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Geldanamycins Trigger a Novel Ron Degradative Pathway, Hampering Oncogenic Signaling*[S]

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Ron, the tyrosine kinase receptor for macrophage-stimulating protein is responsible for proliferation and migration of cells from different tissues. Ron can acquire oncogenic potential by single point mutations in the kinase domain, and dysregulated Ron signaling has been involved in the development of different human cancers. We have previously shown that ligand-activated Ron recruits the negative regulator c-Cbl, which mediates its ubiquitylation and degradation. Here we report that Ron is ubiquitylated also by the U-box E3 ligase C-terminal Hsc70-interacting protein (CHIP), recruited via chaperone intermediates Hsp90 and Hsc70. Gene silencing shows that CHIP activity is necessary to mediate Ron degradation upon cell treatment with Hsp90 inhibitors geldanamycins. The oncogenic Ron^{M1254T} receptor escapes from c-Cbl negative regulation but retains a strong association with CHIP. This constitutively active mutant of Ron displays increased sensitivity to geldanamycins, enhanced physical interaction with Hsp90, and more rapid degradation rate. Cell growth and migration, as well as the transforming potential evoked by Ron^{M1254T}, are abrogated upon Hsp90 inhibition. These data highlight a novel mechanism for Ron degradation and propose Hsp90 antagonists like geldanamycins as suitable pharmacological agents for therapy of cancers where altered Ron signaling is involved.

Dysregulated activation of receptor tyrosine kinases (RTKs)[3] has been extensively documented in different types of human tumors (1) and frequently correlates with poor responsiveness to conventional therapies, pointing at RTKs as potential targets for molecule-based cancer therapy (2, 3). Among the different therapeutic approaches a promising strategy relies on forcing RTKs toward degradative pathways (4).

The Ron tyrosine kinase, receptor for macrophage-stimulating protein (MSP), is a member of the hepatocyte growth factor (HGF) receptor subfamily (5, 6). Ron is expressed in a variety of human tissues, and the engagement by its cognate ligand activates multiple intracellular signaling pathways controlling normal cell proliferation, migration, and adhesion-dependent survival (7–9). A single point mutation (M1254T), targeted to a conserved residue of the tyrosine kinase domain is oncogenic (Ron^{M1254T}), by increasing kinase efficiency and subverting substrate specificity (10, 11). Growing evidence indicates that Ron can be involved in cancer development and progression in humans (12, 13) and in murine models (14, 15). Therefore, targeting Ron expression by forcing its down-regulation may help to elucidate its role in tumor development and progression.

It has been reported that many kinases that are deregulated in human cancers are dependent on the chaperone activity of the Heat shock protein 90 (Hsp90) for their conformational maturation and stability (16). Hsp90 is a ubiquitous chaperone protein abundantly expressed in mammalian cells, where it performs housekeeping functions assisting in the folding, activation, and assembly of a variety of proteins (17). Hsp90 functions in concert with several co-chaperone proteins that modulate its chaperone activity (18). The co-chaperone E3 ubiquitin ligase C-terminal Hsc70-interacting protein (CHIP) has been reported to participate in Hsp90 multichaperone complexes, being involved in ubiquitylation and degradation of client proteins (19, 20).

Geldanamycins are a class of benzoquinone ansamycin antibiotics, able to compete with ADP/ATP in the nucleotide binding pocket of Hsp90, inhibiting its ATP-dependent chaperone activity and thus directing the ubiquitin-mediated proteasomal degradation of the client proteins (21, 22). By leading to depletion of important effector proteins that contribute to deregulated signaling, geldanamycin (GA) and its less toxic derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) exhibit potent anti-tumor activity against human cancer cells, both *in vitro* and in tumor xenografts (23, 24). Indeed, based on promising preclinical evaluations, 17-AAG is currently in clinical trials as a single agent or in combination with other chemotherapeutics (25, 26).

Sensitivity to benzoquinone ansamycins has been described for several RTKs. It has been reported that treatment of breast
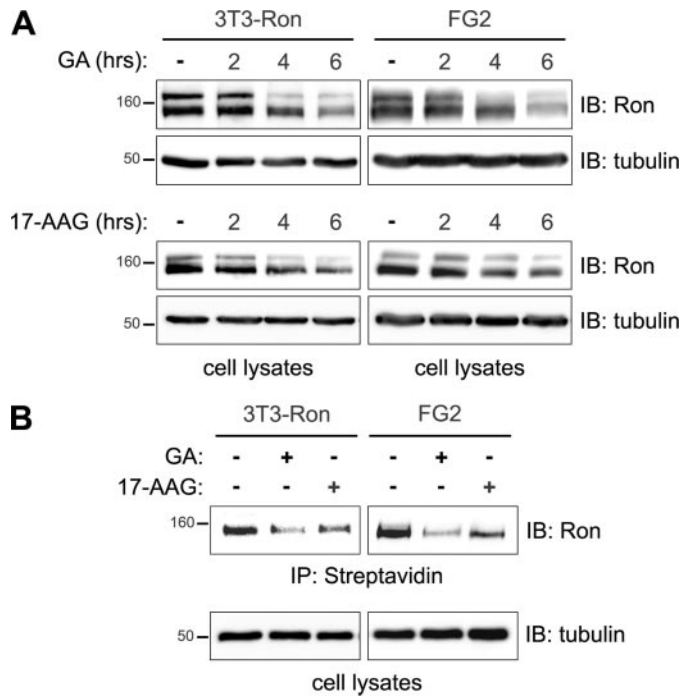
FIGURE 1. **Geldanamycins induce degradation of both precursor and mature cell surface Ron.** *A*, 3T3-Ron and FG2 cells were treated with vehicle (−) or 1 μM GA or 1 μM 17-AAG for the indicated times. Cell lysates were analyzed by immunoblotting (*IB*) with a polyclonal antibody against Ron C-terminal domain, recognizing both the 170-kDa precursor and the 150-kDa β-chain of the receptor. α-Tubulin immunoblotting was used as loading control. *B*, 3T3-Ron and FG2 cells were cell surface-biotinylated and then cultured in medium with vehicle (−) or 1 μM GA, or 1 μM 17-AAG for 6 h. After immunoprecipitation (*IP*) of cell lysates with streptavidin-Sepharose, surface-labeled mature Ron was detected by anti-Ron immunoblotting. All results shown are representative of at least four independent experiments.
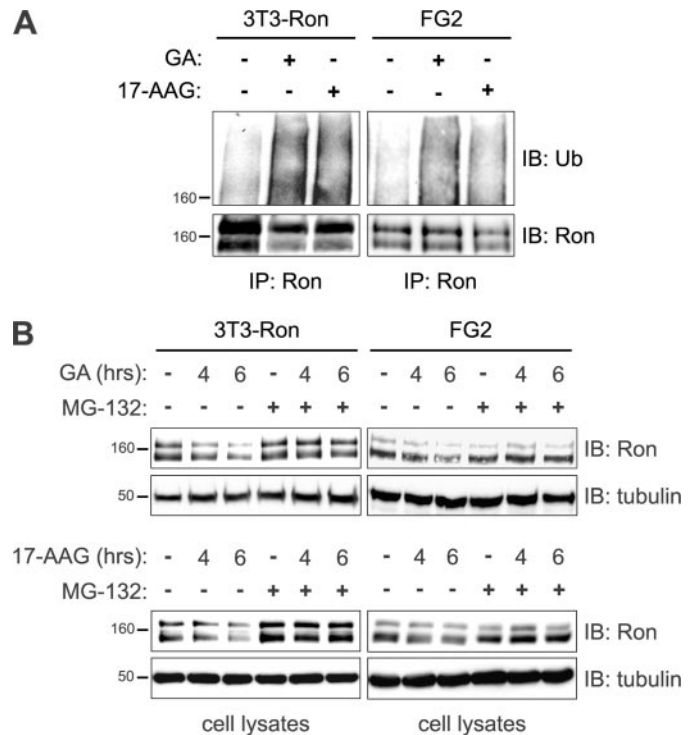

