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Interaction of Hsp90 with the Nascent Form of the Mutant Epidermal Growth Factor Receptor EGFRvIII*

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EGFRvIII is a mutant epidermal growth factor that promotes aggressive growth of glioblastomas. We made a plasmid that directed the expression of an EGFRvIII with three copies of the Flag epitope at its amino terminus. Flag-tagged EGFRvIII was expressed at the same levels as unmodified EGFRvIII, and showed the same subcellular localization. However, the Flag epitope could only be detected on EGFRvIII present in the endoplasmic reticulum; the epitope was covalently modified during trafficking of the receptor through the Golgi so that it was no longer recognized by anti-Flag antibody. This property was exploited to selectively purify nascent EGFRvIII from glioblastoma cells. Nascent EGFRvIII was found to copurify with a set of other proteins, identified by mass spectrometry as the two endoplasmic reticulum chaperones Grp94 and BiP, and the two cytosolic chaperones Hsc70 and Hsp90. The Hsp90-associated chaperone Cdc37 also co-purified with EGFRvIII, suggesting that Hsp90 binds EGFRvIII as a complex with this protein. Geldanamycin and radicicol, two chemically unrelated inhibitors of Hsp90, decreased the expression of EGFRvIII in glioblastoma cells. These studies show that nascent EGFRvIII in the endoplasmic reticulum associates with Hsp90 and Cdc37, and that the Hsp90 association is necessary to maintain expression of EGFRvIII.

Glioblastoma multiforme is an incurable disease with a median survival time that is typically less than 1 year. Surgery and radiation have been shown to increase survival times, but the disease responds poorly to chemotherapy. One of the most common genetic abnormalities found in glioblastoma is amplification of the gene for EGFR.[1] Along with amplification, the EGFR gene is frequently mutated. The most common mutation is a deletion of exons 2–7. This deletion is in-frame and results in the expression of a truncated EGFR, known as EGFRvIII, in which amino acids 6–273 of the extracellular domain are replaced by a single glycine residue (1–4). Multiple studies have demonstrated that EGFRvIII is expressed in approximately half of all glioblastomas (5–8), and there is evidence that its expression is associated with a poorer prognosis (9). Consistent with this, EGFRvIII has been shown to greatly enhance the tumorigenicity of human glioblastoma cells grown as xenografts in nude mice (10).

Studies in mouse models of glioblastoma show that EGFRvIII cooperates with mutations at the INK4a/ARF locus to promote the formation of glioblastoma (11). Thus, whereas EGFRvIII expression in normal mice does not give rise to tumors, expression of EGFRvIII in INK4a/ARF(−/−) mice does give rise to tumors with a high frequency. The INK4a/ARF locus contains genes coding for two different proteins (12). One of these is Ink4a, a repressor of cyclin-dependent kinase 4 (cdk4); the other is Arf, which has a role in regulating p53 levels. EGFRvIII will also form tumors in mice if cdk4 is overexpressed at the same time, indicating that these two pathways cooperate in the formation of glioblastoma. It is likely that these two pathways also cooperate in the formation of human glioblastoma, as there is a high concurrence of EGFR overexpression and mutations at the INK4a/ARF locus in these tumors (13).

The mutation in EGFRvIII has been shown to generate a receptor with many properties that are distinct from those of normal EGFR (reviewed in Refs. 14–16). EGFRvIII is unable to bind epidermal growth factor and transforming growth factor α (two activating ligands for EGFR) (10, 17, 18), and has a constitutive, ligand-independent tyrosine kinase activity (19, 20). In addition, EGFRvIII is internalized slowly compared with normal EGFR after ligand activation (19). EGFRvIII appears to preferentially use a different subset of downstream signaling pathways compared with normal EGFR (18, 21), and shows differential sensitivity to EGFR tyrosine kinase inhibitors (22).

To better characterize the properties of EGFRvIII, we have generated an epitope-tagged version of the receptor that can be purified rapidly and efficiently. Here we describe the characterization of the epitope-tagged receptor, its purification, and the identification of proteins that co-purify with the receptor.

MATERIALS AND METHODS

Plasmid Constructs—Plasmid containing the cDNA for the Moloney murine leukemia virus ecotropic receptor was from Dr. L. Albritton, University of Tennessee, Memphis, TN. The insert was removed by digestion with *Bam*HI and *Sal*I and subcloned into the retroviral vector pWZL-Hygro (obtained from Dr. S. Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). To construct the vector expressing triple

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; EGFRvIII, mutant epidermal growth factor receptor lacking amino acids 6–273 of the extracellular domain; PBS, phosphate-buffered saline; MS, mass spectroscopy; cdk4, cyclin-dependent kinase 4.

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Flag-tagged EGFRvIII, the cDNA for EGFRvIII was excised from pLERNL (10) (obtained from Dr. W. Cavenee, Ludwig Institute for Cancer Research, La Jolla, CA) by *Sal*I digestion and subcloned into the *Sal*I site of a modified version of the vector T7-blue (Novagen, Inc., Madison, WI) in which the unique *Sph*I site had been removed. Site-directed mutagenesis was then used to create a silent *Sph*I restriction site at codons 43–44 of the mutant receptor, using the oligonucleotide SPHV3 (CGCATGCTCGGACGCACGAGCCGTGATC). This plasmid was then used as a template for two PCRs, using two primer pairs: KPNV3 (CGGTACCACGCTGCAGACGCG) and TF1 (GATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCCAGAGCCCGACTCGCCGGGCA); and TF2 (GATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGCTGGAGGAAAAGAAAGGT) and SPHV3 (see above). The two PCR products were gel-purified and joined in a third PCR using the KPNV3 and SPHV3 primer pair and the two initial PCR products as templates. The final PCR product was subcloned and sequenced. The *Kpn*I/*Sph*I fragment was excised and ligated into T7-blue containing EGFRvIII cDNA with the silent *Sph*I site, which had also been digested with *Kpn*I and *Sph*I. The full-length cDNA for EGFRvIII containing the triple Flag tag sequence was then excised with *Sal*I and ligated into *Sal*I digested pLERNL. Clones showing the correct orientation by restriction digest were designated pLRNLtftΔ.

