Activation of STAT3 by EGFR Variant III: Tyrosine Residue 1068 is the Primary STAT3 Docking Site on EGFR

Jane Zhang, Timothy S. Schaefer, Robert M. Umek and Jacob N. Wohlstadter

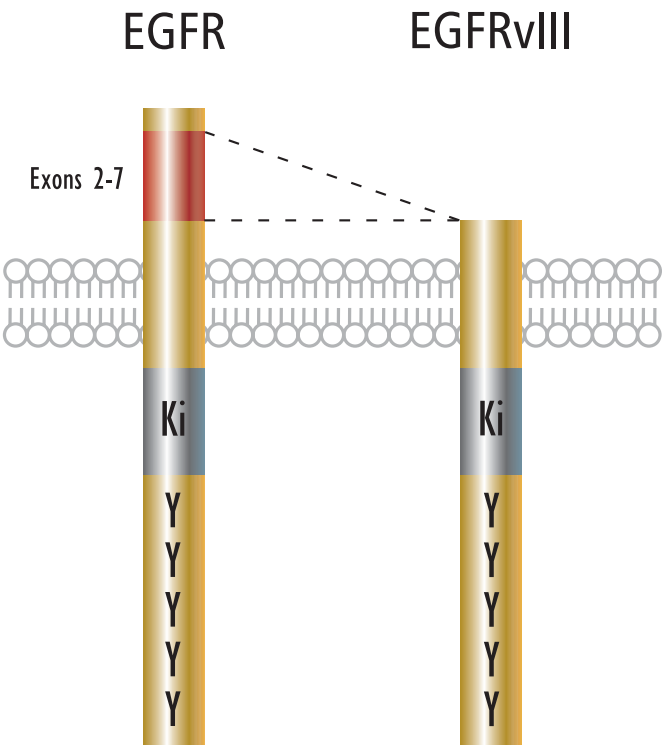
One of the hallmarks of glioblastoma multiforme (GBM) is the amplification and rearrangement of the epidermal growth factor receptor. One such rearranged receptor, termed variant III (vIII), has an internal deletion in the extracellular domain of the protein. This modification results in a receptor that is unable to bind ligand, constitutively activated, and as a result activates downstream signaling pathways. Our results demonstrate that EGFRvIII is phosphorylated on tyrosine to a greater extent than wild-type receptor using pan-and phospho-specific antibodies. We also show that enforced expression of EGFRvIII results in the activation of the transcription factor STAT3 to a greater extent than wild-type receptor as determined by phosphospecific antibodies and transcriptional activation assays. Using a proprietary assay to assess the interaction between immobilized receptor and STAT3 we determined that tyrosine 1068 is the primary association site for STAT3 on EGFR with a panel of mutants containing individual or combinations of the alterations in the receptor's known autophosphorylation sites. The importance of this residue in STAT3 signaling was also confirmed independently by the activation of STAT3 transcriptional activity by receptors containing tyrosine at position 1068. This panel of mutants will allow us to continue to determine the role of STAT3 activity and that of other proteins activated in EGFR-initiated signaling pathways.

Background

EGFR amplification occurs frequently in glioma and is often correlated with a shorter relapse time and a decreased rate of survival. These amplifications are generally accompanied by gene rearrangements. The most commonly observed rearrangement is an in-frame deletion of the extracellular ligand-binding domain (exons 2-7) that renders the resulting receptor constitutively activated. This, in turn, initiates the activation of downstream signaling pathways. These pathways include the phosphatidylinositol 3-kinase (PI 3-kinase), mitogen activated protein kinase (MAP kinase), and c-Jun N-terminal kinase (JNK). Glioma cells that express EGFRvIII have increased rates of proliferation and decreased levels of apoptosis that results in enhanced tumorigenicity when grown in animals. Levels of one of the key regulators of apoptosis, bcl-XL, are dramatically increased in EGFRvIII expressing cells. Bcl-XL is transcriptionally regulated by STAT3α, a gene originally discovered as member of the STAT family of normally latent transcription factors activated by EGF treatment. It has subsequently been shown that STAT3α is activated following the association with phosphorylated tyrosines located in the cytoplasmic domain of the EGF receptor tyrosine kinase. Cells that express activated STAT3α possess many of the same properties of those that express EGFRvIII, including an increase in proliferative capacity, and a reduced level of apoptosis due to the increased expression of c-myc and bcl-XL, respectively. Thus the disruption of the normal balance between proliferative and apoptotic signals by aberrant STAT3α activation may contribute to the tumorigenic properties of EGFRvIII expressing cells. Since STAT3α is known to be a downstream component in EGF-mediated signaling, we hypothesize that STAT3α is the key effector molecule in the signals imparted by the EGFRvIII gene rearrangement.