FIGURE 2. **The ubiquitin-proteasome pathway is involved in Ron degradation induced by geldanamycins.** *A*, proteins from lysates of 3T3-Ron and FG2 cells treated with vehicle (−) or 1 μM GA, or 1 μM 17-AAG for 15 min were immunoprecipitated with Ron antibodies. Anti-ubiquitin immunoblotting (*IB*) was used to detect the ubiquitylated receptor molecules. The immunoprecipitated (*IP*) receptor was detected by Ron immunoblotting. *B*, 3T3-Ron and FG2 cells were treated with vehicle (−) or 1 μM GA or 1 μM 17-AAG in the presence or absence of the proteasome inhibitor MG-132 (20 μM). Cells were incubated with MG-132 for 1 h before drug addition. Equal quantities of lysates from cells harvested at the indicated times were analyzed by anti-Ron immunoblotting. Anti-α-tubulin immunoblotting was used as loading control. Representative results from three independent experiments are shown.

and other cancer cells with GA causes rapid ubiquitylation of cell surface HER2/ErbB2 molecules, followed by their proteasome-dependent degradation (27). A recent study demonstrated the ability of GA to deplete mature EGFR protein harboring kinase domain mutations (28). Moreover, geldanamycins have been shown to down-regulate the HGF receptor (Met) and to prevent HGF-mediated tumor cell motility and invasion (29, 30).

We have previously shown that activated Ron recruits to its multifunctional docking site the negative regulator c-Cbl, which mediates ligand-dependent Ron ubiquitylation and down-regulation, through its E3 ubiquitin ligase activity (31). Here we report that Ron forms a multichaperone complex containing Hsp90, Hsc70, and the E3 ligase CHIP. We also show that GA and its derivative 17-AAG induce degradation of both precursor and membrane-exposed forms of Ron. This occurs by impairing Ron association with Hsp90 and by promoting CHIP-mediated receptor ubiquitylation.

The oncogenic Ron^{M1254T} escapes from c-Cbl-mediated negative regulation but is efficiently destabilized by geldanamycins, which hinder growth and migration as well as transforming activity of the oncogenic receptor.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Geldanamycin, 17-AAG, MG-132, and concanamycin A were purchased from Alexis (Montreal,

Canada). Human recombinant MSP was obtained by R&D Systems (Minneapolis, MN). Polyclonal antibodies against Ron C-terminal domain (c-20) and monoclonal anti-Hsc70 (B-6) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-Hsp90 antibody was from BD Transduction Laboratories; monoclonal anti-α-tubulin (B-5-1-2), anti-phospho-Erk1/2 (MAPK-YT), and anti-FLAG (M2) were from Sigma; polyclonal phospho-Akt (Ser^{473}) was from Cell Signaling Technology (Beverly, MA); monoclonal anti-Cbl (7G10) was from Upstate Biotechnology, Inc. (Charlottesville, VA), monoclonal anti-ubiquitin (FK2) was from Stressgen (San Diego, CA); and CHIP rabbit polyclonal antiserum was from Calbiochem (Merck). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were purchased from GE Healthcare (Uppsala, Sweden).

Preparation of Plasmid Constructs—pMT2-Ron and pMT2-Ron^{M1254T} were described previously (10). c-Cbl, c-Cbl70z, CHIP, CHIPK30A, and CHIPΔU-box were prepared in pcDNA3 (32, 33). GST-CHIP and GST-CHIPΔU-box in pGEX4T-2 vector were kindly provided by Dr. H. Band (Brigham and Women's Hospital, Boston, MA). pCCLsin-.PPT.hPGK.GFP.Wpre transfer plasmid was used to express two independent small interfering RNAs (siRNAs) (5'-ACCA-CGAGGGTGATGAGGA-3' and 5'-GAAGCGAGATATCC-CTGAC-3'), targeting CHIP transcripts or an unrelated

sequence as negative control. The expression was under the transcriptional control of the H1 promoter derived from pSUPER plasmid (34). The vector carries an independent GFP expression cassette, to allow for the identification of transfected cells.

Cell Culture and Transfection—Cells were purchased from American Type Culture Collection. FG2 cells were maintained in RPMI 1640, COS-7, and NIH-3T3 cells in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% fetal bovine serum (Invitrogen) in a 5% CO_2-humidified atmosphere. NIH-3T3 cells stably expressing Ron or Ron[M1254T] were obtained as described previously (10). Transient transfection of COS-7 cells was performed with DEAE-dextran using the CellPhect transfection kit (GE Healthcare). LipofectaminePlus (Invitrogen) was used for transfection of FG2 cells with siRNAs constructs according to the manufacturer's recommendations.

Immunoprecipitation and Immunoblotting—Total cellular proteins were extracted by solubilizing the cells in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing protease and phosphatase inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 mM sodium fluoride). Whole-cell lysates were clarified by centrifugation (14,000 × g, 10 min), quantified with the BCA protein assay reagent kit (Pierce) and dissolved in Laemmli sample buffer. For immunoprecipitation, cells were lysed in solubilization buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100) with protease inhibitors. 500-μg aliquots of clarified cell lysates were incubated with 1 μg of the indicated antibody immobilized on protein A-Sepharose 4B packed beads (GE Healthcare) for 2 h at 4 °C. After extensive washes with lysis buffer, precipitated proteins were dissolved in Laemmli sample buffer. Proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with respective antibodies. For ubiquitin immunoblotting, proteins were transferred to polyvinylidene difluoride membranes, incubated for 30 min in 20 mM Tris-HCl containing 6 M guanidine hydrochloride and 5 mM 2-mercaptoethanol, and then probed with ubiquitin antibodies. Detection was performed by the ECL system (GE Healthcare) and the Chemidoc exposure system (Bio-Rad). Image analysis was performed with Quantity One (Bio-Rad).

In Vitro Ubiquitylation Assay—The GST-CHIP and GST-CHIPΔU-box fusion proteins were expressed in *E. coli* and affinity-purified as described by the manufacturer's protocol. Receptors were immunoprecipitated from 800-μg aliquots of cell lysates with protein A-Sepharose beads. Following purification, Sepharose beads were extensively washed and incubated in a 50-μl reaction for 90 min at 37 °C with 275 ng of purified E1, 400 ng of E2 (UbcH5a), 5 ng/μl biotin NH$_2$-terminal ubiquitin, 10 ng/μl ubiquitin (Boston Biochem Inc., Cambridge, MA), and 5 μg of the indicated GST fusion proteins in a buffer containing 50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl$_2$, 2 mM ATP, 2 mM dithiothreitol. After extensive washes the ubiquitylated receptors were detected by SDS-PAGE and Western blot-
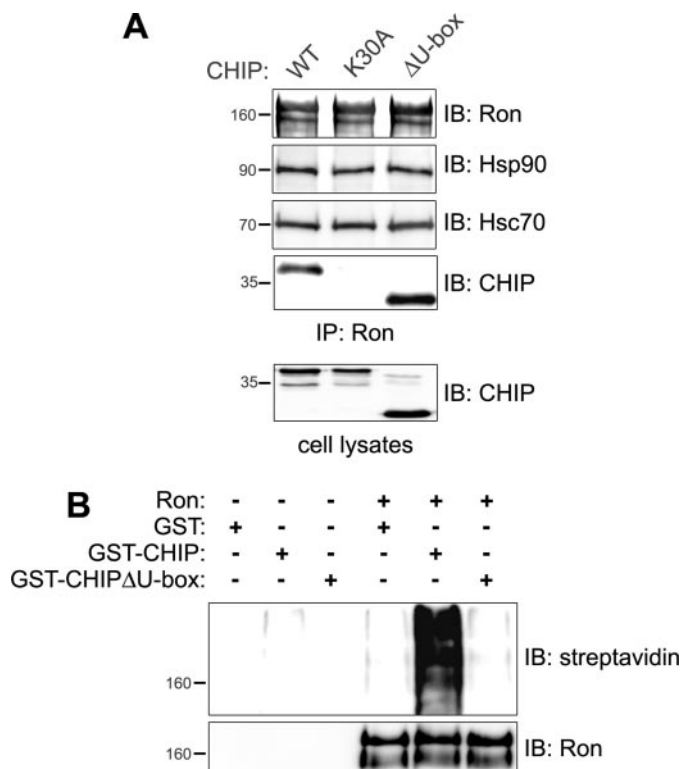

