Fibroblast Growth Factor Receptor 3 (FGFR3) Is a Strong Heat Shock Protein 90 (Hsp90) Client

IMPLICATIONS FOR THERAPEUTIC MANIPULATION

Received for publication, November 23, 2010, and in revised form, March 30, 2011 Published, JBC Papers in Press, April 12, 2011, DOI 10.1074/jbc.M110.206151

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Fibroblast growth factor receptor 3 (FGFR3) is a key regulator of growth and differentiation, whose aberrant activation causes a number of genetic diseases including achondroplasia and cancer. Hsp90 is a specialized molecular chaperone involved in stabilizing a select set of proteins termed clients. Here, we delineate the relationship of Hsp90 and co-chaperone Cdc37 with FGFR3 and the FGFR family. FGFR3 strongly associates with these chaperone complexes and depends on them for stability and function. Inhibition of Hsp90 function using the geldanamycin analog 17-AAG induces the ubiquitination and degradation of FGFR3 and reduces the signaling capacity of FGFR3. Other FGFRs weakly interact with these chaperones and are differentially influenced by Hsp90 inhibition. The Hsp90-related ubiquitin ligase CHIP is able to interact and destabilize FGFR3. Our results establish FGFR3 as a strong Hsp90 client and suggest that modulating Hsp90 chaperone complexes may beneficially influence the stability and function of FGFR3 in disease.

Fibroblast growth factor receptors (FGFRs) are responsible for coordinating numerous developmental and cellular processes such as cellular differentiation and growth (1). Germ line and somatic mutations in FGFRs give rise to genetic disorders of skeletal development and cancer (2, 3), which reflect increased or misregulated FGFR signals. FGFR3 is mutated or abnormally expressed in the most common form of human dwarfism, achondroplasia, and cancers, notably in superficial bladder cancer and multiple myeloma (4, 5). Interestingly, identical mutations are found in bone growth disorders and cancer, suggesting that they share common pathogenetic features and may respond to similar therapeutic approaches (6).

Hsp90 is a molecular chaperone that is abundantly and ubiquitously expressed within cells (7, 8). It is involved in initial protein folding as well as in stabilizing proteins with unstable domains. For kinases, the dedicated co-chaperone Cdc37 helps recruit and control Hsp90 association with the folding complex. Some kinases depend on Hsp90 constitutively for their stability; these “strong clients” include ErbB2 and AKT (9, 10). Others require Hsp90 only when rendered constitutively active by mutation, i.e. EGFR and B-RAF (11, 12). Inhibition of Hsp90 function using small molecule inhibitors alters chaperone complex composition and promotes the association of E3-ubiquitin ligases such as CHIP (carboxyl terminus of Hsp70-interacting protein), leading to client ubiquitination and degradation (13).

Many Hsp90-stabilized kinases are oncogenic, which has led to the development of Hsp90 inhibitors for cancer therapy. Indeed, geldanamycin derivatives such as 17-AAG and other Hsp90 inhibitors have progressed to phase II clinical trials (14). Hsp90 inhibitors have also been investigated in preclinical models of genetic diseases of mutated Hsp90 clients such as the androgen receptor in spinal and bulbar muscular atrophy (Kennedy disease) (15, 16).

Here, we define the relationship of FGFR3 and the FGFR family with Hsp90 and its co-chaperone Cdc37. Our data establish FGFR3 as a strong Hsp90 client in contrast to the other FGFR family members. Inhibition of Hsp90 function alters the chaperone complexes associated with FGFR3 and reduces the stability and signaling capacity of the receptor. Our results suggest that Hsp90 is an important regulator of FGFR3 function and that Hsp90 inhibitors may be useful for treating FGFR3-mediated diseases.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—The following antibodies and reagents were purchased from Santa Cruz Biotechnology: FGFR1 (C-15), FGFR2 (C-17), FGFR3 (C-15), FGFR3 (B9), FGFR4 (C-16), EGFR (528), EGFR (1005), Neu (3BS), Neu (CB11), Hsp90 (F-8), Hsp70 (W27), Cdc37 (C-11), FRS2 (H91), pERK (E-4), ERK1 (K-23), normal rabbit and mouse IgG, and goat anti-mouse IgM HRP. Other antibodies include Sigma cytoplasmic anti-FGFR3 (F-0425, used for untagged immunoprecipitation), Sigma actin (A-2066), Invitrogen V5 (R98025), Abcam GFP (ab290), Cell Signaling pFRS2 (3864) and phospho-FGFR (3471), Covance anti-ubiquitin P4D1 (MS-257P), Biomol anti-polyubiquitin FK1 (PW8805), and BD Biosciences PY20 (610000). The generation of TREX Tet-On cell lines was performed following the manufacturer’s instructions (Invitrogen). Cell lines and transfections were created with plasmids containing mouse FGFR1–4 (IIIc form) and human...
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ErbB1 and ErbB2. FLAG-CHIP was kindly provided by Gen Sobue (17). Mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). The specific residue number for the swapping mutations is as follows for human sequence (if different, mouse amino acid number is shown in parentheses): FGFR1 D554G, FGFR2 D557G (D576G), FGFR3 G533D (G527D), FGFR3 G548D (G542D), and FGFR4 E543G (E540G). Chimeric kinase domain FGFR3/FGFR2 receptors were generated using PCR and blunt ligation. The boundaries used to define the kinase domains were modeled after the manuscript of Chen et al. (18). The human amino acid boundaries are (corresponding mouse amino acids are in parentheses): FGFR3 kinase domain Leu-457–Asp-758 (Leu-451–Asp-752), FGFR2 kinase domain Tyr-466–Glu-767 (Tyr-485–Glu-786), FGFR3 N-terminal lobe/hinge Leu-457–Asn-562 (Leu-451–Asn-556), FGFR2 N-terminal lobe/hinge Tyr-466–Asn-571 (Tyr-485–Asn-590), FGFR3 C-terminal lobe/kinase insert Leu-563–Asp-758 (Leu-557–Asp-752), and FGFR2 C-terminal lobe/kinase insert Leu-572–Glu-767 (Leu-591–Glu-786).