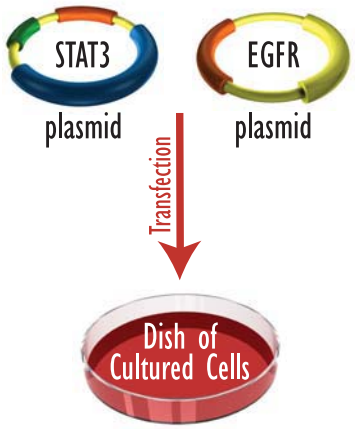
Structural Differences Between EGFR and EGFRvIII

- Frequently observed mutation in GBM
- In frame deletion of exons 2-7
- Results in constitutively activated receptor
- Activation of downstream signaling pathways (JNK, PI-3-kinase, MAP kinase)

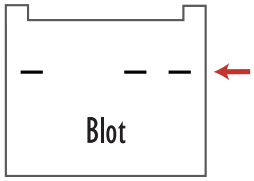


Transient Transfection Schematic

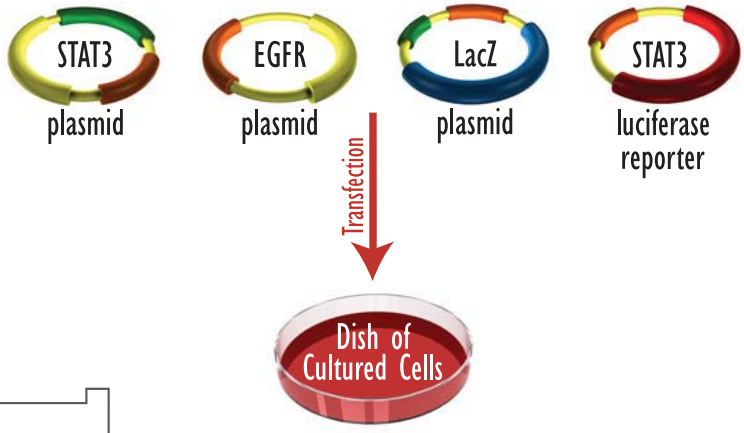
Followed by Immunoblot



1. Harvest cells
2. Prepare cell lysate
3. Electrophoresis
4. Immunoblot with specific antibodies

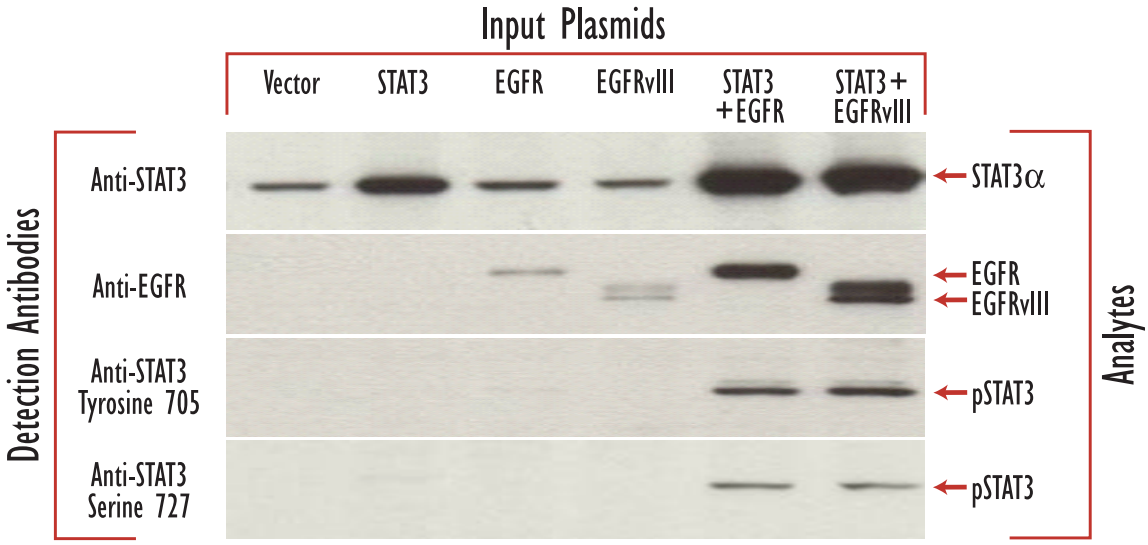


Followed by Transcription Assays



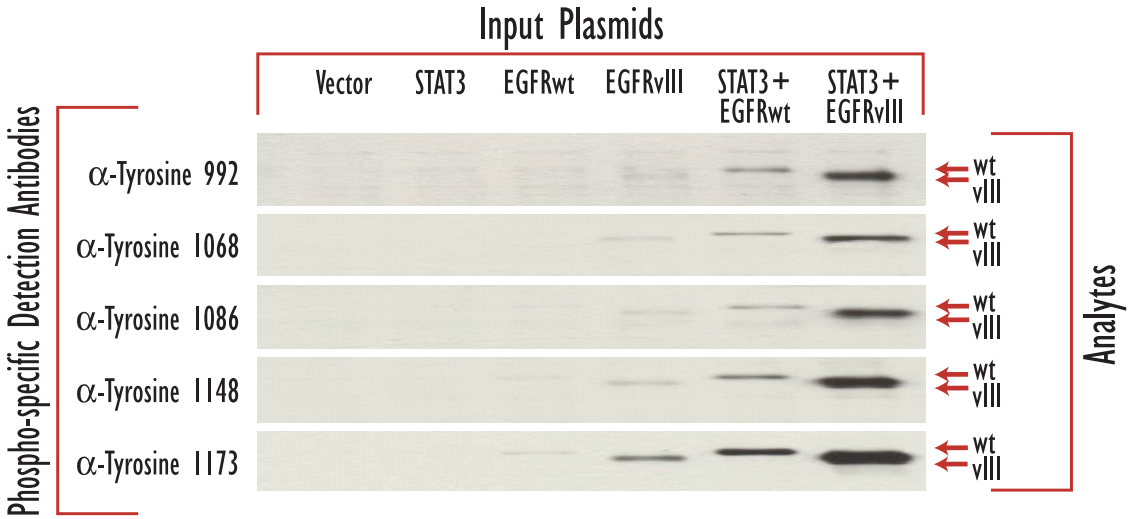
1. Harvest cells
2. Prepare cell lysate
3. Luciferase assay
4. β-gal assay