Antibodies—Anti-Flag M2 antibody and anti-Flag M2 affinity gel were from Sigma. Antiphosphotyrosine antibody was from Transduction Labs (Lexington, KY). Antibody to the carboxyl terminus of EGFR was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Golgin 97 antibody was from Molecular Probes, Inc. (Eugene, OR). Anti-Sec61α antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-cdk4 (Ab-6) and anti-Cdc37 antibodies were from Neomarkers (Fremont, CA). Anti-Hsp90 and anti-p60/Hop antibodies were from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). To make rabbit polyclonal antibodies specific for EGFRvIII, a multiple antigenic peptide containing four copies of the sequence LEEKKGNYVVTDHG (the amino terminus of EGFRvIII plus a glycine spacer) was synthesized and purified on a Superdex peptide column (Amersham Biosciences). This was then used to immunize rabbits. Polyclonal antibodies were purified from rabbit serum using the peptide LEEKKGNYVVTDH-biotin (23) bound to Ultralink immobilized streptavidin gel (BioLynx, Inc., Brockville, ON).

Cell Lines—U87MG and U87MGΔEGFR cell lines were obtained from Dr. W. Cavenee, Ludwig Institute for Cancer Research, La Jolla, CA. 293T cells were obtained from Dr. J. Bell, Ottawa Regional Cancer Centre, Ottawa, Canada. All cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum.

Transfections and Transductions—Replication-incompetent retroviruses were made using the three plasmid transient transfection systems described by Soneoka *et al.* (24). Transduction of U87MG glioblastoma cells was done by incubating the cells with virus in the presence of 8 μg/ml Polybrene for 2 h. U87MGecoR cells, which express the ecotropic receptor for Moloney leukemia virus, were made by transduction with amphotropic retrovirus followed by selection in 75 μg/ml hygromycin B. U87MGtftΔ cells, which express triple Flag-tagged EGFRvIII, were made by transduction of U87MGecoR cells with ecotropic retrovirus followed by selection in 400 μg/ml G418.

Flow Cytometry—Cells growing in tissue culture flasks were detached using Cell Dissociation buffer (Invitrogen), pelleted by centrifugation, and resuspended in PBS containing 1% bovine serum albumin at a concentration of 4×10^6 cells per ml. 200-μl aliquots of cells were then incubated on ice with the indicated antibodies for 20 min. Cells were pelleted again, resuspended in 100 μl of PBS containing 1% bovine serum albumin, and incubated with secondary antibody (either fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody, or fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody, both from Jackson Laboratories, Bar Harbor, ME) for 20 min on ice. Cells were pelleted again and resuspended in 500 μl of PBS containing 1% bovine serum albumin. Flow cytometry was performed using a BD LSR flow cytometer (BD Biosciences, San Jose, CA).

Immunofluorescence—Cells were grown on gelatin-subbed coverslips in 35-mm dishes. Cells were then washed with cold PBS and fixed with 4% paraformaldehyde in PBS for 1 h. Where indicated, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were blocked with 5% normal goat serum for 30 min and then incubated with primary antibody for 1 h at room temperature. Cells were washed three times with PBS and incubated with secondary antibody (either Cy3-labeled goat anti-rabbit antibody used at 3.75 μg/ml or Cy3-labeled goat anti-mouse antibody used at 2.8 μg/ml, both from Jackson Laboratories) for 1 h at room temperature. Cells were washed again, counterstained with Hoechst 33258, and mounted on slides. Immunofluorescence was performed using a Zeiss Axioskop2 fluorescence microscope and images were captured with a CCD camera. Images were deconvolved using Axiovision 3.0 software (Carl Zeiss, Inc., Thornwood, NY). For double labeling, immunofluorescence was performed as above using anti-Sec61 rabbit polyclonal antibody as an endoplasmic reticulum marker, or anti-Golgin 97 mouse monoclonal antibody as a Golgi marker. After incubation with first and second antibodies, cells were washed and incubated for 1 h at room temperature with anti-Flag M2 antibody that had been labeled with Zenon One Alexa Fluor 488 IgG_1 labeling reagent (Molecular Probes, Inc.). Cells were then washed, fixed again with 4% *para*-formaldehyde for 15 min at room temperature, washed, and mounted.

Immunopurification of EGFRvIII—U87MGtftΔ cells or control U87MGecoR cells were plated at 2.5×10^6 in 15-cm tissue culture dishes (five dishes per cell line). After 3 days, media in the dishes was changed to media containing 0.5% fetal calf serum. The following day, cells were washed once with ice-cold PBS and scraped into the same buffer (50 ml total for each cell line). Cells were then pelleted and resuspended in 1 ml of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 20 mM NaF, 1 mM Na_3VO_4, 1 mM *para*-nitrophenyl phosphate, 1 μg/ml each of leupeptin, pepstatin, and aprotinin, 5 mM benzamidine, 1 mM β-mercaptoethanol, pH 7.5). Cells were homogenized by passage once through a 27-gauge needle, and insoluble debris was removed by centrifugation at 12,000 rpm ($15,000 \times g$) in an Eppendorf 5417R centrifuge for 10 min at 4 °C. The supernatant was transferred to a fresh tube and assayed for total protein content using Coomassie Plus Protein Assay Reagent (Pierce). Equal amounts of total protein from the two cell lines were then made up to the same volume with lysis buffer (~1 ml). 50 μl of the original stock suspension of anti-Flag M2 affinity gel was washed three times with lysis buffer and added to each tube. The tubes were then mixed with gentle rocking at 4 °C for 2 h. The resin was then pelleted and washed four times with 1 ml of lysis buffer. Bound proteins were eluted from the affinity gel by the addition of 50 μl of 2× Laemmli buffer without reducing agent (4% (w/v) sodium dodecyl sulfate, 0.1% (v/v) glycerol, 125 mM Tris-HCl, pH 6.8) followed by heating at 90 °C for 5 min. The affinity gel was then removed by centrifugation and the supernatant was transferred to a fresh tube.