The generation of the COS7 stable cell lines has been previously described (19). Because the effective dose of 17-AAG (InvivoGen) is affected by cell number (20), all HEK 293-based cell lines were plated at 2 × 10^5 cells/cm^2 and RT112 cells were plated at 2.4 × 10^5 cells/cm^2. Celastrul, PD173074, and radicicol were purchased from Calbiochem. Quercetin and cycloheximide were purchased from Sigma. Brefeldin A and lactacystin were purchased from Enzo Biochem. FGFR2 and FGFR9 were purchased from R&D Systems. Moosa Mohammadi generously provided us with FGFR1. Biotinylation reagents obtained from Thermo Fisher Scientific were as follows: EZ-link Sulfo-NHS-Ss-biotin (21331) and NeutrAvidin-agarose resin (2920). Thermo Fisher Scientific were as follows: EZ-link Sulfo-NHS-Ss-biotin (21331) and NeutrAvidin-agarose resin (2920).

**Immunofluorescence**—Cells were treated as indicated, fixed in 4% paraformaldehyde for 20 min, mounted with PermaFluor-Thermo Fisher Scientific, and imaged by confocal microscopy.

**Immunoprecipitation**—Cells either transiently transfected or induced with tetracycline for 48 h (or as notated), were treated with drugs as indicated, and rinsed in 1× PBS, and immunoprecipitation was performed as described by Sweeney et al. (21), with the replacement of inhibitors with 1× Roche Complete Protease Inhibitors and Sigma Phosphatase Inhibitor Mixture 2. For denaturing immunoprecipitation, cells were prepared following the protocol by VanSlyke et al. (22) with slight modifications. Briefly, cells were rinsed 1× in PBS and lysed in SDS denaturation buffer (5 mM Tris–HCl, 5 mM EDTA, 5 mM EGTA, 0.6% SDS, 10 μM iodoacetamide, 200 μM PMSF, 1× Roche Protease Inhibitor Mixture). Lysates were incubated at 100 °C for 3 min and passaged through a QIAshredder (Qiagen). The lysates were then diluted with 2.5 volumes of immunoprecipitation dilution buffer (100 mM NaCl, 20 mM sodium borate, 15 mM EDTA, 15 mM EGTA, 0.7% BSA, 1.2% Triton X-100, 10 μM iodoacetamide, 200 μM PMSF) and centrifuged to remove insoluble protein. Samples were immunoprecipitated following standard protocol. Following electrophoresis and transfer to PVDF membranes, membranes were treated for 30 min at 4 °C in denaturing buffer (23) (6 M guanidine HCl, 20 mM Tris–HCl, pH 7.5, 5 mM 2-mercaptoethanol), rinsed well, and processed as a normal Western blot.

**Biotinylation**—RT112 cells were rinsed in PBS and labeled for 20 min at 4 °C on ice with 250 μg/ml biotin. Cells were washed three times in TBS and then incubated in complete media (RPMI + 10% FBS) with either 1 μM 17-AAG or DMSO for indicated times at 37 °C. Cells were lysed, protein was quantified, and equal amounts were affinity-purified with avidin-agarose beads.

**Half-life Determination**—For cycloheximide experiments, cells were treated as indicated in the presence of 100 μM cycloheximide. Cells were lysed, immunoprecipitated, and subjected to Western analysis. For 35S half-life determination, transfected 293 cells were subjected to pulse-chase analysis as described previously with the addition of DMSO or 1 μM 17-AAG in the chase (24). For brefeldin A (BFA) experiments, cells were pulsed (25 min) and chased in the presence of 6 μg/ml BFA. Half-lives were calculated by subtracting background densitometry (captured from phosphor screen) and fitting to an one-phase decay curve using GraphPad software.