FIGURE 3. **CHIP serves as an E3 ligase for Ron and interacts with the receptor by a chaperone intermediate.** *A*, COS-7 cells were transiently transfected with Ron and either CHIP or different CHIP mutants. 72 h after transfection, cells were lysed, and equal protein amounts were immunoprecipitated (*IP*) with Ron antibodies. Association of Hsp90, Hsc70, and CHIP to the Ron immunocomplex was detected with the appropriate antibodies. Anti-CHIP immunoblotting (*IB*) of cell lysates was used to control transfection efficiency. *B*, proteins from 3T3-Ron cell lysates were immunoprecipitated with Ron antibodies or preimmune rabbit serum. Immunoprecipitates were subjected to *in vitro* ubiquitylation assay in the presence of GST or GST-CHIP fusion proteins, biotin-labeled ubiquitin, E1 and E2 enzymes. The ubiquitylated receptors were detected by Western blotting with streptavidin-horseradish peroxidase. All results shown are representative of at least three independent experiments. *WT*, wild type.

ting with horseradish peroxidase-conjugated streptavidin (GE Healthcare).

Cell Surface Biotinylation Assay—Surface proteins were labeled for 30 min at 4 °C with 0.5 mg/ml Ez-Link® sulfo-NHS-Biotin (Pierce) in buffer A (1.3 mM CaCl$_2$, 0.4 mM MgSO$_4$-7H$_2$O, 5 mM KCl, 138 mM NaCl, 5.6 mM D-glucose, 25 mM HEPES, pH 7.4). After extensive washes with Dulbecco's modified Eagle's medium containing 0.6% bovine serum albumin and 20 mM HEPES, pH 7.4, cells were lysed, and proteins were subjected to immunoprecipitation with streptavidin-Sepharose (GE Healthcare). Protein samples were analyzed by SDS-PAGE and immunoblotting.

Cell Proliferation Assay—Cells were plated on 96-well plates at a density of 4×10^4/well and cultured in appropriate medium supplemented with 10% fetal bovine serum in the presence or absence of 100 nM 17-AAG. Cells were fixed in 11% glutaraldehyde 0, 24, 48, and 72 h after drug addition and stained in crystal violet. Staining was solubilized in 10% acetic acid, and absorbance at 595 nm was measured with a microplate reader.

Cell Migration Assay—Cell motility was assayed using 8-μm pore size Transwell® chambers (Corning Glass). The lower side of the membrane was coated with 10 μg/ml fibronectin for 2 h and then blocked with 0.2% bovine serum albumin. Cells were
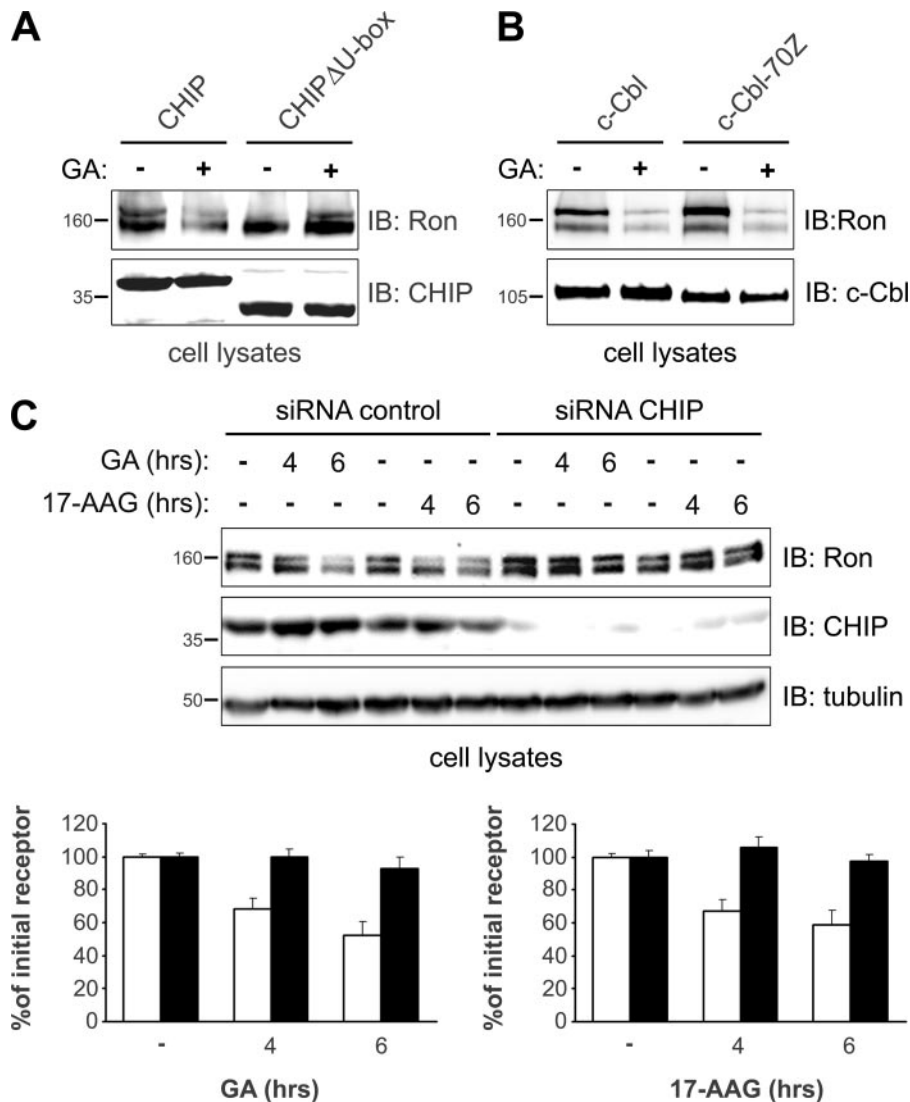
A

B

C

FIGURE 4. **The ubiquitin ligase activity of CHIP is necessary to mediate Ron degradation induced by geldanamycins.** *A*, COS-7 cells were transiently transfected with Ron and either CHIP or the ubiquitylation-defective CHIP Δ U-box. 72 h after transfection, cells were treated with vehicle (−) or 1 μM GA for 6 h and equal amounts of cell lysates were analyzed by immunoblotting (*IB*) with Ron and CHIP antibodies. Representative results from three independent experiments are shown. *B*, COS-7 cells were transiently transfected with Ron and either c-Cbl or c-Cbl-70Z. 72 h after transfection, lysates from cells treated with vehicle (−) or 1 μM GA for 6 h were analyzed by immunoblotting with Ron and c-Cbl antibodies. Representative results from two independent experiments are shown. *C*, FG2 cells were transiently transfected with siRNAs targeted to CHIP transcript or to an unrelated sequence. 48 h after transfection, cells were treated with vehicle (−) or 1 μM GA or 1 μM 17-AAG. Equal quantities of lysates from cells harvested at the indicated times were analyzed by immunoblotting with Ron antibodies. CHIP silencing was verified by immunoblotting with appropriate antibodies and anti-α-tubulin immunoblotting was used as loading control. Densitometry of Ron immunoblotting was performed and values, normalized to the relative α-tubulin control bands, were plotted as percentages of vehicle-treated cells. *White columns*, unrelated siRNA; *black columns*, CHIP siRNA. Each data point represents the mean ± S.E. of three independent experiments.

detached with 1 mM EDTA and resuspended with 2% fetal bovine serum. 1×10^5 cells were plated on the upper side and allowed to migrate for 6 h in the presence of 100 nM 17-AAG toward the lower chamber containing appropriate medium supplemented with 10% fetal bovine serum. Cells remaining in the upper chamber were mechanically removed, and those that migrated to the lower side were fixed and stained as described above.

