SUPPORTING INFORMATION Kim et al.

MATERIALS AND METHODS

Size Exclusion Chromatography for WT and L834R mutant EGFR

Size exclusion chromatography (Superdex 200) was used to examine the oligomeric state of WT and L834R MT EGFR. Flag-affinity purified EGFR was concentrated to ~25-80 μM and loaded onto Superdex 200 10/300 (GE Healthcare, Piscataway, NJ) using ACTA Purifier FPLC system (GE Healthcare, Piscataway, NJ). Column was equilibrated with a buffer containing 50mM TrisCl (pH 7.4), 150 mM $NaCl_2$, 0.01 % Tween-20. Flow-rate was 0.4 mL/min. Absorbance at 280 nm was monitored and elution peaks were analyzed by SDS-PAGE. Standard curve was generated using BSA and ovalbumin (BSA2: BSA dimer, BSA1: BSA monomer, Ova2: Ovalbumin dimer, Ova1: Ovalbumin monomer).

Expression and purification of his-tagged EGFR

To prepare his-tagged EGFR, DNA encoding residues 672-1186 of human EGFR was cloned into pFAST BAC HT (Invitrogen) which contains an N-terminal 6-His tag. Recombinant bacmid were transfected into *Sf9* cells to produce recombinant baculovirus which were used to infect *Sf9* cells for protein expression. The supernatant containing his-tagged EGFR was prepared as flag-tagged EGFR and loaded onto Ni-NTA-agorose resin (Quiagen). After washing with buffer containing 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 20 mM imidazole extensively, the protein was eluted in 500 mM imidazole. Superdex 200 10/300 was used as a final purification step as in Experimental Procedures.

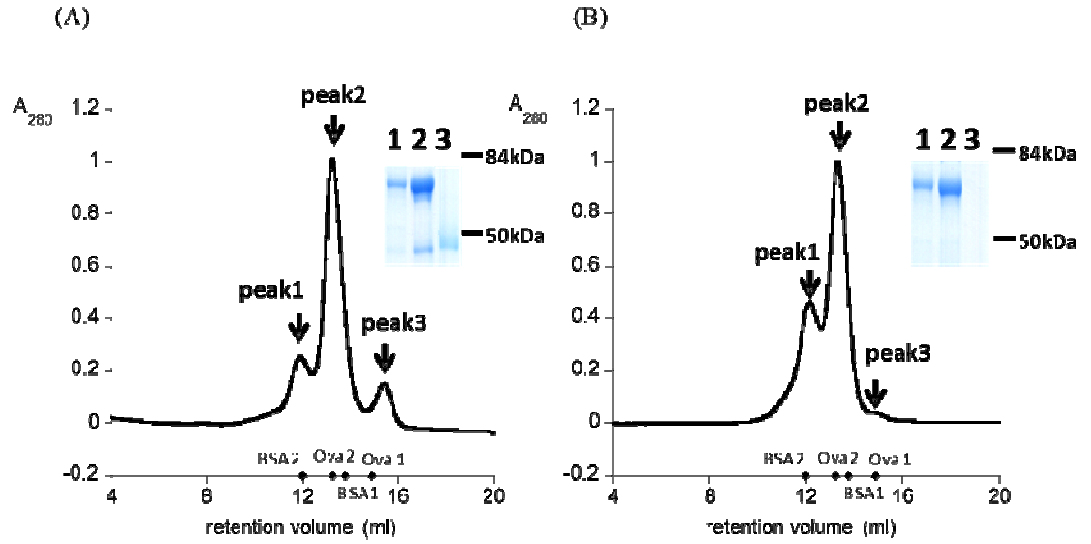
Preparation of small unilamellar vesicles and autophosphorylation assay of EGFR on vesicles

Homogeneous small unilamellar vesicles were prepared from DOPC and DOGS-NTA-Ni according to Zhang et al (3). His-tagged wild type EGFR was mixed with vesicles containing 5 mole percent of DOGS-NTA-Ni. The final concentration of EGFR and DOGS-NTA-Ni in the assay was 3.5 μM and 12.5 μM, respectively. EGFR on vesicle was used for radiolabeled autophosphorylation assay as described in Experimental Procedures in the paper and the activity was compared to the EGFR in solution. The same concentration of EGFR in the absence of vesicles was assayed as a control. Phosphorylated EGFR was quantified as in Experimental Procedures.