**Mass Spectrometry**—One-dimensional SDS-PAGE bands were subjected to in-gel digestion with trypsin. Identification of tryptic peptides was performed by a liquid chromatography mass spectrometer equipped with an electrospray ionization source. Proteins were identified from MS/MS spectra by a database search of the identified peptides using the software provided with the instrumentation.

**Cell Viability and Signaling Assays**—Serum starvation of the RT112 cell line was in DMEM, whereas the Hela cells were starved in DMEM + 0.1% FCS. For MTT proliferation assays, cell lines were plated at appropriate densities (RT112, 1 × 10^5; Hela, 7.5 × 10^5; and rat chondrosarcoma cells, 5 × 10^5 cells per well) in a 96-well dish and incubated overnight. Drugs or vehicle were added as indicated and a MTT assay was performed (Roche Applied Science). For FGFR experiments, cells were pretreated with 17-AAG for 4 h before the addition of growth factor.

**RESULTS**

**FGFR3 Interacts with Hsp90 and Co-chaperones**—To screen for proteins that interact with activated FGFR3, we transiently transfected GFP tagged constitutively activated FGFR3 (CA-FGFR3, K650E mutation of thanatophoric dysplasia type II) or GFP alone into 293 cells (25). Immunoprecipitation (IP) of FGFR3 pulled down multiple species of the receptor and other distinct bands (Fig. 1A). Prominent bands from several replicates were identified by N-terminal sequencing and mass spectrometry. These results were confirmed by coimmunoprecipitation and Western blotting, which consistently showed that Hps90 and Cdc37 immunoprecipitated with wild-type (WT-FGFR3), CA-FGFR3, and kinase-dead FGFR3 (KD-FGFR3, K508A) (Fig. 1B) (26). Although Hsp70 was frequently pulled down nonspecifically in negative controls, it was usually enriched in FGFR3-specific pulldowns (Fig. 1, A and E). In the reverse immunoprecipitation, Hsp90 pulled down immature (120 kDa) and mature
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(130 kDa) forms of WT-, CA-, and KD-FGFR3, suggesting that Hsp90 can interact with the receptor throughout its maturation and trafficking in the secretory pathway (Fig. 1C) (27). Immunoprecipitation of Cdc37 and blotting for FGFR3 revealed that Cdc37, unlike Hsp90, interacted more strongly with CA-FGFR3 (Fig. 1D). The tighter association was with the 120-kDa immature high mannose endoplasmic reticulum form of the mutant receptor, in contrast to the 130-kDa cell surface form, suggesting that that the chaperone complexes associating with immature CA-FGFR3 may be enriched in Cdc37.

To determine whether Hsp90 interacts with endogenous FGFR3, FGFR3 was immunoprecipitated from RT112 bladder cancer cells, which express high levels of wild-type FGFR3 (28). The immunoprecipitation pulled down Hsp90 and Cdc37 and was enriched for Hsp70 (Fig. 1E). In the reverse immunoprecipitation, Hsp90 pulled down endogenous FGFR3 but not the weak Hsp90 client EGFR (Fig. 1F). These data demonstrate that FGFR3 can interact with Hsp90 chaperone complexes regardless of whether the receptor is expressed endogenously (Fig. 1E) or exogenously with a C-terminal tag (Fig. 1A, GFP and Fig. 1B, V5) or untagged (supplemental Fig. S2A).

Hsp90 inhibitors typically interfere with the Hsp90-client interactions (29, 30). Indeed, a concentration-dependent decrease of Hsp90 coimmunoprecipitating with FGFR3 was detected for the two structurally dissimilar Hsp90 inhibitors 17-AAG, and radicicol, which correlated with their affinities for Hsp90 (Fig. 1G) (31, 32). Celastrol, an inhibitor of the Cdc37-Hsp90 interaction, also disrupted binding, whereas quercetin, which alters Hsp90 complexes but not Hsp90 client binding, did not disrupt Hsp90 coimmunoprecipitation (33, 34).

**FGFRs Differentially Associate with Hsp90 Complexes**—Hsp90 interacts with kinase clients through their kinase domain (8, 30). Because the amino acid sequence for this domain is highly conserved for FGFR family members (Fig. 2A), we tested whether other FGFRs interact with Hsp90 and Cdc37. When untagged FGFRs (with the exception of FGFR4 due to lack of an immunoprecipitation antibody) were expressed and immunoprecipitated with receptor specific C-terminal antibodies, Hsp90 readily pulled down (supplemental Fig. S2A).