Transforming Assay—A focus-forming assay was performed on NIH-3T3 fibroblasts (5×10^5 cells) with 5 μg of recombi-

nant plasmid as described previously (10). Two days after transfection, 17-AAG was added to cultures and renewed every 48 h. Cell cultures were maintained at confluence and screened for focus formation 10 ± 18 days after transfection. Spontaneous formation of foci was negligible.

RESULTS

Geldanamycins Target Ron for Degradation—To investigate the sensitivity of Ron to Hsp90 inhibitors, we performed a time course experiment on NIH-3T3 fibroblasts (3T3-Ron) and pancreatic carcinoma FG2 cells, expressing recombinant and endogenous Ron, respectively. After cell exposure to GA for different times, we observed a robust reduction of both precursor and mature form of the receptor within 6 h. It is noteworthy that Ron protein levels declined with similar rates in both cell lines upon GA treatment. Similar effects, at a slightly lesser extent, were observed with the same concentration of the less toxic GA derivative 17-AAG (Fig. 1*A*). Ron destabilization upon Hsp90 inhibition was obtained also by using a chemically unrelated Hsp90 inhibitor, the macrolactone antibiotic radicicol (supplemental Fig. 1).

It has been shown for other RTKs (platelet-derived growth factor receptor and epidermal growth factor receptor) (35) that only the newly synthesized receptor molecules are destabilized by GA. We evaluated if Ron depletion following GA or 17-AAG exposure was due to impaired maturation of the nascent chains only or if also cell surface-exposed receptors were targeted for degradation. By surface biotinylation of 3T3-Ron and FG2 cells, followed by Ron immunoprecipitation, we observed an accelerated decrease of cell surface mature Ron after exposure to GA or 17-AAG for 6 h (Fig. 1*B*). This indicates that GA and 17-AAG, albeit with minor efficacy, are able to destabilize the receptor even after its exposure to the plasma membrane. In NIH-3T3 cells expressing a kinase-defective Ron mutant (31), GA-induced receptor degradation was retained (supplemental Fig. 2), demonstrating that the kinase activity is not required for sensitivity of Ron to GA.

GA-induced degradation of the client proteins is reported to involve the ubiquitin-proteasome pathway (36). Thus, we ana-
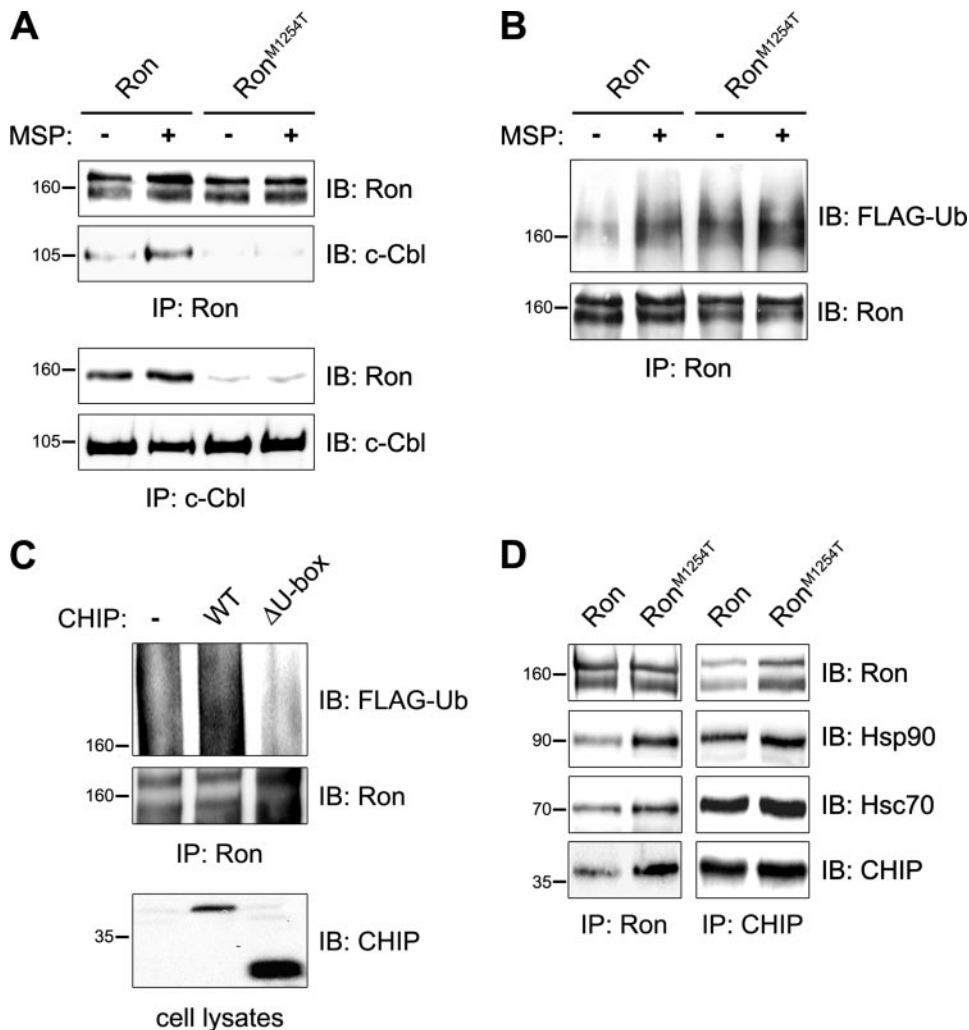
FIGURE 5. **CHIP mediates c-Cbl independent oncogenic Ron^{M1254T} ubiquitylation.** *A*, COS-7 cells were transiently transfected with Ron or Ron^{M1254T}, along with c-Cbl and FLAG-tagged ubiquitin. 72 h after transfection, serum-starved cells were treated with vehicle ($-$) or 300 ng/ml MSP for 20 min, and equal protein amounts of cell lysates were immunoprecipitated (*IP*) with Ron (*top*) or c-Cbl (*bottom*) antibodies. Associated c-Cbl or Ron were detected with the appropriate antibodies. *B*, Ron immunoprecipitation described in *A* was followed by immunoblotting (*IB*) with FLAG antibodies to detect ubiquitylated receptors. The immunoprecipitated receptor was detected by Ron immunoblotting. *C*, proteins from cell lysates of COS-7 transiently transfected with Ron^{M1254T} and FLAG-tagged ubiquitin, along with CHIP or CHIPΔU-box or empty vector, were subjected to immunoprecipitation with Ron antibodies and analyzed by anti-FLAG and anti-Ron immunoblotting. Anti-CHIP immunoblotting on cell lysates was used to control transfection efficiency. *D*, COS-7 cells were transiently transfected with Ron or Ron^{M1254T} along with CHIP. 72 h after transfection equal protein amounts of cell lysates were immunoprecipitated with Ron or CHIP antibodies and presence of CHIP, Ron, Hsp90, or Hsc70 in the immunocomplexes was detected with the appropriate antibodies. Ron, Ron^{M1254T}, and CHIP expression was monitored by immunoblotting. All results shown are representative of at least three independent experiments.

duced kinase-independent degradation, involving the ubiquitin-proteasome pathway.