EGFR and EGFRvIII Increase STAT3 Phosphorylation on Tyrosine 705 and Serine 727



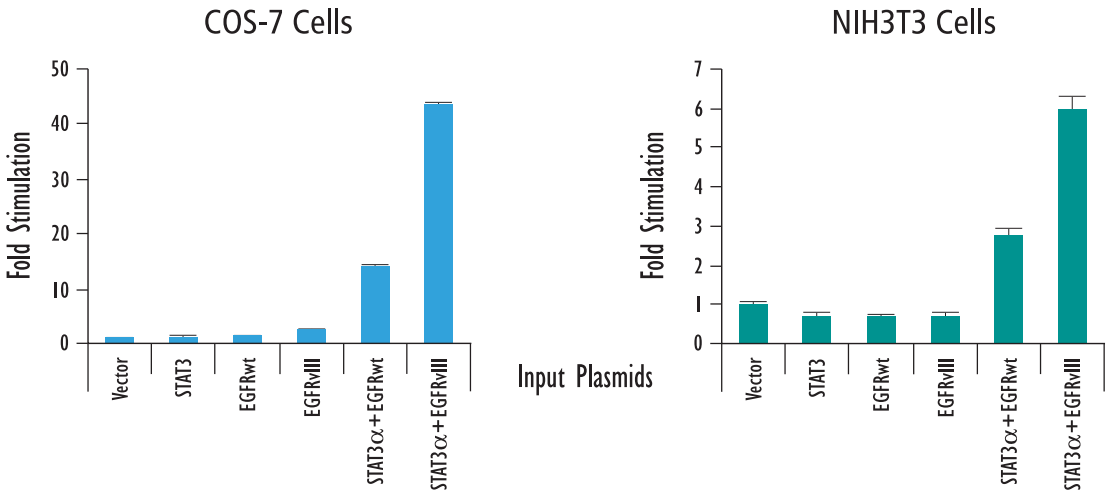
To confirm expression of the STAT and EGFR expression vectors and to determine the phosphorylation status of STAT3, COS-7 cells were transiently transfected with the indicated expression plasmids. Whole-cell lysates were prepared and equal amounts were separated on SDS-polyacrylamide gels. Proteins were transferred to nitro-cellulose membrane and immunoblotted with the antibodies indicated to the left of the gel image. The identity of the proteins is indicated to the right of the gel image.

Co-expression of STAT3 Increases EGFR and EGFRvIII Autophosphorylation



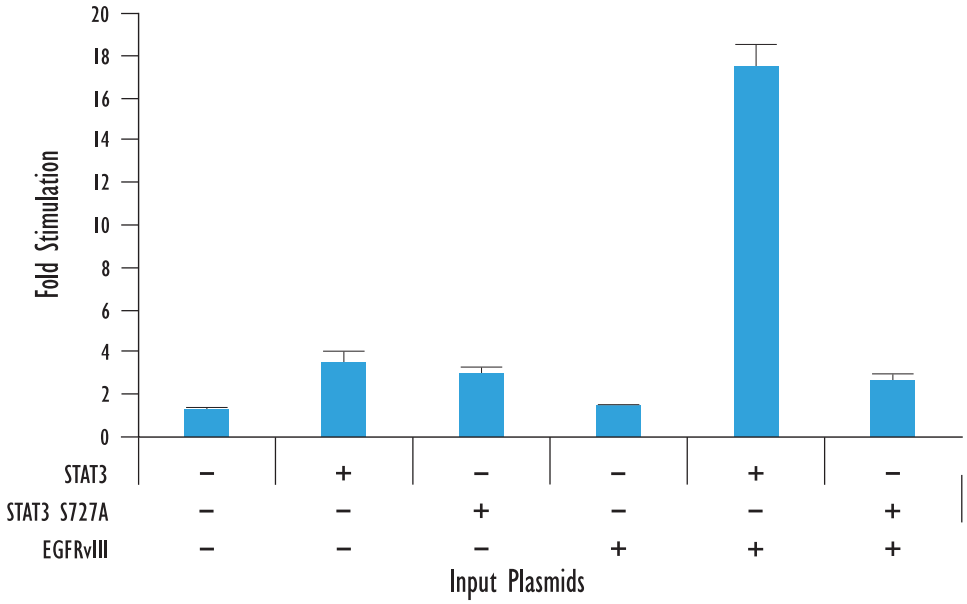
To assess the extent of phosphorylation of EGFR and EGFR vIII alone or when cotransfected with STAT3, COS-7 cells were transiently transfected with the indicated expression plasmids. Whole-cell lysates were prepared and equal amounts were separated on SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane and immunoblotted with the antibodies indicated on the left of the gel. The mobility of wild-type and variant III receptors is indicated with arrows.