FGFR3 consistently pulled down more Hsp90 in these experiments; however, no direct comparison could be made due to differences in the affinities of the receptor-specific antibodies. For a more accurate comparison, we tagged the receptors with a C-terminal V5 epitope. Expression and immunoprecipitation of equal amounts of lyase revealed that FGFR3 associated more strongly with Hsp90 than the other FGFRs (Fig. 2B). Similarly, Cdc37 pulled down more strongly with FGFR3 and was faintly observed for FGFR1 and FGFR4 but not for FGFR2.

The kinase domain αC–α4 loop is important for Hsp90 binding to some kinases, notably the EGFR/ErbB family of receptor tyrosine kinases (10, 35). Binding is favored by the lack of negatively charged residues in the loop. In fact, the presence of a single aspartic acid residue in the loop distinguishes the weak client EGFR from the strong client ErbB2, which has a glycine in the same position. Sequence alignment of the FGFRs alongside EGFR and ErbB2 shows that the FGFRs lack negatively charged residues in this loop and have a glycine in the key position (Fig. 2A). To test whether this glycine residue plays a similar role in the FGFR3-Hsp90 interaction as it does for the ErbB2-Hsp90 interaction, we mutated FGFR3 glycine Gly-533 to an aspartic acid and compared the two receptor proteins in pull-down assays. Mutation of this residue did not disturb Hsp90 or Cdc37 association with FGFR3 (Fig. 2C).

Further examination of the N-terminal lobe of the kinase domain of FGFR revealed a difference that could potentially explain the differential binding of Hsp90 to FGFRs. Residue 548 in the adjacent FGFR3 β4–β5 loop is a glycine in contrast to aspartic acid in FGFR1 and FGFR2 or glutamic acid in FGFR4 (Fig. 2A). We mutated this residue to aspartic acid in FGFR3 and to glycine in the other FGFRs (Fig. 2D). Swapping of these residues did not significantly alter the binding pattern except for that of FGFR4 which decreased rather than increased binding.

To narrow down the binding region of Hsp90 and Cdc37 to FGFR3 and to confirm its specific association with the kinase domain, we carried out domain swapping experiments between FGFR3 and the weakest Hsp90 binder FGFR2. Swapping of the entire kinase domain of FGFR2 into FGFR3 prevented strong
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FIGURE 2. FGFRs differentially associate with Hsp90 complexes. A, sequence alignment of part of the EGFR, ErbB2, and FGFR family kinase domains. Gray shading indicates identical/related residues. Boxed residues and the asterisk mark the conserved glycine (FGFR3 Gly-533 in humans). Double asterisks indicate a negatively charged residue (Gly-548) absent in FGFR3 relative to the other FGFRs. Secondary structures are identified below by black boxes based on the analysis by Chen et al. (18). B, transfection of empty vector (V) or FGFR1–4 V5 in 293 cells followed by V5 IP and blotting. An arrow denotes the Cdc37 band running just below the IgG heavy chain. C, transfection in 293 cells of empty vector (V), WT-FGFR3 (WT), G533D FGFR3 (533), or G548D FGFR3 (548) followed by IP and blotting. — Avg indicates the averaged ratio of the amount of Hsp90 pulldown by mutated receptor relative to the wild-type receptor. D, 293 transfection of empty vector (V) or FGFR1–4 with a swapping mutation of the respective residue found in FGFR3 (548) as indicated by an asterisk (R1/2, D to G; R4, E to G; R3, G to D). E, 293 transfection followed by IP and blotting of FGFR3 (R3), FGFR2 (R2), or chimeras FGFR3/kinase-FGFR2 (N), FGFR3/N-terminal lobe-hinge-FGFR2 (N), and FGFR3/C-terminal lobe-kinase insert-FGFR2 (C).

association of Hsp90 and Cdc37 with chimeric FGFR3 (Fig. 2E). Interestingly, the chimeric kinase receptor displayed an increase in its apparent steady state stability and altered the ratio of biosynthetic species to mimic that of FGFR2. Further swapping of the individual lobes of the kinase domain revealed that Hsp90 and Cdc37 did not bind to the FGFR3 chimera with the N-terminal lobe/hinge of FGFR2 but did bind to FGFR3 containing the FGFR2 C-terminal lobe/kinase insert. These results indicate that Hsp90 and Cdc37 strongly bind to the N-terminal lobe/hinge of FGFR3. Taken together, our results suggest that Hsp90 binding to FGFRs is distinct from that of the EGFR family as has been observed for other Hsp90 kinase clients (36, 37).