Mass Spectrometry—Dithiothreitol was added to the affinity gel eluates to a final concentration of 10 mM, and the eluates were then boiled for 5 min. Acrylamide was then added to a final concentration of 1% (w/v) and eluates were incubated for 1 h at room temperature. Samples (40 μl per lane) were then loaded onto 10% SDS-polyacrylamide gels and electrophoresed. Gels were stained with GelCode Blue Stain reagent (Pierce), destained with H_2O, and photographed. Stained bands were cut of gels and stored in aqueous 1% acetic acid. For mass spectrometry analysis, bands were then washed extensively with deionized water, shrunk with acetonitrile, re-swollen with 50 mM ammonium bicarbonate containing sequencing grade, modified trypsin (10 ng/μl) from Promega (Madison, WI). Sufficient 50 mM ammonium bicarbonate was added to cover the gel pieces (typically 30 μl). The tubes were sealed and incubated overnight at 37 °C. The digest solutions were removed and the gel pieces were extracted with 50 μl of 5% acetic acid and then with 50 μl of 5% acetic acid in 50% aqueous methanol. The extracts were pooled with the digest solutions and concentrated to ~10 μl.

The extracts were analyzed by rapid LC-MS/MS using a CapLC high performance liquid chromatography pump (Waters Associates, Millford, MA) coupled to a Q-TOF2 hybrid quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK). The entire samples were injected onto a 0.3×5-mm C_{18} micro precolumn cartridge (Dionex/LC-Packings, San Francisco, CA). The peptides were retained while the sample solution was washed to waste. The trap was then brought online with the mass spectrometer and the peptides were eluted with a rapid gradient supplied by the CapLC pump (5–80% acetonitrile, 0.2% formic acid in 6 min, 1 μl/min flow rate). The mass spectrometer was set to operate in automatic MS/MS acquisition mode and tandem mass spectra were acquired on doubly and triply protonated precursor ions only. These spectra were then searched against the Swiss-Prot/TrEMBL and NCBI nr protein sequence data bases using Mascot Daemon™ (Matrix Science Ltd., London, UK), an algorithm that uses mass spectrometry data to identify proteins in primary sequence data bases.

Immunoprecipitations—Small scale immunoprecipitations were carried out using the same basic procedure as described above for immunopurification of EGFRvIII, with the following changes: cells growing in two 10-cm tissue culture dishes were washed once with cold PBS and then scraped directly into 0.5 ml of lysis buffer; cleared extracts were incubated for 45 min at 4 °C with 5 μg of His-tagged MR1dsFv antibody

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 C   P   A   S   R   A   L   D   Y   K
TGC CCG GCG AGT CGG GCT CTG GAC TAC AAA