EGFR and EGFRvIII Increase STAT3 Transcription Activity



Cells were transiently transfected with a Stat3-responsive luciferase plasmid and the indicated expression vectors and a plasmid expressing β-galactosidase. Cell lysates were prepared and lucifierase and β-galactosidase activity were determined. Luciferase values were normalized to β-galactosidase values. Each condition was performed in triplicate and normalized to cells containing only the reporter vectors.

STAT3 Transcription Activation by EGFRvIII Requires STAT3 Serine 727



COS-7 cells were transiently transfected with a STAT3-responsive luciferase plasmid, indicated expression vectors, and a plasmid expressing β-galactosidase. STAT3 S727A contains a point mutation in which serine 727 is converted to alanine. Cell lysates were prepared and luciferase and β-galactosidase activity was determined. Luciferase values were normalized to β-galactosidase values. Each condition was performed in triplicate and normalized to cells containing only the reporter vectors.

EGFR Autophosphorylation Sites

Amino Acid Context

992	DVVDADE**Y**LIPQQGFFSSP
1068	TFLPVPE**Y**INQSVPKRPAG
1086	GSVQNPV**Y**HNQPLNPAPSR
1148	ISLDNPD**Y**QQDFFPKEAKP
1173	STAENAE**Y**LRVAPQSSEFI

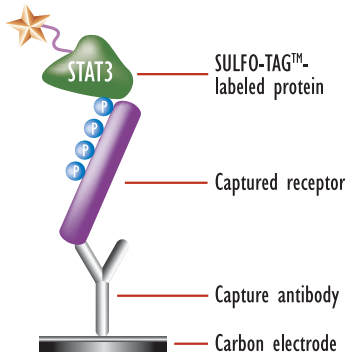
The positions of the five primary EGFR autophosphorylation sites are in red. Note that Tyrosine 1068 and 1086 are located within YXXQ motifs where X is any amino acid.

EGFR Mutant Panel

1	992F
2	1068F
3	1086F
4	1148F
5	1173F
6	1068F, 1086F
7	1045F, 1068F, 1086F
8	1068F, 1086F, 1148F
9	1068F, 1086F, 1173F
10	1068F, 1086F, 1148F, 1173F
11	992F, 1068F, 1086F, 1148F
12	992F, 1068F, 1086F, 1173F
13	992F, 1068F, 1086F, 1148F, 1173F
14	992F, 1148F, 1173F
15	992F, 1068F, 1148F, 1173F
16	992F, 1086F, 1148F, 1173F

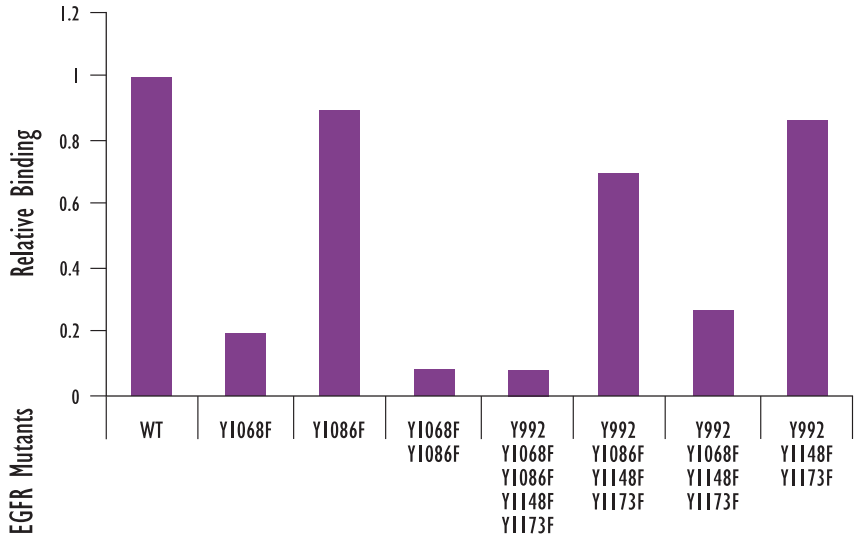
Using site-directed mutagenesis, we prepared a panel containing individual and combinations of mutations in the five primary autophosphorylation sites. The mutations were introduced into both the full length wild-type and variant III receptors. The F designates the specific tyrosine residues that were replaced with phenylalanine.