FGFR3 Requires Hsp90 Function for Stability—Altering the chaperone-client interaction through inhibition of Hsp90 function rapidly destabilizes strong Hsp90 client proteins (8). To determine the dependence of FGFR3 stability on Hsp90 function, we examined endogenous receptor stability in the presence of 17-AAG. FGFR3 stability was compared with that of the strong and weak clients ErbB2 and EGFR, respectively, which are also endogenously expressed in the RT112 cells. FGFR3 was destabilized rapidly by 17-AAG, even more so than ErbB2 (Fig. 3A). A comparison of untagged, stably induced receptors in 293 cells showed that FGFR3 is less sensitive to 17-AAG than ErbB2, but more sensitive than EGFR (supplemental Fig. S2B). These results suggest that cellular context may influence the dependence of FGFR3 stability on Hsp90, as has been reported for other Hsp90 clients (9). Confocal imaging of FGFR3-GFP revealed decreased fluorescence after addition of 17-AAG (Fig. 3B) further supporting the need of Hsp90 function for FGFR3 stability.

To further document the loss of FGFR3 stability associated with Hsp90 inhibition and to exclude that it is mediated by Hsp90 effects on transcription and translation, we performed 35S-labeled pulse-chase half-life analysis. A substantial decrease in FGFR3 half-life was observed consistently in the presence of Hsp90 inhibitors when the receptor was expressed either transiently (Fig. 3C) or stably (supplemental Fig. S2D). This reduction was dose-dependent (supplemental Fig. S2D). These results were validated for endogenous FGFR3 in RT112 cells. Because immunoprecipitation of FGFR3 in the RT112 cells pulled down a nonspecific radioactively labeled band, which confounded interpretation, cycloheximide was used to determine the half-life in this cell line. The half-life of FGFR3 was decreased by 50%, from 4 to 2 h in the presence of 17-AAG (Fig. 3D). Originally, we hypothesized that CA-FGFR3 would be destabilized by 17-AAG more than WT-FGFR3. Although we found a variable response depending on the cell line tested, CA-FGFR3 was destabilized less than WT-FGFR3 in three paired transfection experiments (WT-FGFR3 destabilized by an average of 51 ± 3% of vehicle and CA-FGFR3 31 ± 7%). In contrast, in our stable COS7 cell lines, where adaptation to CA-FGFR3 results in a similar half-life for the two receptors, the half-life decreased to a similar degree (supplemental Fig. S2D). These findings support the idea that, like ErbB2, FGFR3 is a strong Hsp90 client and requires Hsp90 function for stability even when constitutively activated.

Pulse-chase analysis was used to assay the half-lives of the other FGFRs in the presence of 17-AAG (Fig. 3E). As for FGFR3, the half-life of FGFR4 decreased in response to Hsp90 inhibition. FGFR1 displayed a small increase, whereas FGFR2...
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A. FGFR3 Is a Strong Hsp90 Client

B. CHIP Can Interact and Influence Stability of FGFR3

C. FGFR3 Requires Hsp90 Function for Stability

D. FGFR3 Is a Strong Hsp90 Client

E. Hsp90 Inhibition Results in FGFR3 Ubiquitination

F. CHIP Can Interact and Influence Stability of FGFR3—CHIP

FIGURE 3. FGFR3 requires Hsp90 function for stability. A, time course of 1 μM 17-AAG or vehicle (DMSO, D) in whole cell lysates from RT112 cells. B, immunofluorescence of stably expressed WT-FGFR3-GFP in COS7 cells in the presence of DMSO or 1 μM 17-AAG for 3 h. C, 35S-labeled half-life of transfected WT-FGFR3 and CA-FGFR3 in 293 cells in the presence or absence of 1 μM 17-AAG. The graph represents background subtracted densitometry relative to the percentage remaining at time 0. D, cycloheximide half-life analysis of immunoprecipitated and blotted FGFR3 in the presence or absence of 0.5 μM 17-AAG in RT112. The graph represents background subtracted band densitometry expressed as the percent remaining relative to 0.5 h. E, representative 35S-labeled pulse-chase half-life analysis scan of transfected FGFR1–4 V5 in 293 cells and chased in the presence or absence of 1 μM 17-AAG. The graph represents the average percent change in half-life in response to 1 μM 17-AAG relative to DMSO control. Error bars are mean ± S.D. (n = 3).