Ron Associates with a Chaperone Complex Containing the E3 Ubiquitin Ligase CHIP—We aimed at identifying the ubiquitin ligase responsible for GA-induced Ron ubiquitylation. Among several ligases, we focused our attention on CHIP, since this E3 enzyme mediates degradation of signaling proteins, relying on the association with chaperone proteins Hsp90 and Hsc70 (20, 37). We verified if Ron and CHIP were recruited in stable complexes with these chaperone proteins. We co-transfected COS-7 cells with cDNAs encoding both Ron and CHIP. Immunoblotting analysis on Ron immunoprecipitates demonstrated that the receptor associates with endogenous Hsp90 and Hsc70 as well as with CHIP (Fig. 3*A*).

CHIP has the ability to bind Hsc70 by means of the amino-terminal tetratricopeptide domain, whereas its E3 ubiquitin ligase activity is mediated by its carboxyl-terminal U-box domain (38). To better characterize the interaction between the receptor and the ubiquitin ligase, we tested the ability of CHIP proteins harboring a mutation in the tetratricopeptide (K30A) or lacking the U-box (ΔU-box) domain to interact with Ron. The K30A mutant, which does not bind to either Hsp90 or Hsc70 (20), failed to co-immunoprecipitate with Ron. This suggests that these chaperone intermediates are involved in the Ron-CHIP interaction. Conversely, the deletion of the U-box domain did not impair the complex formation (Fig. 3*A*).

To verify whether CHIP could directly mediate Ron ubiquitylation, we performed an *in vitro* ubiquitylation assay on immunocomplexes from 3T3-Ron fibroblasts, by using purified GST-CHIP fusion proteins in the presence of biotinylated ubiquitin and E1 and E2 enzymes. Wild type (GST-CHIP), but not U-box-deleted (GST-CHIPΔU-box), fusion protein catalyzed the receptor ubiquitylation. No ubiquitylation was observed in the presence of GST protein alone as well as when the reaction was performed in absence of immunoprecipitated Ron (Fig. 3*B*). This demonstrates that CHIP can serve as an E3 ligase for Ron. Taken together, these results indicate that in live cells Ron forms a complex with

lyzed Ron ubiquitylation upon GA or 17-AAG treatment of 3T3-Ron and FG2 cells. In these conditions, a marked ubiquitylation of the receptor was observed as early as 15 min after drug addition (Fig. 2*A*). This indicates that receptor ubiquitylation is an early step in GA-induced Ron degradation and suggests that a specific E3 ligase is involved. Moreover, pretreatment of both cell lines with the proteasome inhibitor MG-132 impaired Ron depletion induced by geldanamycins (Fig. 2*B*), indicating that this destabilizing effect on Ron requires proteasomal activity. Conversely, when cells were pretreated with the lysosomal inhibitor concanamycin A, GA retained full activity on Ron (supplemental Fig. 3). Our results show that cell surface-exposed mature Ron is destabilized by GA-in-
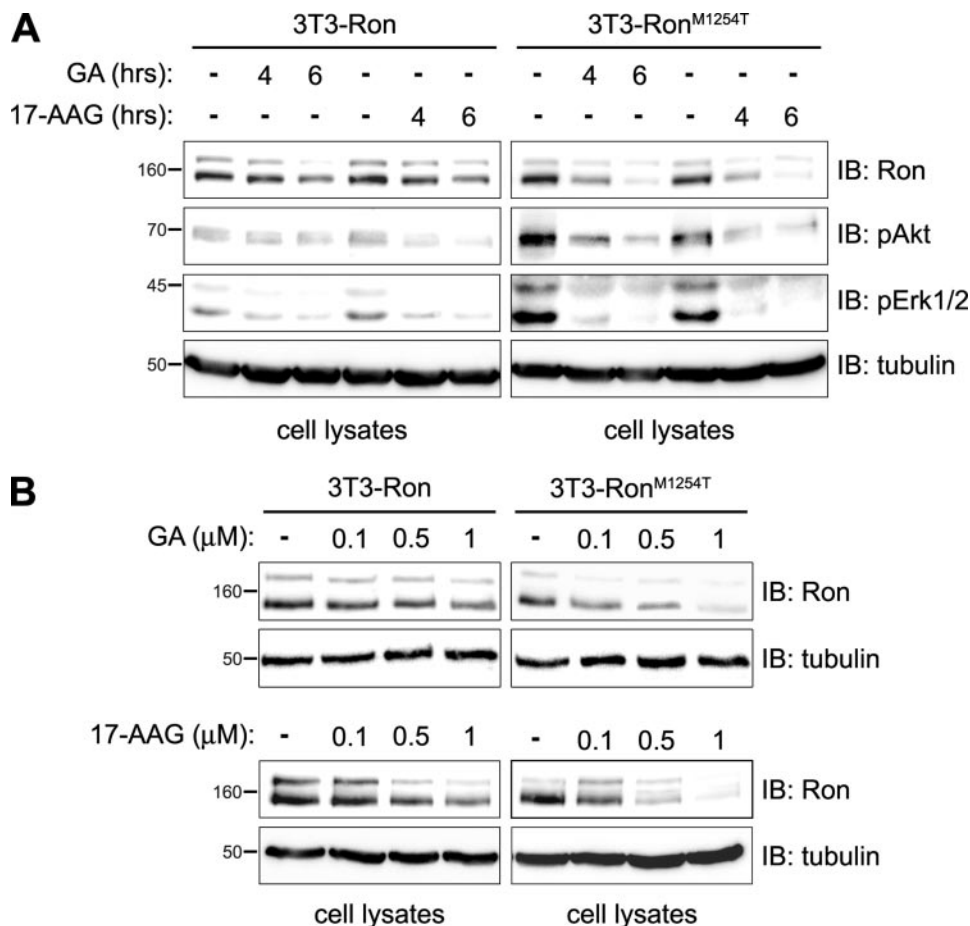
A

B

FIGURE 6. **Oncogenic Ron^{M1254T} displays higher sensitivity to geldanamycins than wild-type receptor.** *A*, 3T3-Ron and 3T3-Ron^{M1254T} cells were treated with vehicle (−) or 1 μM GA, or 1 μM 17-AAG for the indicated times. Equal amounts of cell lysates were analyzed by immunoblotting (*IB*) with Ron, phospho-Akt, and phospho-Erk1/2 antibodies. α-Tubulin immunoblotting was used as loading control. *B*, 3T3-Ron and 3T3-Ron^{M1254T} cells treated with vehicle (−) or increasing amounts of GA, or of 17-AAG for 6 h. Equal amounts of cell lysates were analyzed by immunoblotting with Ron antibodies. Anti-α-tubulin immunoblotting was used as loading control. All results shown are representative of at least four independent experiments.

the chaperones Hsp90 and Hsc70, which mediate receptor association with the E3 ubiquitin ligase CHIP.

CHIP Mediates Ron Degradation Induced by Geldanamycins—We evaluated the involvement of CHIP in GA-induced Ron degradation by using COS-7 cells expressing the wild-type E3 ligase or the deletion mutant CHIPΔU-box, which despite its lack of ubiquitin ligase activity still associates with the receptor. Overexpression of the dominant negative CHIPΔU-box resulted in abrogation of GA-induced Ron degradation (Fig. 4*A*).