MSD® Immuno-Receptor Association Assay Format



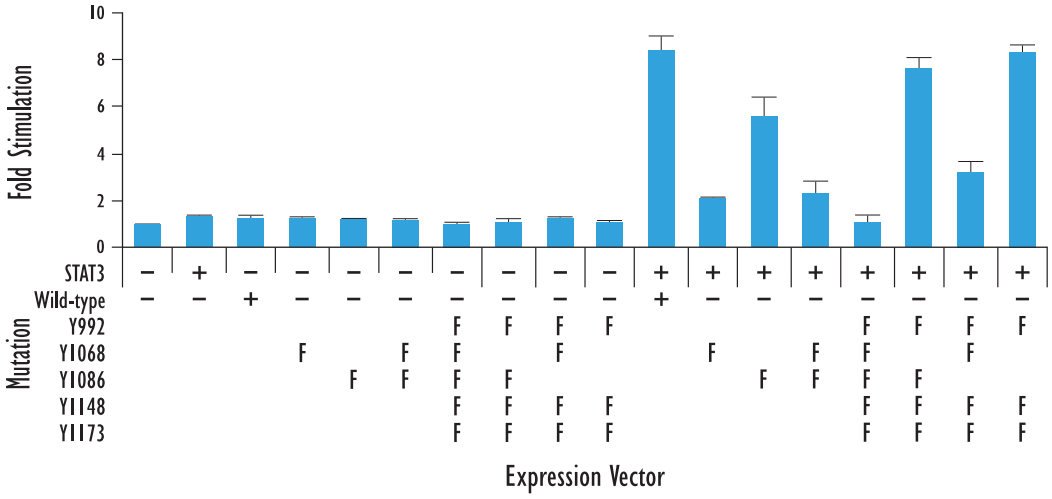
As an alternative to conventional co-immuno-precipitation assays (CoIPs), we have developed an assay using MSD MULTI-ARRAY® plates. In brief, an anti-EGFR capture antibody is applied the surface of a 96-well MULTI-ARRAY plate. Cell lysates containing the receptor are then added to the well followed by the addition of MSD SULFO-TAG-labeled recombinant STAT3. After a 1 hour incubation and a final wash, MSD Read Buffer is added and the plate is imaged on a SECTOR™ Imager 6000. The light generated from the labeled protein on the surface of the electrode can be measured by MSD's DISCOVERY WORKBENCH® Software.