FIGURE 4. Hsp90 inhibition results in FGFR3 ubiquitination. A, time course of total ubiquitin blotting of immunoprecipitated FGFR3 from RT112 in the presence of 0.5 μM 17-AAG or DMSO (D) for the indicated times. B, transiently expressed V5 tagged FGFR1–4 (R1–R4) in 293 cells for 48 h. Cells were treated for 1 h with 1 μM 17-AAG (17) or vehicle (D) followed by IP and blotting for polyubiquitin (Poly Ub). C, cotransfection (24 h) of WT- or CA-FGFR3-V5 with empty vector (V), CHIP-FLAG (C), or H260Q-FLAG (Q) in 293 cells, followed by V5 IP, and probed as indicated. D, equal protein loaded from whole cell lysates of cotransfected (48 h) WT-FGFR3-V5 with CHIP-FLAG (C), H260Q-FLAG (Q), or empty vector (E) 24 h, cells were cotransfected with WT-FGFR3-V5 and empty vector (V), CHIP-FLAG (C), or H260Q-FLAG (Q). After 24 h, cells were treated for 1 h with 1 μM 17-AAG or DMSO followed by IP and polyubiquitin blotting (Poly Ub). E, transfection and IP of WT-FGFR3-V5 in the presence of 5 μM lactacystin (L) or vehicle (D, DMSO) for 5 h, with the last hour in the presence of 1 μM 17-AAG (17) or vehicle (D, DMSO).

shown a modest increase in half-life. Although rare, an increase in half-life in response to Hsp90 inhibition has been reported (38, 39). The trend we detected for half-life is consistent with what we observed for Hsp90 binding (Fig. 2B). Despite differences in experimental conditions, i.e. cell type and means of receptor expression, FGFR3 was consistently more sensitive to 17-AAG-mediated destabilization than the other FGFRs with FGFR2 as the most resistant.

Inhibition of Hsp90 Results in Ubiquitin-mediated Degradation of FGFR3—Inhibition of Hsp90 function alters co-chaperone complex composition and promotes the incorporation of E3-ubiquitin ligases, leading to client ubiquitination and degradation (13). To determine whether FGFR3 undergoes ubiquitination after Hsp90 inhibition, we treated RT112 cells with 17-AAG and immunoprecipitated FGFR3 under denaturing conditions. FGFR3 displayed a time-dependent increase in total ubiquitination after 17-AAG treatment but not with vehicle control (Fig. 4A). A coincidental decrease in total FGFR3 levels was observed at later time points. These results are consistent with the observed decrease in half-life and are characteristic of strong Hsp90 clients.

We next determined whether 17-AAG induces ubiquitination of other FGFR family members. For direct comparison, tagged FGFRs were transfected and immunoprecipitated under denaturing conditions following treatment with 17-AAG for 1 h. FGFR3 and FGFR4 displayed increases in polyubiquitination, with CHIP showing a small increase and CHIP-FLAG showing no response (Fig. 4B). Although not comparable among receptors, similar trends were observed in response to 17-AAG treatment for untagged receptors (supplemental Fig. S2C). These responses to Hsp90 inhibitors echo the observed relative associations of Hsp90 with the receptors and the changes in half-life in response to Hsp90 inhibition.

CHIP Can Interact and Influence Stability of FGFR3—CHIP is an E3-ubiquitin ligase that binds to the C-terminal tail of Hsp70 and Hsp90 and catalyzes the ubiquitination of client proteins (13). Upon treatment with an Hsp90 inhibitor, Hsp90 complexes incorporate CHIP leading to client ubiquitination and degradation. We were unable to convincingly detect endogenous CHIP in FGFR3-Hsp90 coimmunoprecipitations, which we attributed to the technical limitations of the available antisera (data not shown). Thus, we performed coimmunoprecipitation studies with transiently expressed FGFR3 and FLAG-
tagged CHIP. As a control we used dominant-negative H260Q-CHIP-FLAG (H260Q), which has a mutated E2 interaction site, thereby preventing ubiquitin conjugation but not client binding (40). After immunoprecipitation of FGFR3, CHIP-FLAG was barely detectable above background, but the catalytically inactive H260Q-FLAG immunoprecipitated with both WT- and CA-FGFR3 (Fig. 4C). Increased detection of H260Q in co-immunoprecipitations has been reported for other Hsp90 clients such as ErbB2 and has been attributed to “enzymatic trapping” of H260Q-CHIP (41).

We next examined the extent to which CHIP can destabilize FGFR3 by cotransfected CHIP or H260Q with WT-FGFR3 for 48 h. WT-FGFR3 was stabilized when co-transfected with CHIP but not H260Q (Fig. 4D). Our working model predicts that this destabilization results from CHIP-mediated ubiquitination of FGFR3 and that this would be enhanced by Hsp90 inhibition. To test this prediction, CHIP or H260Q was cotransfected with WT-FGFR3 in the presence or absence of 17-AAG for 24 h (Fig. 4E). We did not observe an increase in ubiquitination upon cotransfection with CHIP, presumably due to a rapid destabilization of this ubiquitinated population over the 24 h of cotransfection. Most significantly however, H260Q did not destabilize FGFR3 and protected it from 17-AAG induced ubiquitination (Fig. 4E). Although other ubiquitin ligases can compensate for ubiquitination of Hsp90 clients in the absence of CHIP (42, 43), our results suggest that for many Hsp90 clients, CHIP can affect FGFR3 stability.