We previously reported that the E3 ubiquitin ligase c-Cbl physically interacts with Ron, promoting its ligand-dependent ubiquitylation and down-regulation (31). To verify the role for c-Cbl in receptor destabilization driven by GA, we performed the parallel experiment in COS-7 transfected with c-Cbl or the dominant negative c-Cbl-70Z (39). Impairment of c-Cbl activity had no effect on GA-induced Ron degradation (Fig. 4*B*).

To confirm the key role of CHIP in GA-mediated destabilization, we analyzed cells deprived of CHIP by expression of targeted siRNAs. FG2 cells were engineered by means of vectors to express siRNAs designed to selectively inactivate CHIP transcripts or targeted to an unrelated sequence as control.

Cells expressing CHIP-targeted siRNAs displayed markedly reduced levels of CHIP. In these conditions, Ron was refractory to the degradation induced by GA, whereas in cells expressing control siRNAs, receptor degradation still occurred. Similar results were obtained with the same concentration of 17-AAG (Fig. 4*C*). We conclude that the ubiquitin ligase activity of CHIP is necessary to mediate Ron degradation induced by geldanamycins.

CHIP Is Responsible for Oncogenic Ron^{M1254T} c-Cbl-independent Ubiquitylation—We next addressed the role of CHIP-chaperone complex on the negative regulation of the oncogenic mutant Ron^{M1254T}. This mutant harbors a point mutation responsible for constitutive activation of the kinase and for overcoming the requirement for the multifunctional docking site of the Ron receptor (10, 11).

In COS-7 cells co-expressing c-Cbl and Ron^{M1254T}, the mutant receptor failed to co-immunoprecipitate the ubiquitin ligase, even upon MSP stimulation. Likewise, in the reciprocal experiment, wild-type Ron, but not Ron^{M1254T}, was present in c-Cbl immunoprecipitates of the same cells (Fig. 5*A*). On the basis of these results, we evaluated if the lack of association with c-Cbl could affect Ron^{M1254T} ubiquitylation. In COS-7 cells co-transfected with wild-type or mutant receptor along with c-Cbl and a tagged form of ubiquitin (FLAG-Ub), the ubiquitylation of Ron^{M1254T} was preserved and was ligand-independent (Fig. 5*B*).

To verify if Ron^{M1254T} ubiquitylation *in vivo* relies on the ubiquitin ligase activity of CHIP, we overexpressed CHIP or the defective ligase CHIPΔU-box in COS-7 cells. Endogenous CHIP was sufficient to promote Ron^{M1254T} ubiquitylation, which was increased by overexpression of recombinant CHIP and almost totally abrogated in cells overexpressing CHIPΔU-box. This demonstrates that CHIP is a functional E3 ubiquitin ligase for this oncogenic receptor (Fig. 5*C*).

On the basis of these data, we sought evidence of the association of Ron^{M1254T} with the chaperone complex containing CHIP. We co-transfected COS-7 cells with cDNAs encoding either Ron^{M1254T} or wild-type Ron and CHIP. Hsp90, Hsc70, and CHIP were more abundant in Ron immunocomplexes from cells expressing the oncogenic receptor, and the stronger interaction between CHIP and Ron^{M1254T} was confirmed by the reciprocal experiment (Fig. 5*D*).

These data altogether indicate that Ron and Ron^{M1254T} differentially interact with the E3 ligase CHIP, which is responsi-
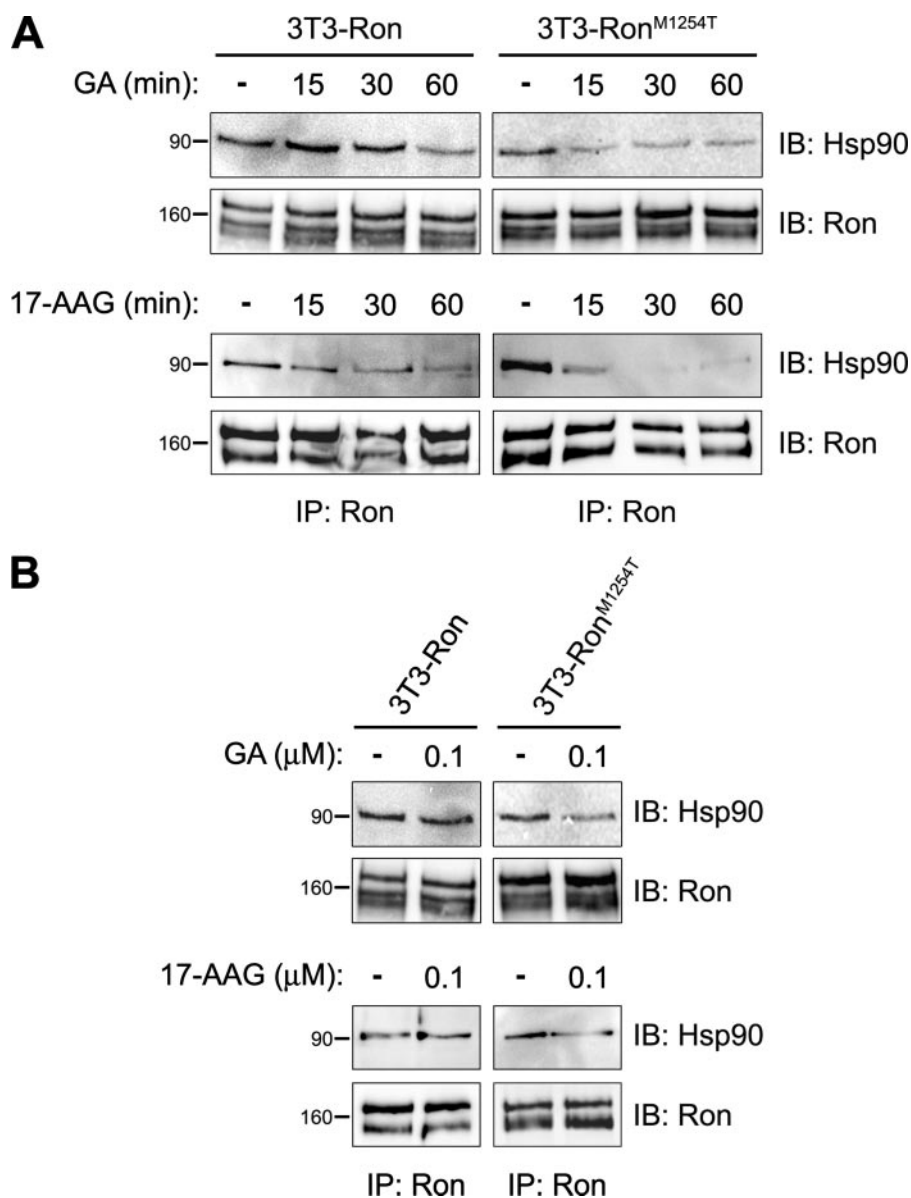
FIGURE 7. **Hsp90 dissociation from Ron^(M1254T) is more rapid and occurs at lower geldanamycin concentrations compared with wild-type receptor.** *A*, 3T3-Ron and 3T3-Ron^(M1254T) cells were treated with vehicle (−) or 1 μM GA or with 1 μM 17-AAG for the indicated times. Equal protein amounts of cell lysates were subjected to immunoprecipitation (*IP*) with Ron antibodies, and associated Hsp90 was detected by immunoblotting (*IB*) with the appropriate antibodies. The immunoprecipitated receptor was detected by Ron immunoblotting. *B*, proteins from cell lysates of 3T3-Ron and 3T3-Ron^(M1254T) treated with vehicle (−) or increasing amounts of GA or of 17-AAG for 6 h were subjected to Ron immunoprecipitation. Associated Hsp90 was detected by immunoblotting with the appropriate antibodies. The immunoprecipitated receptor was detected by Ron immunoblotting. All results shown are representative of at least three independent experiments.