 D   H   D   G   D   Y   K   D   H   D
GAC CAT GAC GGT GAT TAT AAA GAT CAT GAC

 I   D   Y   K   D   D   D   D   K   L
ATC GAT TAC AAG GAT GAC GAT GAC AAG CTG

 E   E   K   K   G
GAG GAA AAG AAA GGT
```

FIG. 1. **Nucleotide and amino acid sequence of the amino terminus of triple Flag-tagged EGFRvIII.** The last six codons of the EGFRvIII leader sequence are *underlined* with a *solid line*. The inserted triple Flag tag sequence is shown in *bold*. The normal amino terminus sequence of mature EGFRvIII is *underlined* with a *dashed line*.

fragment (25); 100 μl of Ni-NTA Superflow (Qiagen, Inc., Mississauga, Ontario, Canada) was then added and tubes were incubated with rocking at 4 °C for 1 h; pellets were washed 5–7 times with 750 μl of lysis buffer before adding 50 μl of 2× Laemmli buffer.

Western Blotting—Western blotting was performed as described previously (26). After electrophoretic transfer from the gel, blots were stained with Amido Black to check that equal sample loading and transfer was achieved.

RESULTS

Construction and Expression of Flag-tagged EGFRvIII—To allow efficient purification of EGFRvIII, we constructed a version in which a triple Flag tag sequence was inserted between the leader sequence and the start of the mature protein (Fig. 1). The triple Flag tag amino acid sequence has previously been shown to bind with high affinity to the anti-Flag M2 monoclonal antibody (27). A codon for a single leucine residue was included at the amino terminus of the triple Flag tag sequence to preserve the same signal peptide cleavage site as in normal EGFRvIII. The sequence for tagged EGFRvIII (hereafter designated tftEGFRvIII) was subcloned into a retroviral vector. Initially expression was tested by transient transfection into 293T cells (Fig. 2*A*). Cell lysates were prepared and analyzed by Western blotting, using polyclonal antibody to the carboxyl terminus of EGFR. Unmodified EGFRvIII migrated as a doublet, in agreement with previous results (21). TftEGFRvIII was expressed at the same levels as unmodified EGFRvIII, and also migrated as a doublet, with both bands migrating at a slightly higher apparent molecular weight, as expected given the additional 23 amino acids added at the amino terminus. From this result it can also be inferred that tftEGFRvIII is glycosylated to the same extent as unmodified EGFRvIII. TftEGFRvIII was also found to be autophosphorylated to the same extent as EGFRvIII, showing that the addition of the epitope tag did not affect its constitutive tyrosine kinase activity. As expected tftEGFRvIII, but not EGFRvIII, was detected by the anti-Flag antibody M2. We also performed a test immunoprecipitation of tftEGFRvIII expressed by transient transfection in 293T cells. TftEGFRvIII could be reduced to undetectable levels in cell lysates by incubation with M2 antibody affinity gel, showing that the binding was very efficient (not shown).

We next made ecotropic replication-incompetent retrovirus containing tftEGFRvIII, and used these to infect U87MG human glioblastoma cells that had been modified to express the ecotropic receptor for Moloney murine leukemia virus. Infected cells were selected for resistance to G418 and drug-resistant populations of cells (designated U87MGtftΔ cells) were used for further experiments. Western blot analysis showed that these cells also expressed tftEGFRvIII and autophosphorylated tftEGFRvIII at levels similar to those seen in U87MG cells infected with a retrovirus containing the cDNA for unmodified EGFRvIII (Fig. 2*B*). Flow cytometry, using an antibody that recognizes the amino terminus of EGFRvIII, showed that these two cell populations also expressed similar amounts of cell-surface EGFRvIII (Fig. 3, *panels c* and *d*). However, we were

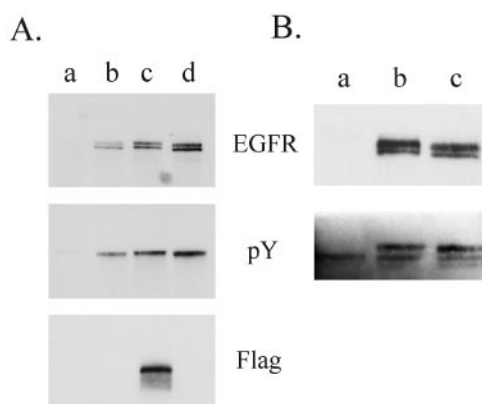

FIG. 2. **Expression of tftEGFRvIII in 293T cells and glioblastoma cells.** *A*, 293T cells were transiently transfected with: *lane a*, no DNA; *lanes b* and *d*, pLERNL, which expresses EGFRvIII; *lane c*, pLRNLtftΔ, which expresses triple Flag-tagged EGFRvIII. Total cell lysates were analyzed by Western blotting with antibodies to EGFR, phosphotyrosine, and Flag epitope. *B*, U87MGecoR glioblastoma cells (*lane a*) were transduced with retroviral vectors containing cDNA for either triple Flag-tagged EGFRvIII (*lane b*) or unmodified EGFRvIII (*lane c*). Total cell lysates were then analyzed by Western blotting using antibodies to EGFR and phosphotyrosine. We have shown previously that the upper phosphotyrosine band, seen in *lanes b* and *c*, represents autophosphorylated EGFRvIII (21).

unable to detect the triple Flag epitope on the surface of U87MGtftΔ cells by flow cytometry (Fig. 3, *panels e* and *f*).

Immunofluorescence on permeabilized and nonpermeabilized cells using the antibody to the amino terminus of EGFRvIII showed that the subcellular distribution of tftEGFRvIII and EGFRvIII were the same (not shown). As with the flow cytometry, we were unable to detect Flag epitope on the surface of nonpermeabilized U87MGtftΔ cells. Immunofluorescence of permeabilized cells shows that the triple Flag epitope is detectable within the cell (Fig. 4). The pattern of staining suggested that the localization was mainly in the endoplasmic reticulum. This was confirmed by double immunofluorescence with anti-Flag antibody and an antibody to the endoplasmic reticulum protein Sec 61α, which showed a clear overlap in the staining pattern (Fig. 4, *panels d–f*). Double immunofluorescence with an antibody to the Golgi marker protein Golgin 97 showed that while some of the triple Flag epitope was present in the Golgi, it was not present throughout the Golgi (Fig. 4, *a–c*); this suggests that Flag epitope immunoreactivity is lost during the passage of tftEGFRvIII through this organelle.