CHIP-targeted proteins are commonly reported to be degraded by proteasomes (13). Adding the proteosomal inhibitor lactacycin to FGFR3 enhanced receptor polyubiquitination, and this effect was further enhanced by the addition of 17-AAG (Fig. 4F). These findings are consistent with a role for the proteasome in the degradation of FGFR3 in response to 17-AAG.

**Hsp90 Inhibition Reduces FGFR3 Signaling Output**—As receptors typically initiate signaling from the cell surface, we next asked whether inhibition of Hsp90 function destabilizes FGFR3 residing at this location. Surface receptors of RT112 cells were biotinylated, incubated with or without 17-AAG, and analyzed following affinity purification of biotin-labeled proteins. Inhibition of Hsp90 accelerated degradation of biotinylated FGFR3 (Fig. 5A) as was also observed for the strong client ErbB2 but not for the weak client EGFR. These data suggest that Hsp90 inhibitors effectively reduce the cell surface population of FGFR3.

Some mutations directly activate FGFR3 kinase activity, eliminating the need for the receptor to dimerize or reach the cell surface for signaling. To determine whether Hsp90 inhibitors can destabilize intracellularly retained receptors, we carried out 35S-labeled pulse-chase analysis of FGFR3 in the presence of BFA, which prevents anterograde trafficking from the endoplasmic reticulum thereby trapping newly synthesized and labeled FGFR3. A chase in the presence of 17-AAG and BFA showed reduced stability of this population of FGFR3 over time (Fig. 5B). We also observed a progressive increase in the molecular weight of FGFR3, presumably due to continued exposure of the retained receptor to glycosylating enzymes residing in the endoplasmic reticulum as a consequence of the BFA blockade.

One would expect the decreased FGFR3 levels resulting from Hsp90 inhibition to be accompanied by reduced FGFR3 signal output. To confirm this expectation, we assayed FRS2 and ERK1/2 for phosphorylation as proximal and distal readout markers of MAPK signaling, respectively, in RT112 cells. Both FRS2 and ERK1/2 were constitutively phosphorylated in the absence of FGF stimulation despite serum starvation for 20 h (Fig. 5C). Treatment with 17-AAG for 1.5 h abolished phosphorylation of both markers and also significantly reduced phosphorylation after 10 min of ligand stimulation (Fig. 5C).

![Figure 5. Loss of Hsp90 function reduces FGFR3 bioactivity.](image-url)
As the viability of RT112 cells depends on FGFR3 function, we reasoned that destabilization of FGFR3 by Hsp90 inhibitors would reduce their viability (44). MTT assays, which measure respiring cells and over time reflect a combination of proliferation and survival, were performed in the presence of increasing concentrations of inhibitors (Fig. 5D). A dose-dependent reduction in cell viability was observed for 17-AAG. These cells showed a similar response to radicicol and to the FGFR kinase inhibitor PD173074.

We next asked whether Hsp90 inhibition alters the responses of other cell types to FGF stimulation. HeLa cells undergo growth arrest when serum-starved; they display a proliferative response to FGF stimulation. HeLa cells showed a similar response to radicicol and to the FGFR kinase inhibitor PD173074 (supplemental Fig. S2E). This protocol had minimal effects on the mitogenic response to serum stimulation.

Finally, we examined rat chondrosarcoma cells, which, similar to chondrocytes, reduce proliferation in response to FGF stimulation. They display a reduction in proliferation as measured by reduced viability and proliferation in response to FGF9 for 48 h as reported previously (45). Low concentrations of 17-AAG blocked this reduction in a dose-dependent manner (Fig. 5E). As expected, inhibition of FGFR3 kinase activity with PD173074 also increased proliferation. Similar results were seen with FGFR2 (supplemental Fig. S2F). We conclude that Hsp90 inhibitors can block FGFR3 signal output and can modulate FGFR3 signaling pathways and even reverse its growth inhibitory signals.

DISCUSSION

The data presented here establish FGFR3 as a strong Hsp90 client. The degree to which FGFR3 is stabilized by Hsp90 is underscored by the rapid degradation of both intracellular and cell surface receptors and a subsequent loss in signal output following Hsp90 inhibition. The dependence of FGFR3 on Hsp90 contrasts with that of the other FGFR family members that interact and respond to 17-AAG differently. Overall, our findings provide a new context for understanding FGFR3 biology and for treating FGFR3-related disease.

We found that Hsp90 association was stronger for FGFR3 compared with other FGFRs, although their stability was influenced by Hsp90 inhibition. We consistently observed a hierarchy of destabilization by 17-AAG treatment, with FGFR3 > FGFR4 > FGFR1 > FGFR2. Interestingly, FGFR2 exhibited little binding to Hsp90, but its half-life was increased in the presence of 17-AAG, a phenomenon that has been reported for other proteins and illustrates the variable nature of Hsp90-client survival (38, 39).