Interestingly, Ron^(M1254T) degradation was paralleled by an evident dephosphorylation of Akt and Erk1/2 effectors (Fig. 6*A*). We further evaluated the relative sensitivity of wild-type and oncogenic Ron to these inhibitory drugs in a dose-response experiment. GA or 17-AAG concentration as low as 0.1 μM was efficient in degrading the mutant Ron^(M1254T) after 6 h of treatment, whereas at least a 1 μM concentration was required to induce detectable degradation of the wild-type receptor (Fig. 6*B*).

The destabilizing effects of GA have been attributed to altered association of Hsp90 with its client proteins (40). Therefore, we tested Ron interaction with Hsp90 upon GA or 17-AAG treatment of 3T3-Ron and 3T3-Ron^(M1254T) cells in a short term experiment (up to 60 min). In both cell types, the receptor co-precipitated with Hsp90, and geldanamycins caused an evident decrease in the amount of Hsp90 associated with Ron immunocomplexes. However, the dissociation of the Ron^(M1254T)-Hsp90 complex occurs earlier, starting within 15 min of drug exposure, as compared with the Ron-Hsp90 complex (Fig. 7*A*). Consistently, by using the lowest effective concentration of GA and 17-AAG (0.1 μM) able to induce degradation of Ron^(M1254T) but not of wild-type Ron, we observed dissociation of Hsp90 from the mutant receptor only (Fig. 7*B*). These results indicate that the oncogenic M1254T substitution in the Ron receptor is associated with increased sensitivity to geldanamycins and with a more dynamic interaction with Hsp90.

Geldanamycins Hamper Growth, Migration, and Transforming Activity of Oncogenic Ron—The amino acid substitution M1254T confers to Ron *in vitro* transforming potential, including growth and migration in a ligand-independent way (10). We tested if the GA derivative suitable for clinical use 17-AAG could hamper these biological effects in 3T3-Ron^(M1254T). In a 72-h proliferation assay, the higher proliferation rate of 3T3-Ron^(M1254T) cells compared with 3T3-Ron cells was considerably reduced in the presence of low concentrations (0.1 μM) of 17-AAG. As expected, the growth rates of nontransformed 3T3 and 3T3-Ron cells were similar, and, consistently, the reduction of growth rate observed in these cells upon 17-AAG was com-

ble for the c-Cbl- and ligand-independent ubiquitylation of the oncogenic receptor.

Oncogenic M1254T Substitution Increases Ron Sensitivity to Geldanamycins—Since oncogenic Ron^(M1254T) is recruited into the CHIP-chaperone complex even more efficiently than wild-type Ron, we tested the activity of GA and 17-AAG on the stability of the wild-type receptor and of its oncogenic counterpart.

The treatment of 3T3-Ron and 3T3-Ron^(M1254T) cells with both inhibitors revealed an accelerated degradation rate for the mutant receptor, as compared with wild-type Ron (Fig. 6*A*).
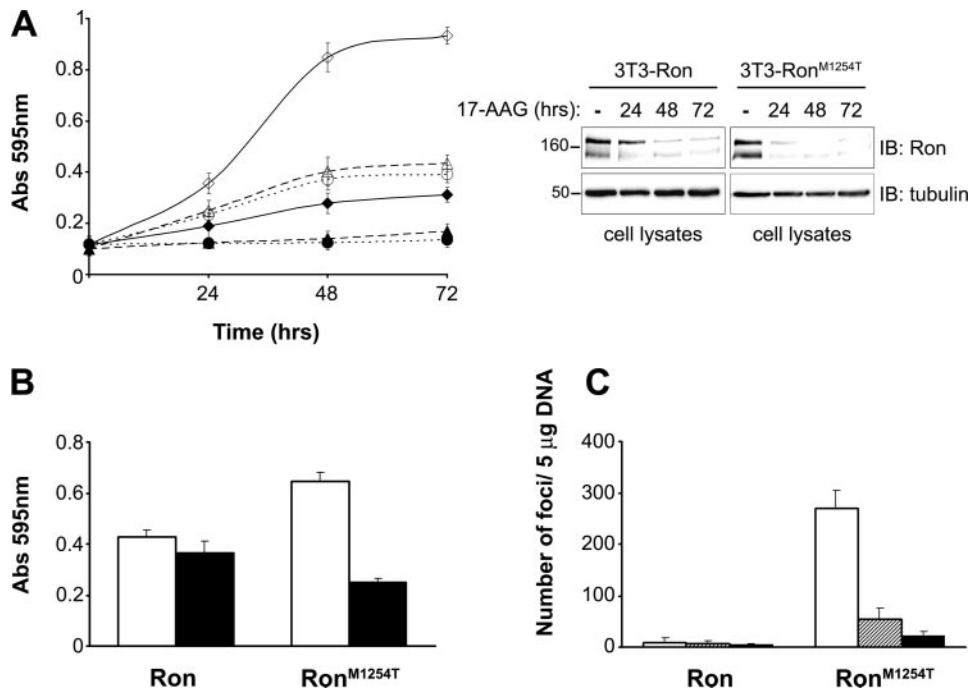
FIGURE 8. **17-AAG potently affects growth, migration, and transformation of cells expressing oncogenic Ron^{M1254T}.** *A*, NIH-3T3 fibroblasts (*dotted curve*), 3T3-Ron (*dashed curve*), and 3T3-Ron^{M1254T} (*solid curve*) cells were plated on 96-well plates and cultured in medium supplemented with 10% fetal bovine serum in presence of vehicle (*white symbols*) or 100 nM 17-AAG (*black symbols*). After 0, 24, 48, and 72 h of drug addition, cells were fixed and stained in crystal violet, and absorbance at 595 nm was measured. OD is expressed as mean $A_{595} \pm$ S.E. of three independent experiments (*left panel*). Protein levels from 3T3-Ron and 3T3 Ron^{M1254T} cell lysates were determined by immunoblotting (*IB*) at same times and conditions of the growth curve (*right panel*). *B*, 3T3-Ron and 3T3-Ron^{M1254T} cells were plated on the *upper side* of 8-μm pore size Transwell® chambers and allowed to migrate for 6 h in the presence of vehicle (*white columns*) or 100 nM 17-AAG (*black columns*) toward the lower chamber containing appropriate medium supplemented with 10% fetal calf serum and coated with fibronectin. Cells that migrated toward the lower chamber were fixed and stained as described in *A*. OD is expressed as mean $A_{595} \pm$ S.E. of three independent experiments. *C*, NIH-3T3 fibroblasts were transfected as described under "Experimental Procedures" with Ron- or Ron^{M1254T}-encoding plasmid. Two days after transfection, vehicle (*white columns*), 10 nM 17-AAG (*hatched columns*), or 50 nM 17-AAG (*black columns*) were added and renewed every 48 h. Cell cultures were maintained at confluence and screened for focus formation 10 $\pm$ 18 days after transfection. Spontaneous formation of foci was negligible. All of the experiments were performed in triplicate, and mean $\pm$ S.E. number of foci is displayed.

parable. Immunoblotting analysis of Ron protein levels under the same experimental conditions confirmed the higher sensitivity of oncogenic Ron^{M1254T} to 17-AAG, even if degradation of also the wild-type receptor occurred upon the prolonged drug exposure (Fig. 8*A*).

In a migration assay, performed in the presence of serum, 3T3-Ron^{M1254T} cells migrated more efficiently than 3T3-Ron cells. The presence of 17-AAG hampered the haptotactic migration of both cell types, and the inhibition was by far more evident in cells expressing the Ron oncogenic form (Fig. 8*B*).