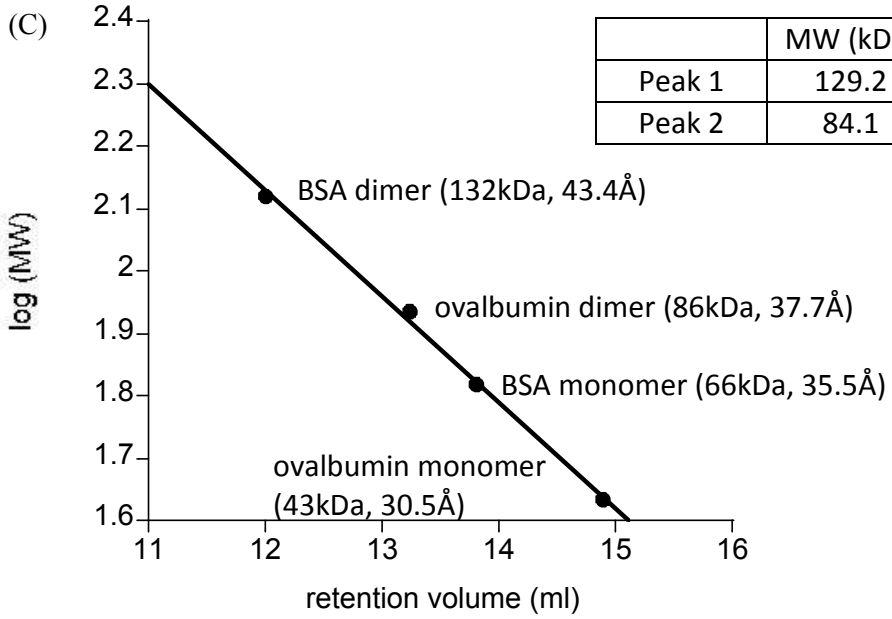
Identification of Tyrosine Phosphorylation Sites by ESI LC-MS/MS. Phosphorylated EGFR quenched at different reaction times was subjected to trypsin and/or glutamic-C digestion according to manufacturer's protocol. We used a 5:1 ratio of enzyme to trypsin to ensure more than 90% proteolytic coverage. The reaction was performed in 100 mM NH_4HCO_3, pH 7.8 overnight at 37 ºC. The digested peptides were separated on a reverse phase column (Aquasil C18 column (100x4.6 mm, 3μm), Thermo Scientific, Waltham, MA) using a Shimadzu HPLC (Shimadzu, Norwell, MA) coupled to a Finnigan LCQ DECA ESI-ion trap mass spectrometer (Thermo Scientific, Waltham, MA). A 60 min

linear gradient was used (Solvent A: 0.1% Formic acid, 0.01% TFA in water; Solvent B: 0.1% Formic acid, 0.01% TFA in 50% ACN). The elution was performed with a flow rate of 0.2 ml/min. The peptides eluted from the column were detected and fragmented in an automated fashion using data dependent analysis in the positive ion mode. The spray voltage was kept at 5 kV and the capillary temperature at 250 ºC. The sheath gas (nitrogen) was set at 60 units. Peptide identification was done using the software Bioworks 3.1 (Thermo Scientific, Waltham, MA). Peptides with Xcorr value of over 1.0 were confirmed as true signal. Representative spectra for peptide LC/MS identification and MS/MS fragmentation to determine peptide sequence and site of tyrosine phosphorylation are illustrated in Figure S4A-H.

Figure S1. Size Exclusion Chromatography for (A) WT and (B) L834R MT EGFR. (C) Standard curve using BSA and Ovalbumin (BSA2: BSA dimer, BSA1: BSA monomer, Ova2: Ovalbumin dimer, Ova1: Ovalbumin monomer). (D) Data from SEC-LS.



(C)



	MW (kDa)	Stokes radius (Å)
Peak 1	129.2	43.2
Peak 2	84.1	38.0

(D) Data from SEC-LS

WT	Retention volume (ml)	MW(kDa) calculated by ASTRA	MW for monomer (kDa)
Peak 1	12.1	135	60
Peak 2	13.2	66.2	60
Peak 3	14.9	36.5	-

L834R	Retention volume (ml)	MW(kDa) calculated by ASTRA	MW for monomer (kDa)
Peak 1	12.1	130	60
Peak 2	13.3	68.7	60

Figure S2. Concentration dependency of WT (A) and L834R (B) EGFR. Wild type or L834R EGFR was autophosphorylated in the presence of γ-^{32}P-ATP and $MnCl_2$. The incorporation of radiolabeled ATP to EGFR was quantified using Molecular Imager FX and plotted against increasing times. Data were fitted to equation 1 (Materials and Methods) to determine the rate of autophosphorylation.