Western blot analysis of tftEGFRvIII expressed in either 293T cells or glioblastoma cells showed that antibody to the carboxyl terminus of EGFR recognized two bands (Figs. 2 and 5). In contrast, antibody to the Flag epitope only recognized a single band. Careful comparison of blots that were run on the same gel showed that the Flag antibody recognized the lower of the two bands recognized by the EGFR antibody (Fig. 5*A*). The different EGFRvIII bands likely represent receptors with different glycosylation status. (EGFRvIII contains eight of the 12 potential *N*-linked glycosylation sites present in EGFR.) To investigate this further, we treated U87MGtftΔ cells with tunicamycin, which blocks the core *N*-linked glycosylation of proteins that occurs in the endoplasmic reticulum. Western blot analysis showed that tunicamycin-treated cells contained a low molecular weight form of tftEGFRvIII that is probably nonglycosylated receptor (Fig. 5*B*). This form was smaller than the lower band of the tftEGFRvIII doublet, suggesting that the lower doublet band is at least partly glycosylated. The lower band of the tftEGFRvIII doublet was absent after tunicamycin treatment, whereas the upper band was still present. A likely explanation for this was that the lower band represented

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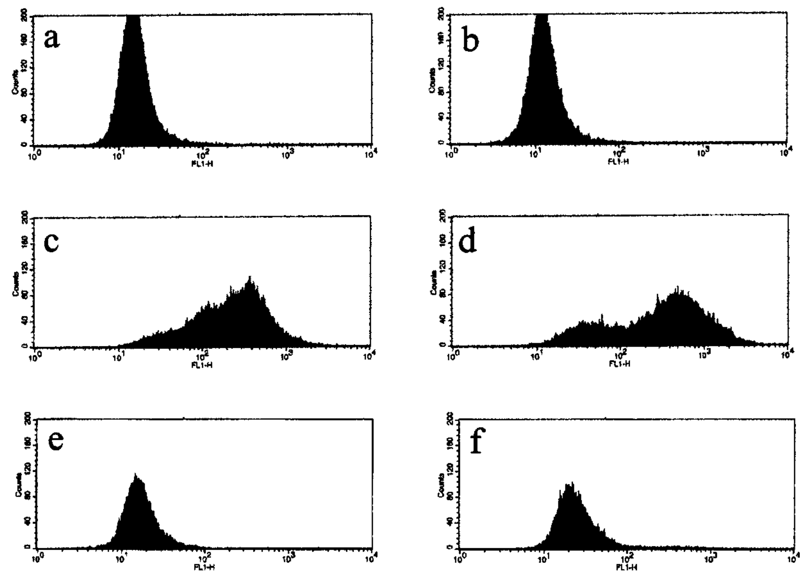

FIG. 3. **Analysis of U87MGtftΔ cells by flow cytometry.** *a*, U87MGecoR cells labeled with affinity purified rabbit IgG specific for EGFRvIII; *b*, U87MGtftΔ cells labeled with nonimmune rabbit IgG; *c*, U87MGtftΔ cells labeled with affinity purified rabbit IgG specific for EGFRvIII; *d*, U87MGecoR cells transduced with unmodified EGFRvIII and labeled with affinity purified rabbit IgG specific for EGFRvIII; *e*, U87MGtftΔ cells labeled with nonimmune mouse IgG_1; *f*, U87MGtftΔ cells labeled with anti-Flag mouse monoclonal IgG_1 antibody.

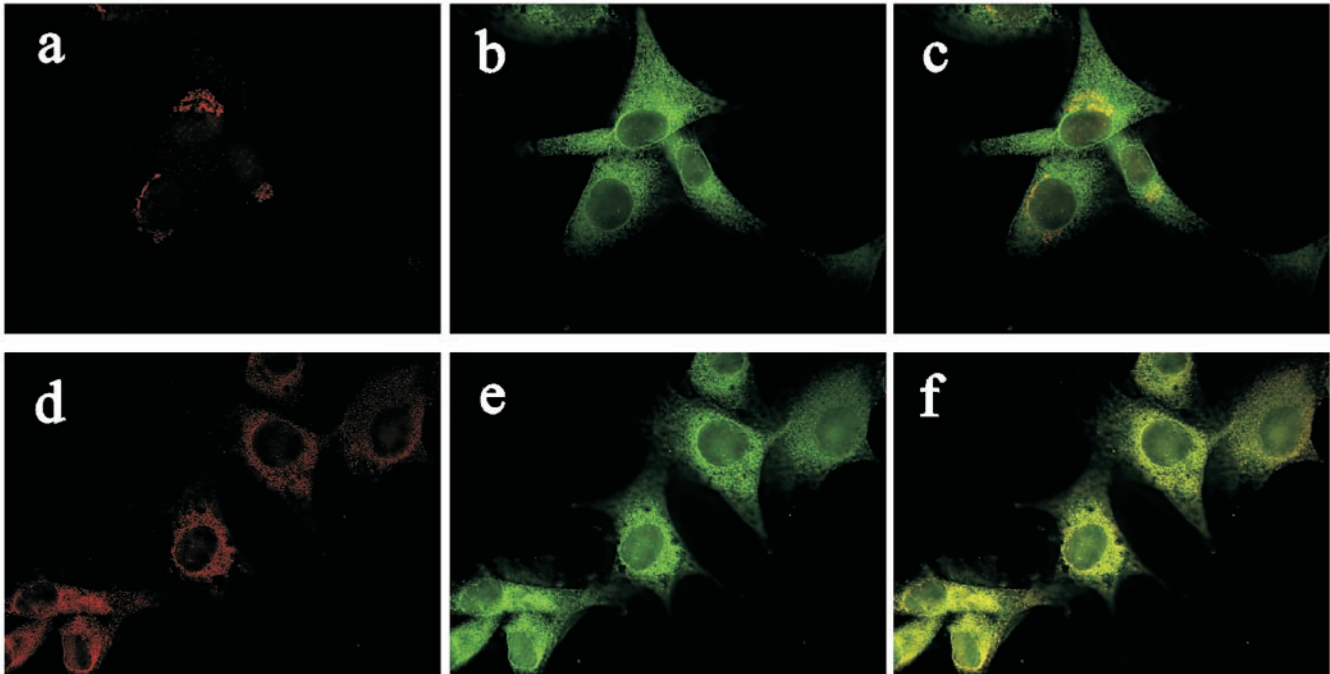

FIG. 4. **Analysis of tftEGFRvIII expression by immunofluorescence.** All images are of U87MGtftΔ cells and have been deconvolved as described under "Materials and Methods." *a*, cells stained with anti-Golgin 97 antibody, a Golgi marker, and Cy3-labeled secondary antibody; *b*, the same cells stained with Alexa Fluor 488-labeled anti-Flag antibody; *c*, merged images from *a* and *b*. The presence of some *yellow color* shows that there is some colocalization of the two proteins. However, Flag epitope is not detected throughout the Golgi, as *red color* is still evident; *d*, cells stained with anti-Sec61 antibody, an endoplasmic reticulum marker, and Cy3-labeled secondary antibody; *e*, the same cells stained with Alexa Fluor 488-labeled anti-Flag antibody; *f*, merged images from *d* and *e*. The absence of any *red color* in the merged images shows that the Flag epitope is present in its immunoreactive form throughout the endoplasmic reticulum.

core-glycosylated tftEGFRvIII, whereas the upper band represented tftEGFRvIII in which the carbohydrate had been converted to complex oligosaccharides, a process that occurs in the Golgi. After tunicamycin treatment, no new core-glycosylated tftEGFRvIII would form, but any that was present at the initiation of tunicamycin treatment would be converted into tftEGFRvIII containing complex oligosaccharide. We also treated U87MGtftΔ cells with the α-mannosidase I inhibitor deoxymannojirimycin (28), which inhibits the formation of complex oligosaccharides (Fig. 5*C*). A 24-h treatment with deoxymannojirimycin resulted in loss of the upper tftEGFRvIII band, whereas levels of the lower band were enhanced. This supports the idea that the lower tftEGFRvIII band, which is recognized by the anti-Flag antibody, is tftEGFRvIII that has not yet undergone conversion of its carbohydrate residues to complex oligosaccharides in the Golgi.

The results from flow cytometry, immunofluorescence, and Western blot analysis show that tftEGFRvIII is expressed at the same levels and in the same localization pattern as non-tagged EGFRvIII, but that the triple Flag epitope is covalently modified (by an unknown mechanism) in the Golgi apparatus so that it is no longer recognized by the anti-Flag antibody. This provides a system in which nascent EGFRvIII in the endoplasmic reticulum can be selectively purified for characterization.

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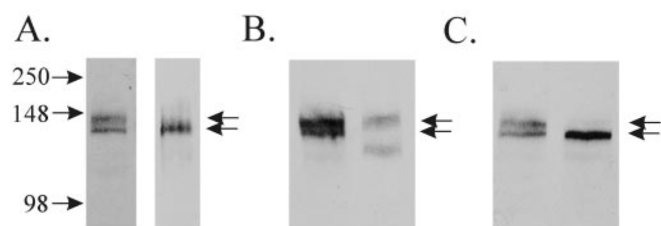

FIG. 5. **Western blot analysis of tftEGFRvIII from cells treated with glycosylation inhibitors.** *A*, total cell extract from U87MGtftΔ cells probed with antibody to the carboxyl terminus of EGFR (*left panel*) or with anti-Flag antibody (*right panel*). Molecular weight markers are shown on the *left*. The migration position of the EGFRvIII doublet is marked with a *pair* of *arrows* on the *right* in *A–C*. *B*, total cell extracts from U87MGtftΔ cells either treated with vehicle alone (*left lane*) or treated for 24 h with 1 μg/ml tunicamycin (*right lane*), and probed with antibody to the carboxyl terminus of EGFR; *C*, total cell extracts from U87MGtftΔ cells either treated with vehicle alone (*left lane*) or treated for 24 h with 2.5 mM deoxymannojirimycin (*right lane*), and probed with antibody to the carboxyl terminus of EGFR.

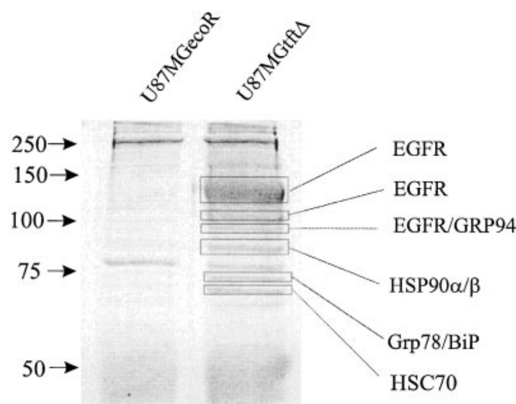