We have previously shown that Ron^{M1254T} has a strong transforming activity when expressed in NIH-3T3 fibroblasts (10). A focus-forming assay was performed in the presence or absence of low doses of 17-AAG. As expected, Ron^{M1254T}-transfected cells displayed a high transforming activity, which was completely abolished by 17-AAG treatment (Fig. 8*C*).

We conclude that the clinically relevant inhibitor 17-AAG is a potent negative regulator of cell proliferation, migration, and transformation, typically induced by the Ron^{M1254T} oncogenic form.

DISCUSSION

Ubiquitylation and down-regulation represent a major deactivation pathway for RTKs, whose impairment may be a mechanism in cancer (41).

Dysregulated signaling of Ron, the tyrosine kinase receptor for MSP, due to over-activation or loss of negative regulation, is involved in tumor progression and metastasis. Overexpression and subsequent aberrant activation of Ron have been observed in primary breast carcinomas (42), non-small cell lung tumors (43), and colorectal adeno-carcinomas (44). Silencing *ron* gene expression by RNA interference has been shown to hamper *in vivo* tumor formation of established colorectal carcinoma cells (45). Moreover, it has been reported that Ron expression is positively associated with histological grading, larger size, and tumor stage in bladder cancer specimens (13).

We have previously demonstrated a ligand-induced c-Cbl-dependent mechanism for the down-regulation of Ron (31). Here we identify a novel degradation pathway for Ron. This mechanism involves proteasomal activity, the Hsp90/Hsc70 chaperones and the U-box ubiquitin ligase CHIP. The whole process of receptor degradation is triggered by the small molecule inhibitors of Hsp90 benzo-quinone ansamycins via dissociation of the Ron-chaperone complex. The requirement of this complex for Ron stability is confirmed by the destabilizing effects observed also with the Hsp90 inhibitor radicicol, which is chemically unrelated to geldanamycins.

It has been reported that Hsp90 and its cohort of co-chaperones play a regulatory role in conformational maturation and in maintenance of structural integrity of a variety of cellular proteins (17). We show here for the first time that Ron is present in a stable but dynamic complex with the Hsp90/Hsc70-based chaperone machinery, also containing the E3 ligase CHIP. As shown for ErbB2 (20, 40), this complex is required for maintaining Ron receptor stability. This is confirmed by the activity of the Hsp90-inhibitory drugs geldanamycin or 17-AAG, which force disruption of the Ron-Hsp90 interaction, followed by receptor ubiquitylation and degradation. Moreover, conversely to what occurs with c-Cbl-mediated degradation (31), geldanamycin does not require the Ron kinase activity for its action, since the "kinase-dead" Ron^{K1114M} is highly sensitive to this Hsp90 inhibitor.

Geldanamycins Induce CHIP-mediated Ron Degradation

The effect of geldanamycin has been restricted to the ability of destabilizing newly synthesized EGFR or platelet-derived growth factor receptor molecules (35, 46). On the other hand, it has been also demonstrated that geldanamycin enhances the loss of mature cell surface ErbB2 protein (40, 47), suggesting a lack of univocal mean to target different receptors. Our results clearly indicate that mature Ron exposed at plasma membrane is a target of geldanamycin, as in the case of ErbB2. This is in accordance with the association of mature Ron with the E3 ligase CHIP, which has been characterized as a mediator of geldanamycin action for ErbB2 degradation (20, 47). However, based on our data, we cannot exclude that also the uncleaved precursor of the Ron receptor may be affected by geldanamycins.

Several evidences indicate that the ternary complex of the substrates with chaperones and ubiquitin ligase is targeted to the proteasome for degradation (16). Moreover, recently it has been reported that proteasomal activity is required for ErbB2 internalization but that receptor degradation takes place in lysosomes (48). Our results on Ron confirm that geldanamycin-mediated receptor degradation requires the integrity of the proteasomal pathway but that the lysosomal involvement is dispensable.

A destabilizing effect of geldanamycins has been described for the HGF/SF receptor Met, homologous to Ron (29, 49), but the E3 ligase regulating this process has not been identified yet. Conversely, our results clearly show that Ron is a substrate of CHIP both *in vitro* and *in vivo*. Furthermore, by CHIP RNA interference and by use of a truncated CHIP mutant, we demonstrate that CHIP is necessary for Ron degradation, following Hsp90 inhibition by geldanamycins.

This mechanism of Ron depletion is reminiscent of that shown for ErbB2 (47) and for mutated EGFR (28). Also Ron can harbor oncogenic mutations in a conserved region of the kinase domain (10). One of these substitutions (M1254T) shifts substrate specificity and overcomes the requirement for the multifunctional docking site (11).

We show here that the oncogenic Ron^{M1254T} receptor escapes from c-Cbl mediated down-regulation. Notwithstanding it is efficiently ubiquitylated and becomes degraded upon geldanamycin treatment. Oncogenic Ron degradation occurs even more rapidly than for the nonmutated receptor and at lower doses of the Hsp90 inhibitor. The higher sensitivity of Ron^{M1254T} to geldanamycins may be explained by the stronger interaction of Ron^{M1254T} with Hsp90 and CHIP, compared with wild-type Ron. Similar results were observed also for EGFR mutants (28), v-Src (50), and mutated p53 (51). As hypothesized for other kinases (28), the reason for the greater association of the mutated receptor to the chaperone complex, may reside more on a inherently less stable structure rather than on its altered phosphorylation state.

The effect of Hsp90 inhibition by geldanamycin has been thoroughly investigated and clarified in terms of altered association of the chaperone to its client proteins, which are thus degraded (52). In the case of ErbB2, geldanamycin activity has been associated to a parallel increase of Hsp/Hsc70 association to the receptor (20). We did not observe any reciprocal exchange between Hsp90 and Hsc70 in the binding to either Ron or Ron^{M1254T}. On the other hand, we show

the first evidence of a marked and significant difference in dissociation rate from Hsp90, between wild-type and oncogenic Ron receptors, upon geldanamycins treatment. We hypothesize that the ADP/ATP cycling rate of the Hsp90-Ron^{M1254T} complex is higher than that of Hsp90 complex containing Ron. This may explain the higher sensitivity of the oncogenic mutant to geldanamycins. This highlights Ron^{M1254T} as an ideal target for the antioncogenic activity of these drugs.

Cells expressing oncogenic Ron^{M1254T} display ligand-independent, strong constitutive Erk1/2 and phosphatidylinositol 3-kinase/Akt signaling, leading to elevated levels of growth and migration. The less toxic GA-derivative 17-AAG markedly inhibits these signaling pathways, resulting in a strong reduction of growth and migration rates, induced by oncogenic Ron^{M1254T} signaling. We cannot exclude that degradation of intracellular proteins, possibly Ron^{M1254T} effectors, may also contribute to these effects. Nevertheless, this confirms the very high sensitivity of the oncogenic receptor and of its signaling to geldanamycins, resulting in robust inhibition of dysregulated biological activities.

Recently, it has been demonstrated that geldanamycins inhibit HGF/SF-dependent, urokinase-type plasminogen activator-mediated cell scattering and invasion, thus affecting typical tumor cell properties (30). Consistently, our results demonstrate that 17-AAG abrogates the transforming ability of oncogenic Ron. Even if further studies on xenographed immunodeficient mice are required to substantiate the inhibition of Ron oncogenic properties, the overall effects observed in cultured cells strongly suggest that Ron-dependent tumorigenesis is a sensitive target of geldanamycin or its derivatives. We identified a novel Ron destabilization pathway, which highlights the important role of ansamycin antibiotics as potential pharmacological tools, able to target altered Ron expression and dysregulation in cancers.

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