Binding of STAT3 to EGFR Mutants



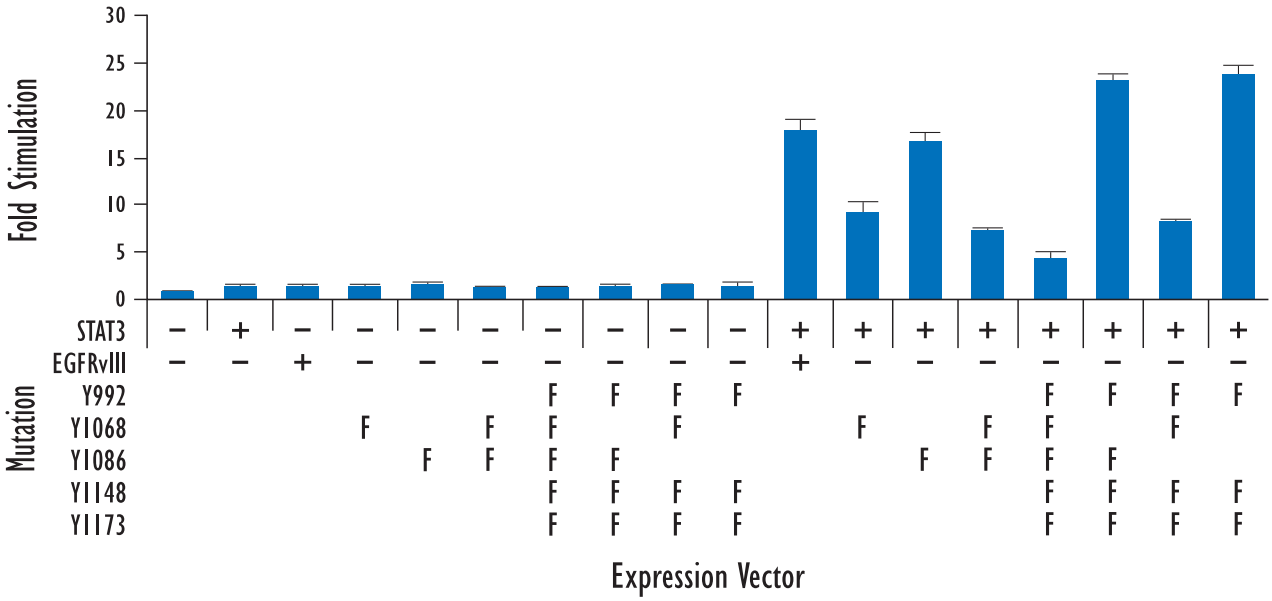
Using MSD technology, we developed an assay that assesses the interaction of STAT3 with a subset of EGFR mutants. Cell lysates from cells expressing the indicated mutant were captured onto the surface of a MULTI-ARRAY plate with a total EGFR antibody. MSD SULFO-TAG-labeled recombinant, dimer-incompetent STAT3 protein was then added to the wells (200 nM final concentration). The plate was incubated at room temperature for 1 hr, washed and MSD Read Buffer added. The plate was analyzed on the SECTOR Imager 6000. Binding relative to wild-type receptor is shown.

Maximal STAT3 Activation Requires EGFR Tyrosine 1068



COS-7 cells were transiently transfected with a STAT3-responsive luciferase plasmid and the indicated expression vectors and a plasmid expressing β-galactosidase. Cell lysates were prepared and luciferase and β-galactosidase activity were determined. Luciferase values were normalized to β-galactosidase values. Each condition was performed in triplicate and normalized to cells containing only the reporter vectors. The positions of specific tyrosine point mutations are indicated by **F**.

Maximal STAT3 Activation Requires EGFRvIII Tyrosine 1068



COS-7 cells were transiently transfected with a STAT3-responsive luciferase plasmid, the indicated expression vectors and a plasmid expressing β-galactosidase. Cell lysates were prepared and luciferase and β-galactosidase activity were determined. Luciferase values were normalized to β-galactosidase values. Each condition was performed in triplicate and normalized to cells containing only the reporter vectors. The positions of specific tyrosine point mutations are indicated by F.

Conclusions

- Enforced expression of both EGFR and EGFRvIII activate STAT3 as judged by detection with phospho-specific antibodies.
- Enforced expression of both EGFR and EGFRvIII activate STAT3 transcriptional activity.
- Variant III Results in the activation STAT3 transcriptional activity to a greater magnitude than wild-type receptor.
- Variant III is autophosphorylated to a greater extent than wild-type when co-expressed with STAT3.
- Maximal STAT3 transcriptional activity by EGFRvIII requires STAT3 serine 727.
- Using MSD technology and a panel of EGFR point mutants, we identified tyrosine 1086 as the predominant STAT3 association site. The use of full-length receptor and full-length STAT3 strengthen the notion that this interaction is biologically relevant.