We detected similar binding of Hsp90 to WT-, CA-, and KD-FGFR3 as has been observed for strong Hsp90 clients such as ErbB2 that interact with Hsp90 independent of activation (30). In contrast to ErbB2, however, we observed that addition of a negatively charged residue into the aC-β4 loop of FGFR3 did not disrupt binding to Hsp90 or Cdc37. This finding illustrates the complex association of Hsp90 chaperone complexes with kinases (8). Along these lines, it has been suggested that Hsp90 binding may be linked to kinase dimer formation (46, 47). Indeed, given the collective observations that Hsp90-Cdc37 chaperone complexes interact with key regions of the N- and C-terminal lobes of kinases (8, 35, 48), and recent insights into the asymmetric nature of FGFR kinase domain transphosphorylation (49), it is conceivable that Hsp90 may play a role in asymmetric FGFR3 kinase dimer formation or its regulation.

We observed physiologic responses to Hsp90 inhibition in two cell types that we attribute largely to antagonism of FGFR3 signaling due to receptor destabilization. In the first case, bladder cancer RT112 cells, which are dependent on FGFR3 output for proliferation and survival, exhibited a dose-dependent decline in viability in response to both 17-AAG and radicicol. The response was remarkably similar to that following treatment with the FGFR tyrosine kinase inhibitor PD173074. Rat chondrosarcoma cells display a cellular phenotype similar to proliferating growth plate chondrocytes, including expression of endogenous FGFR3 and an antiproliferative response to FGF stimulation (45). We found that pretreatment with 17-AAG blunted the FGF-mediated inhibition of proliferation, increasing cellular growth in a dose-dependent fashion. This result is consistent with a direct effect on FGFR3 stability, although we cannot exclude that it was mediated by Hsp90-stabilized pathways unrelated to FGFR3.

We have reported previously that activated FGFR3 is degraded in lysosomes and is directed into this pathway by c-Cbl-mediated ubiquitination (19). In fact, we observed that FGFR3 bearing gain-of-function mutations progress through this pathway less efficiently than WT-FGFR3. Our current results suggest FGFR3 may also be degraded through a proteasomal pathway that utilizes the CHIP ubiquitin ligase and is buffered by Hsp90 stabilization. Little is known about the coordination of these two pathways, but it is conceivable that mutant receptors that escape c-Cbl-mediated degradation are stabilized by Hsp90 and Cdc37, making them susceptible to chaperone inhibitor-mediated degradation. FGFR3 would not be unique as a substrate for both pathways as similar properties have been reported for the Ron tyrosine kinase (29). Furthermore, CHIP has the capacity to target proteins for lysosomal degradation in addition to its role in proteasomal targeting (50).

Although we have established in detail that FGFR3 is a client of Hsp90, others have proposed this relationship for FGFRs and FGFR3. For example, a proteomics screen for proteins that interact with the dimerized transmembrane and intracellular domains of FGFR1 detected Hsp90 and Cdc37 (51). A report that Hsp90 inhibitors destabilize oncogenes implicated in synovial sarcoma included FGFR3 as a kinase that responds to Hsp90 inhibition (52). Most recently, subtypes of FGFR3-driven multiple myeloma were shown to be sensitive to a new Hsp90 inhibitor (53). This study reported that both WT- and CA-FGFR3 interact with Hsp90 and are sensitive to Hsp90 inhibitors. Thus, our findings are consistent with and expand on previously described observations.

Our findings implicate Hsp90 in the pathogenesis of diseases resulting from activating FGFR3 mutations. It follows that Hsp90 inhibition could be an effective therapeutic strategy in these diseases, notably certain cancers and achondroplasia. The use of Hsp90 inhibitors for cancer is not new, as they are being investigated as a means to down regulate multiple oncogenic
kinases (14). Cancers in which oncogenic mutations of FGFR3 are detected, such as superficial bladder cancer and multiple myeloma, would be potential candidates for this strategy. In contrast to cancer, the potential application of this therapeutic strategy to achondroplasia is novel, although it has been used to target mutant proteins for degradation in another genetic disease (15). Spinal and bulbar muscular atrophy is a slowly progressive muscle disease that results from a polyglutamine expansion in the androgen receptor, a known Hsp90 client protein (16). Hsp90 inhibitor treatment of mice modeling spinal and bulbar muscular atrophy reduced the cellular accumulation of the mutant receptor andameliorated most of the clinical disease establishing the paradigm for this therapeutic approach. We are currently pursuing similar studies in mouse models of achondroplasia.

Acknowledgments—We thank Gen Soubre for the CHIP-FLAG construct, Colleen Sweeney for the EGFR and ErbB2 constructs, and Moosa Mohammadi for FGFI.

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