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

Sylvie J. Lavictoire‡§, Doris A. E. Parolin‡§, Alex C. Klimowicz‡§§, John F. Kelly¶, and Ian A. J. Lorimer‡§**

From the 3Ottawa Regional Cancer Centre, Centre for Cancer Therapeutics, Ottawa, Ontario K1H 1C4, Canada, the 4Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada, and the 5Institute of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A OR6, Canada

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. 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 BamHI and StuI 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...
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 SalI digestion and subcloned into the SalI site of a modified version of the vector T7-blue (Novagen, Inc., Madison, WI) in which the unique SplI site had been removed. Site-directed mutagenesis was then used to create a silent SplI restriction site at codons 45–44 of the mutant receptor, using the oligonucleotide SPHV3 (CCGATGTCCGAGGACCAGCCTGACGTATC). This plasmid was then used as a template for two PCR reactions, using two primer pairs: KPN3 (CGGTACCATGGGTGACGAG) and TF1 (GATGACTGATTTTTATATCCGTATGTTTGTGTAATAGCTGAGG). The PCR products were then purified and a third PCR using the KPN3 and SPHV3 primer pair and the two initial PCR products as templates. The final PCR product was subcloned and sequenced. The KpnI/SplI fragment was excised and ligated into T7-blue containing EGFRvIII cDNA with the silent SplI site, which had also been digested with KpnI and SplI. The full-length cDNA for EGFRvIII containing the triple Flag tag sequence was then excised with SalI and ligated into SalI digested pLERNL. Clones showing the correct orientation by restriction digest were designated pLRNFlta.

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 Inc. (Santa Cruz, CA). Anti-EGFRvIII antibody was from Molecular Probes, Inc. (Eugene, OR). Anti-Ste20α antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-cdk4 (Ab-6) and anti-cyclin G antibody were from Neomarkers (Fremont, CA). Anti-Hsp90 and anti-p60/hop antibodies were from StressGen (Ab-6) and anti-Cdc37 antibodies were from Neomarkers (Fremont, CA) in which the unique

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% (v/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 UltraLink immunoblotting streptavidin gel (BioLynx, Inc., Brockville, ON).

Cell Lines—U87MG and U87MG/H9004 cells were obtained from Dr. W. Cavenee, Ludwig Institute for Cancer Research, La Jolla, CA. U87MGtft 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. Flow Cytometry—Cells growing in tissue culture flasks were detached by using Cell Dissociation buffer (Invitrogen), pelleted by centrifugation and subcloned into the SalI site of a modified version of the vector T7-blue (Novagen, Inc., Madison, WI) in which the unique SplI site had been removed. Site-directed mutagenesis was then used to create a silent SplI restriction site at codons 45–44 of the mutant receptor, using the oligonucleotide SPHV3 (CCGATGTCCGAGGACCAGCCTGACGTATC). This plasmid was then used as a template for two PCR reactions, using two primer pairs: KPN3 (CGGTACCATGGGTGACGAG) and TF1 (GATGACTGATTTTTATATCCGTATGTTTGTGTAATAGCTGAGG). The PCR products were then purified and a third PCR using the KPN3 and SPHV3 primer pair and the two initial PCR products as templates. The final PCR product was subcloned and sequenced. The KpnI/SplI fragment was excised and ligated into T7-blue containing EGFRvIII cDNA with the silent SplI site, which had also been digested with KpnI and SplI. The full-length cDNA for EGFRvIII containing the triple Flag tag sequence was then excised with SalI and ligated into SalI digested pLERNL. Clones showing the correct orientation by restriction digest were designated pLRNFlta.

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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. 2A). 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). The EGFRvIII expressed by transient transfection in 293T cells or glioblastoma cells showed that 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 (Figs. 2 and 4). The pattern of staining suggested that the localization was mainly in the endoplasmic reticulum. This was confirmed by double immunofluorescence with antibody to the Flag epitope and an antibody to the endoplasmic reticulum protein Sec61α, which showed a clear overlap in the staining pattern (Fig. 4, panels d–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 antibody to the Flag epitope and an antibody to the endoplasmic reticulum protein Sec61α, which showed a clear overlap in the staining pattern (Fig. 4, panels d–f).

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. 5A). 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. 5B). 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...
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 oligosaccharides. We also treated U87MGtftΔ cells with the α-mannosidase I inhibitor deoxymannojirimycin (28), which inhibits the formation of complex oligosaccharides (Fig. 5C). 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.
Immunopurification of Flag-tagged EGFRvIII—TREGFR
vIII was purified from ~5 × 10^5 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 control lane. Based on comparison with bovine serum albumin standards run on the same gel, 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.

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 Hsp90. To determine whether EGFR–Hsp90 interactions are specific but relatively inefficient, and so were not used 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.

To confirm that the interaction of EGFRvIII with Hsp90 was not an artifact caused by the addition of the epitope tag, we also 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
Effects of the Hsp90 Inhibitors on EGFR\textsubscript{vIII} expression. A, U87MG\textsubscript{EGFR} 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 blotted 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, U87MG\textsubscript{ft\Delta} 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 EGFR\textsubscript{vIII} 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 EGFR\textsubscript{vIII} maturation, most likely because of a covalent modification taking place in the Golgi apparatus. There were two main lines of evidence for this: first, immunofluorescence localization showed loss of Flag epitope immunoreactivity as EGFR\textsubscript{vIII} matures and second, the Flag epitope could only be detected on EGFR\textsubscript{vIII} 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- or O-linked glycosylation (see Fig. 1). The Flag epitope is rich in serine and threonine residues, which could be phosphorylated by kinases, such as AMPK, which are known to bind to the amino-terminal ATP-binding site, although it is chemically unrelated to geldanamycin (37). Radicicol was able to decrease levels of phosphorylated EGFR\textsubscript{vIII} and EGFR\textsubscript{vIII} protein at concentrations of 300 nM or greater (Fig. 8B). 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 EGFR\textsubscript{vIII} and cdk4 (which interacts with Hsp90 but not Grp94), this indicates that the effects of radicicol on EGFR\textsubscript{vIII} are because of inhibition of Hsp90, rather than Grp94.
Hsp90 Interaction with EGFRvIII

Hsp90 interacts with client proteins as part of a complex of cytosolic chaperones Hsc70 and Hsp90. 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 Cyp40, the protein phosphatase PP2A, and 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).

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