FIG. 6. **Immunopurification of tftEGFRvIII and mass spectrometry identification of associated proteins.** TftEGFRvIII was purified from U87MGtftΔ cells as described under "Materials and Methods." As a control, the same purification procedure was also performed on U87MGecoR cells (*left lane*). Immunopurifications were analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining with colloidal Coomassie Blue. Identities of the proteins present in specific bands, determined by mass spectrometry, are shown on the *right*.

Immunopurification of Flag-tagged EGFRvIII—TftEGFRvIII was purified from ~5 × 10^7 U87MGtftΔ cells. As a control, the same purification procedure was performed on the parent glioblastoma cell line that does not express tftEGFRvIII. Immunopurified tftEGFRvIII was analyzed by one-dimensional gel electrophoresis and staining with colloidal Coomassie Blue (Fig. 6). A prominent band of the expected molecular weight for tftEGFRvIII was evident. Based on comparison with bovine serum albumin standards run on the same gel, 1–2 μg of tftEGFRvIII were purified from 5 × 10^7 cells. Seven other bands were also found to reproducibly copurify with tftEGFRvIII. (Additional bands were also detected in some preparations.) Two of these were present in the control lane, and were not studied further. The five other bands were absent in the control lane. Based on comparison with bovine serum albumin standards, these were present at about 1/10th the amount of the tftEGFRvIII band.

Mass Spectrometry Identification of Bands Copurifying with EGFRvIII—Bands from the one-dimensional gel lane of purified tftEGFRvIII were excised and the proteins were identified by in-gel tryptic digestion and mass spectrometric analysis (Fig. 6). The major band was identified as tftEGFRvIII, as were the two bands immediately below it. However, the lower band in this pair also contained as second protein, identified as the endoplasmic reticulum chaperone Grp94. The three other unique protein bands were identified as: 1) a mixture of the closely related cytosolic heat shock/chaperone proteins Hsp90α and Hsp90β; 2) the endoplasmic reticulum heat shock/chaperone protein Grp78, also known as BiP; and 3) the cytosolic heat shock/chaperone protein Hsc70.

In this paper, we have chosen to focus on the interaction of EGFRvIII with Hsp90. To confirm the mass spectrometry identification of Hsp90, we analyzed immunopurified EGFRvIII by Western blots, using an antibody that recognizes Hsp90α and weakly cross-reacts with Hsp90β; a band of the expected size was labeled in the EGFRvIII immunoprecipitate and was absent in the control lane (Fig. 7*A*). To determine whether EGFRvIII had any effects on overall expression levels of Hsp90, Western blots of total cell lysates from U87MGecoR and U87MGtftΔ cells were probed with antibody to Hsp90. Expression levels were the same in the two cell lines (Fig. 7*B*). Thus the presence of Hsp90 in EGFRvIII immunoprecipitates is not because of higher levels of these proteins in cells expressing EGFRvIII, but rather is because of a physical association between the proteins.

To confirm that the interaction of EGFRvIII with Hsp90 was not an artifact caused by the addition of the epitope tag, we also

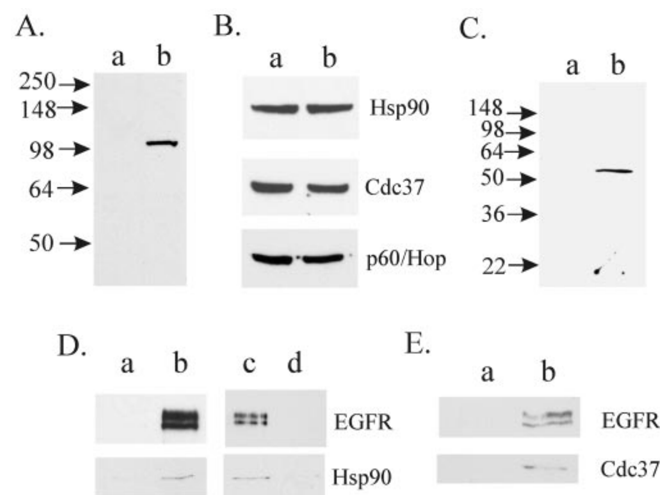

FIG. 7. **Association of Hsp90 and Cdc37 with EGFRvIII.** *A*, Western blot of EGFRvIII immunopurified from U87MGtftΔ cells (*lane b*) or mock immunopurified from U87MGecoR cells (*lane a*) probed with antibody to Hsp90. *B*, Western blots of total cell extracts from either U87MGecoR cells (*lane a*) or U87MGtftΔ cells (*lane b*), probed with antibody to Hsp90 (*top*), Cdc37 (*middle*), or p60/Hop (*bottom*). *C*, Western blot of EGFRvIII immunopurified from U87MGtftΔ cells (*lane b*) or mock immunopurified from U87MGecoR cells (*lane a*) probed with antibody to Cdc37. *D*, immunoprecipitations of nontagged EGFRvIII. Immunoprecipitations were performed with a His-tagged version of a Fv antibody fragment specific for EGFRvIII (MR1dsFv) and immobilized Ni^{2+} resin, as described under "Materials and Methods." *Lanes a* and *b* show an immunoprecipitation in which U87MGΔEGFR cell extracts were incubated without MR1dsFv (*lane a*) or with MR1dsFv (*lane b*) followed by incubation with immobilized Ni^{2+} resin (*both lanes*); *lanes c* and *d* show a second, independent immunoprecipitation in which U87MGΔEGFR cell extract (*lane c*) or U87MG cell extract (*lane d*) were incubated with MR1dsFv and immobilized Ni^{2+} resin. Immunoprecipitates were analyzed by Western blotting for the presence of EGFRvIII and Hsp90. *E*, nontagged EGFRvIII was immunoprecipitated as in *D* and analyzed by Western blotting for the presence of EGFRvIII and Cdc37. *Lane a*, U87MGΔEGFR cell extract incubated in the absence of MR1dsFv; *lane b*, U87MGΔEGFR cell extract incubated in the presence of MR1dsFv.

performed small scale immunoprecipitations of nontagged EGFRvIII and probed these by Western blotting. These immunoprecipitations were done using an engineered antibody fragment that is specific for EGFRvIII. (These immunoprecipitations are specific but relatively inefficient, and so were not used

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for large scale immunopurifications.) For these experiments, we used U87MG and U87MGΔEGFR cells (obtained from Dr. W. Cavenee), which have been described previously (10). Western blot analysis of EGFRvIII immunoprecipitates showed that Hsp90 was present (Fig. 7*D*). Hsp90 was absent in control immunoprecipitations from U87MGΔEGFR cells in which the primary antibody was omitted, and in control immunoprecipitations done with U87MG cells. This shows that Hsp90 also interacts with nontagged EGFRvIII.

