates to the active site. The side chain hydroxyls of two tyrosine residues (Tyr1101 and Tyr1118) are hydrogen bonded to surrounding residues and may stabilize this conformation of the C-terminal tail. Deletion of the Tie2 C-terminal tail increased receptor autophosphorylation and kinase activity, lending further support to a model in which the tail is involved in kinase regulation (44).

The RON receptor studied here is closely related to the MET receptor, for which a crystal structure has been solved which includes the C-terminal tail (45). The tail of MET includes two unphosphorylated tyrosines that correspond to the dityrosine motif studied here. The first tyrosine (Tyr1349 in MET) is in an extended conformation, whereas the second (Tyr1356) is part of a type I β turn. As in the case of Tie2, the MET C-terminal tail approaches the substrate binding site, but it is not clear from the structure whether the tail regulates substrate access or kinase activity. Previous biochemical experiments suggested a role for the MET C-terminal tail in kinase regulation. MET was immunoprecipitated from mammalian cells, and the addition of a peptide mimicking the C terminus inhibited autophosphorylation and phosphorylation of myelin basic protein (23). Delivery of this peptide in intact cells inhibited a variety of MET signaling functions.

Here we provide biochemical evidence for C-terminal regulation of RON, a receptor tyrosine kinase in the MET family. Our experiments were carried out with purified RON, ruling out the possibility that copurifying cellular components were responsible for the effects. The C-terminal tail region of RON, especially the bidentate tyrosine motif, appears to serve an inhibitory role for the MET C-terminal tail in kinase regulation. MET was immunoprecipitated from mammalian cells, and the addition of a peptide mimicking the C terminus inhibited autophosphorylation and phosphorylation of myelin basic protein (23). Delivery of this peptide in intact cells inhibited a variety of MET signaling functions.

include EphB2 (35), FLT3 (FMS-like tyrosine kinase 3) (36), MuSK (37), and c-Kit (38). The crystal structures of the autoinhibited forms of these three kinases have been solved, and
F1353/F1360 and Y1353/Y1360 to RON-CT. We immobilized the two peptides and carried out pull-down experiments with RON-CT. RON-CT bound to both peptides in this experiment, FIG. 5.

**Activity of various RON constructs.** A, purified RON-KIN was analyzed by SDS-PAGE with Coomassie staining (left) or by immunoblotting with anti-RON antibody and alkaline phosphatase-conjugated secondary antibody (right). B, kinase activity of RON-CT, RON-KIN, and RON-2YF was measured with 0.5 mM EGFR peptide at 30 °C using the phosphocellulose paper assay. Note that the scale on the left represents activity for RON-CT and RON-KIN, whereas the lower scale on the right represents activity for RON-2YF. Closed circles, RON-KIN; open squares, RON-2YF; closed squares, RON-CT. C, kinetic analysis of RON-CT (closed circles) and RON-KIN (open squares). Phosphorylation of EGFR peptide was determined with the phosphocellulose paper assay using a range of peptide concentrations (0.05–2.0 mM) and 0.25 mM [\gamma-\text{32P}]ATP. D, kinetic analysis of RON-2YF.

**Fig. 6. RON-KIN inhibition by C-terminal tail peptides.** A, purified RON-KIN was preincubated with various concentrations of the indicated peptides for 10 min followed by a kinase assay with 0.5 mM Abl peptide as a substrate and 0.25 mM [\gamma-\text{32P}]ATP for 30 min. RON activity was measured with the phosphocellulose paper assay. B, RON-KIN was preincubated with peptides for 10 min. Next, 0.25 mM [\gamma-\text{32P}]ATP was added in kinase buffer. After a 40-min incubation, the reaction was terminated by the addition of SDS-sample buffer and analyzed by SDS-PAGE and autoradiography. C, RON-KIN activity was determined in the presence of 0.5 mM EGFR peptide substrate, 0.25 mM [\gamma-\text{32P}]ATP, and 0, 50, 125, or 250 μM concentrations of peptide F1353/F1360. The concentrations of peptide F1353/F1360 are given on the right of the lines.
but RON-CT showed a higher affinity for peptide F1353/F1360 than for peptide Y1353/Y1360.2

Our results suggest a model in which the C-terminal tail (Y1353VQLPAT1360YMNL) interacts with the catalytic domain and partially inhibits RON kinase activity (Fig. 8). Upon ligand stimulation, the C terminus tail would be displaced from its autoinhibitory position. Oligomerization-induced conformational changes together with phosphorylation of the activation loop may trigger this displacement of the C-terminal tail. We also speculate that the C-terminal tail becomes phosphorylated during this process. It is unclear whether this phosphorylation is catalyzed by RON itself or by another kinase. Synthetic peptides containing the C-terminal tail sequence were not phosphorylated by RON, whereas larger C-terminal constructs (GST-RON65 and GST-RON100) were substrates. These larger proteins may contain additional determinants for substrate recognition which are absent in the peptides. In our model, the 2YF substitution in the C-terminal tail results in strengthened binding to the catalytic domain, and this interaction interferes with access of substrates to the active site. This would explain the decreased activity of the RON-2YF construct (Fig. 5D).

Peptide Y1353 was a more effective inhibitor of RON-CT and RON-KIN than peptide Y1360 under all conditions tested (Figs. 4 and 6). These results could indicate that the side chain of unphosphorylated Y1353 is particularly important in autoinhibition. It is also possible, however, that there are N-terminal amino acids present only in peptide Y1353 which strengthen binding to RON in the context of a synthetic peptide (or C-terminal residues present in peptide Y1360 which weaken binding). This might also explain why peptide Y1353 inhibited RON-CT more potently than peptide Y1353/Y1360, which includes both residues of the dityrosine motif (Fig. 4). Peptides Y1353 and Y1353/Y1360 were equally effective as inhibitors of RON-KIN (Fig. 6). The mechanism for autoinhibition remains speculative in the absence of structural information.

After phosphorylation, the bidentate motif interacts with several different signaling proteins to activate multiple downstream signaling pathways (15, 46). Among the SH2 domain-containing proteins that interact with the bidentate motif are Grb2 and the p85 subunit of phosphatidylinositol 3-kinase containing proteins that interact with the bidentate motif are activated in the context of this conformational change (Fig. 9). This might also explain why peptide Y1353 inhibited RON-CT more potently than peptide Y1353/Y1360, which includes both residues of the dityrosine motif (Fig. 4). Peptides Y1353 and Y1353/Y1360 were equally effective as inhibitors of RON-KIN (Fig. 6). The mechanism for autoinhibition remains speculative in the absence of structural information.

FIG. 7. Inhibition in NIH3T3 cells. RON-expressing NIH3T3 cells were incubated with 9 or 90 μM concentrations of peptides (control, Y1353/Y1360, and F1353/F1360) together with Chariot transfection reagent for 3 h. Cell lysates from MSP-treated or untreated cells were analyzed by SDS-PAGE and Western blotting with anti-phosphotyrosine antibody (A, upper panel) and anti-RON antibody (A, lower panel). The position of RON is indicated by the arrow. The blot was reprobed with antibody against phospho-MAPK (B, upper panel) and with anti-MAPK antibody (B, lower panel). Src substrate (AEEEIY-GEFEAKKKK) was used as the control peptide.

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