Hsp90 does not interact with "client" proteins on its own, but rather forms a set of different complexes with other proteins, some of which also have chaperone-like activity (reviewed in Ref. 29). One of these is the protein Cdc37, which appears to promote the interaction of Hsp90 with protein kinases (30). We probed Western blots of immunopurified EGFRvIII with antibody to Cdc37 and detected a single band of the expected size in immunopurified EGFRvIII, which was absent in the control lane (Fig. 7*C*). This suggests that Hsp90 binds EGFRvIII in association with Cdc37. In gels stained with colloidal Coomassie Blue this region is obscured by IgG heavy chain that is present in affinity gel eluates (see Fig. 6), which explains why Cdc37 was not detected in our original screen for proteins binding to EGFRvIII. Cdc37 expression levels were the same in U87MGecoR and U87MGtftΔ cells (Fig. 7*B*, *middle panel*), showing that the presence of Cdc37 in EGFRvIII immunopurifications is not because of overexpression in the latter cell line. Cdc37 was also present in small-scale immunoprecipitations of nontagged EGFRvIII, performed as described above (Fig. 7*E*).

A second protein with chaperone-like activity that associates with Hsp90 is p60/Hop (31, 32). We also probed immunopurified tftEGFRvIII with antibody to p60/Hop; although this antibody readily recognized a single protein in U87MGecoR and U87MGtftΔ cells (Fig. 7*B*, *bottom panel*), we did not detect this protein in immunopurified tftEGFRvIII preparations.

Effects of the Hsp90 Inhibitors on EGFRvIII—The natural product geldanamycin binds in the amino-terminal ATP-binding domain of Hsp90, blocking many of the activities of this protein within the cell (33). To assess the role of the association of Hsp90 with EGFRvIII, we treated U87MGΔEGFR cells with different concentrations of geldanamycin. Overnight treatment of these cells with 1 μM geldanamycin did not induce apoptosis in these cells, as judged by morphological criteria, but did cause the cells to adopt a more flattened appearance with less refractile edges that is more typical of nontransformed cells. This treatment did not induce any gross changes in protein expression patterns, but did induce significant decreases in EGFRvIII protein levels (Fig. 8*A*). As well as inhibiting Hsp90, geldanamycin also is able to inhibit Grp94. Xu *et al.* (34) have reported relative drug binding affinities of 0.3 and 1 μM for geldanamycin binding to Hsp90 and Grp94, respectively. Geldanamycin was able to induce EGFRvIII degradation at concentrations as low as 100 nM, suggesting that its effects are mediated by inhibition of Hsp90, rather than Grp94. This result is consistent with EGFRvIII being a client protein for Hsp90. In agreement with studies in other cell types, geldanamycin also decreased levels of the Hsp90 client protein cdk4 in glioblastoma cells (35); this occurred at the same concentration that decreased expression of EGFRvIII (Fig. 8*A*). Also in agreement with studies in other cell types, geldanamycin did not affect the expression of ERK (36).

The natural product radicicol also binds Hsp90 at its amino-terminal ATP-binding site, although it is chemically unrelated to geldanamycin (37). Radicicol was able to decrease levels of phosphorylated EGFRvIII and EGFRvIII protein at concentrations of 300 nM or greater (Fig. 8*B*). Radicicol also down-regulated expression of cdk4 at a similar concentration without affecting ERK expression. Radicicol has a 5-fold lower affinity for Grp94 than for Hsp90 (37); because the same concentration of radicicol decreases the expression of both EGFRvIII and cdk4 (which interacts with Hsp90 but not Grp94), this indicates that the effects of radicocol on EGFRvIII are because of inhibition of Hsp90, rather than Grp94.

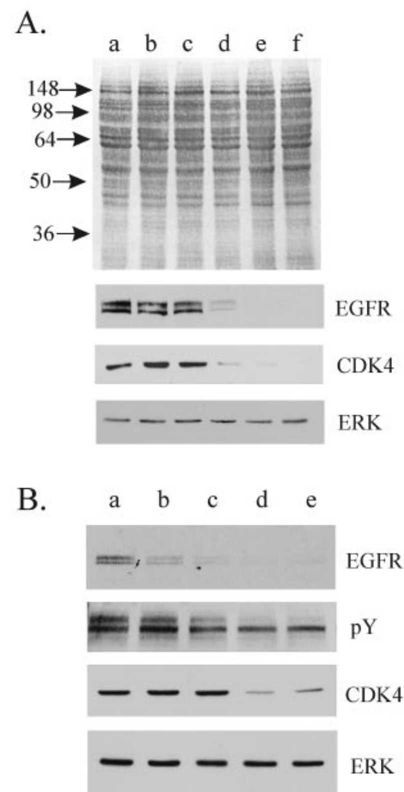

FIG. 8. **Effects of geldanamycin and radicicol on EGFRvIII expression.** *A*, U87MGΔEGFR cells were treated for 24 h with geldanamycin at the following concentrations: *a*, 0 nM; *b*, 10 nM; *c*, 30 nM; *d*, 100 nM; *e*, 300 nM; *f*, 1000 nM. Total cell extracts were then analyzed by Western blotting. The *top panel* shows a Western blot stained for total protein with Amido Black. The *lower three panels* show Western blots probed with antibody to EGFR, cdk4, and ERK, as indicated. *B*, U87MGtftΔ cells were treated with radicicol for 24 h at the following concentrations: *a*, 0 nM; *b*, 100 nM; *c*, 300 nM, *d*, 1000 nM; *e*, 3000 nM. Total cell extracts were then analyzed by Western blotting for EGFR, phosphotyrosine, cdk4, and ERK.

DISCUSSION

We have made and characterized a version of EGFRvIII that contains three copies of the Flag epitope at the amino terminus of the receptor after removal of the leader sequence. Surprisingly, we found that the immunoreactivity of this epitope tag was lost during EGFRvIII maturation, most likely because of a covalent modification taking place in the Golgi apparatus. There were two main lines of evidence for this: first, immunofluoresence localization showed loss of Flag epitope immunoreactivity in the Golgi; second, the Flag epitope could only be detected on EGFRvIII that had not undergone conversion of its carbohydrate into complex oligosaccharides, a process that occurs in the Golgi. We do not know what the covalent modification is at this time. It is very unlikely that it is glycosylation, as the Flag epitope does not contain sites for either *N*-linked or *O*-linked glycosylation (see Fig. 1). The Flag epitope is rich in aspartate residues; one possibility is that the loss of immunoreactivity is because of β-hydroxylation of aspartate residues, a post-translational modification that has been shown to occur in some secreted proteins (38, 39).

The loss of Flag epitope immunoreactivity as EGFRvIII

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passes through the Golgi provides a convenient method for selectively purifying newly synthesized EGFRvIII. We were able to purify the nascent receptor in microgram quantities, and showed that a set of other proteins reproducibly co-purified with it. These were identified by mass spectrometry as the two endoplasmic chaperones Grp94 and Grp78/Bip, and the two cytosolic chaperones Hsc70 and Hsp90.

In this paper we have focused on the association of EGFRvIII with Hsp90, a finding that is of interest from both basic science and clinical perspectives. Hsp90 is a member of the heat shock protein/chaperone family: the major role of this family is to assist in the folding of newly synthesized proteins in the cell, and to assist in protein refolding after environmental insults (40, 41). Hsp90 function has been studied in most detail with respect to its role in steroid receptor function, where it has a clear role both in receptor assembly and in maintenance of the high affinity hormone binding of the mature receptor (29). Hsp90 has also been shown to interact with a number of tyrosine kinases including the Src family members v-Src (42) and Hck (43), erbB2 (34), and with the serine/threonine kinases Raf (44) and cdk4 (35). These studies have led to the idea that Hsp90 may have a general role in supporting signaling by oncogenic protein kinases. Whereas we clearly detect an interaction between EGFRvIII and Hsp90, Xu *et al.* (34) have reported that normal EGFR does not interact with Hsp90. This may indicate that there is a greater degree of association of Hsp90 with EGFRvIII than with normal EGFR. A preferential association of EGFRvIII with Hsp90 would parallel previous studies showing that oncogenic, constitutively active v-Src preferentially interacts with Hsp90 compared with normal cellular Src (42).

In systems where Hsp90 function has been studied in detail, Hsp90 interacts with client proteins as part of a complex of other proteins that often also have chaperone-like activity. The immunophilins FKBP51, FKBP52, and CyP-40, the protein phosphatase PP5, and p60/Hop all appear to bind at a common site on Hsp90 (45). These proteins bind Hsp90 via a domain known as the tetratricopeptide repeat (46). Another Hsp90-interacting protein, Cdc37, binds Hsp90 at a site that may overlap with the tetratricopeptide repeat domain-binding site (30). We found that Cdc37 copurified with nascent EGFRvIII, suggesting that some or all of the Hsp90 associated with EGFRvIII is present as a complex with Cdc37. The binding of Cdc37 and p60/Hop to Hsp90 are mutually exclusive (30). We did not detect p60/Hop in association with immunopurified EGFRvIII, suggesting that the nascent receptor is selective for the type of Hsp90 complex that it interacts with.

Cdc37 was first identified as the product of a cell division cycle start gene in yeast (47). A *Drosophila* homologue of Cdc37 was also identified in genetic screens for mutations that impair signaling by the sevenless receptor tyrosine kinase, which is required for differentiation of the R7 photoreceptor neuron (48). After this, several groups showed that the 50-kDa protein that copurified with v-Src and Hsp90 was the mammalian homologue of Cdc37 (35, 49). Cdc37 also interacts with several other kinases including Raf and cdk4 (30, 35). There is evidence that Cdc37 plays a role in tumor development, as mice engineered to overexpress Cdc37 under control of either the Moloney murine leukemia virus promoter (50) or a probasin promoter (51) develop tumors at a high frequency. Cdc37 is also overexpressed in prostate cancer (51). Biochemically, Cdc37 has been shown to have chaperone activity and, to a limited extent, can substitute for the chaperone activity of Hsp90 (52). Cdc37 is able to rescue expression of a mutant version of the Zap70 kinase that is found in one form of severe combined immunodeficiency disease (53); this may be a parallel to our own work, with Cdc37 helping to rescue the function of a mutant EGFR. Although an earlier study in *Drosophila* suggested a link between Cdc37 and receptor tyrosine kinases (48), the work presented here is the first demonstration of a physical interaction between Cdc37 and a receptor tyrosine kinase.

The Hsp90 inhibitor geldanamycin was able to decrease expression of EGFRvIII at a concentration similar to its reported affinity for Hsp90 (34). The interaction with Hsp90 therefore appears to be essential to maintain high level expression of EGFRvIII. Geldanamycin treatment also reduced levels of cdk4 in glioblastoma cells, in agreement with results seen in other cell types. EGFRvIII cooperates with cdk4-activating mutations (either mutation of the cdk4 repressor Ink4a, or overexpression of cdk4) to induce the formation of glioma (11). Hsp90 inhibition is therefore a means to simultaneously inactivate two key signaling pathways that are aberrantly activated in glioblastoma. Inhibitors of Hsp90 are currently under evaluation as cancer therapeutics, and our study suggests that EGFRvIII may be a novel target for the antitumor activity of these drugs (54). The ability of Hsp90 inhibitors to block two key signaling pathways in glioblastoma may give them a therapeutic advantage over agents such as EGFR tyrosine kinase inhibitors, which target a single pathway.

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Interaction of Hsp90 with the Nascent Form of the Mutant Epidermal Growth Factor Receptor EGFRvIII

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