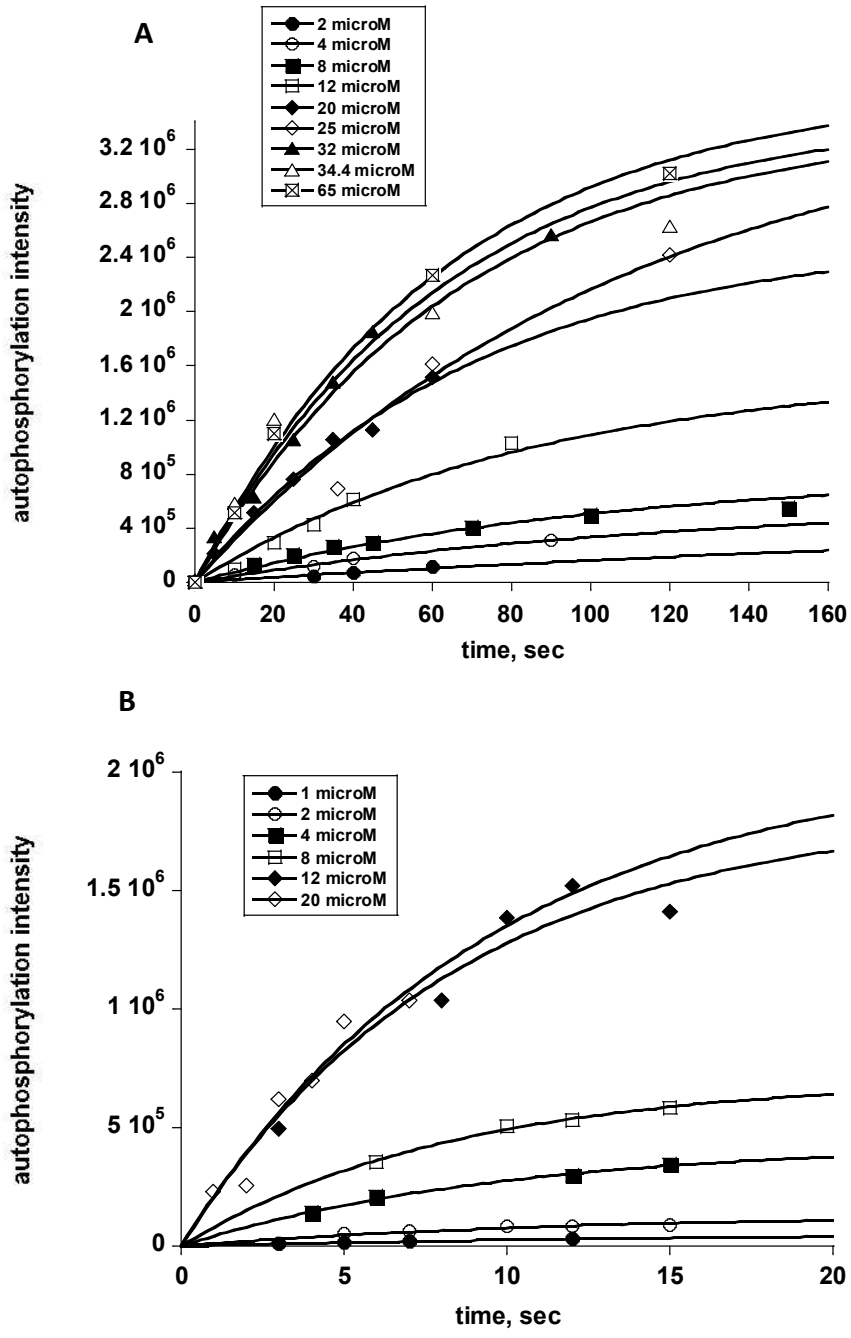


Figure S3. Rate of WT EGFR autophosphorylation on vesicles. Wild type EGFR was phosphorylated on vesicle in the presence of γ-^{32}P-ATP and $MnCl_2$. Final concentration of EGFR and DOGS-NTA-Ni were 3.5 μM and 12.5μM, respectively. The incorporation of radiolabeled ATP to EGFR was quantified using Molecular Imager FX and plotted against increasing times. Data were fitted to equation 1 (Materials and Methods) to determine the rate of autophosphorylation.

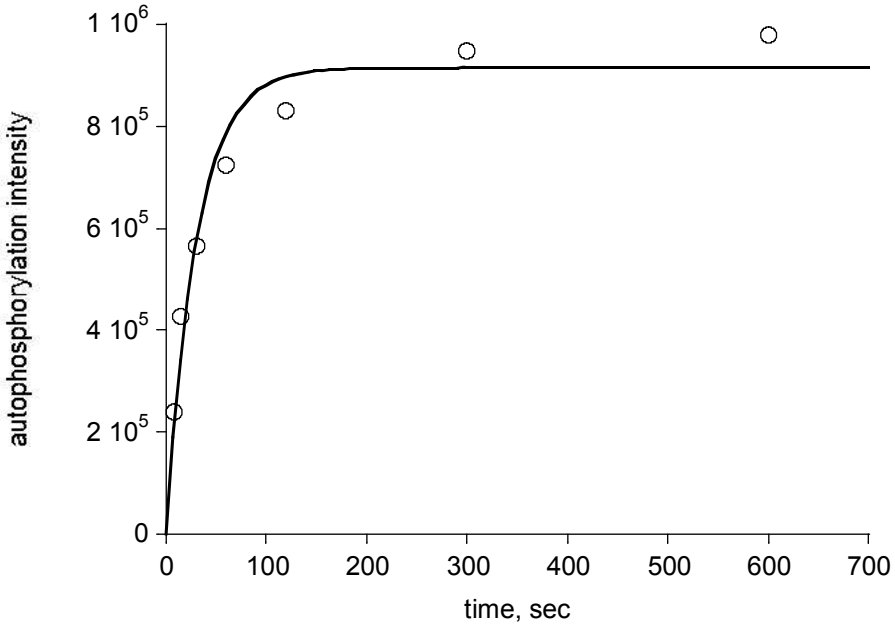


Fig S4A-H. Identification of Tyrosine Phosphorylation Sites by ESI LC-MS/MS

Fig. S4A.

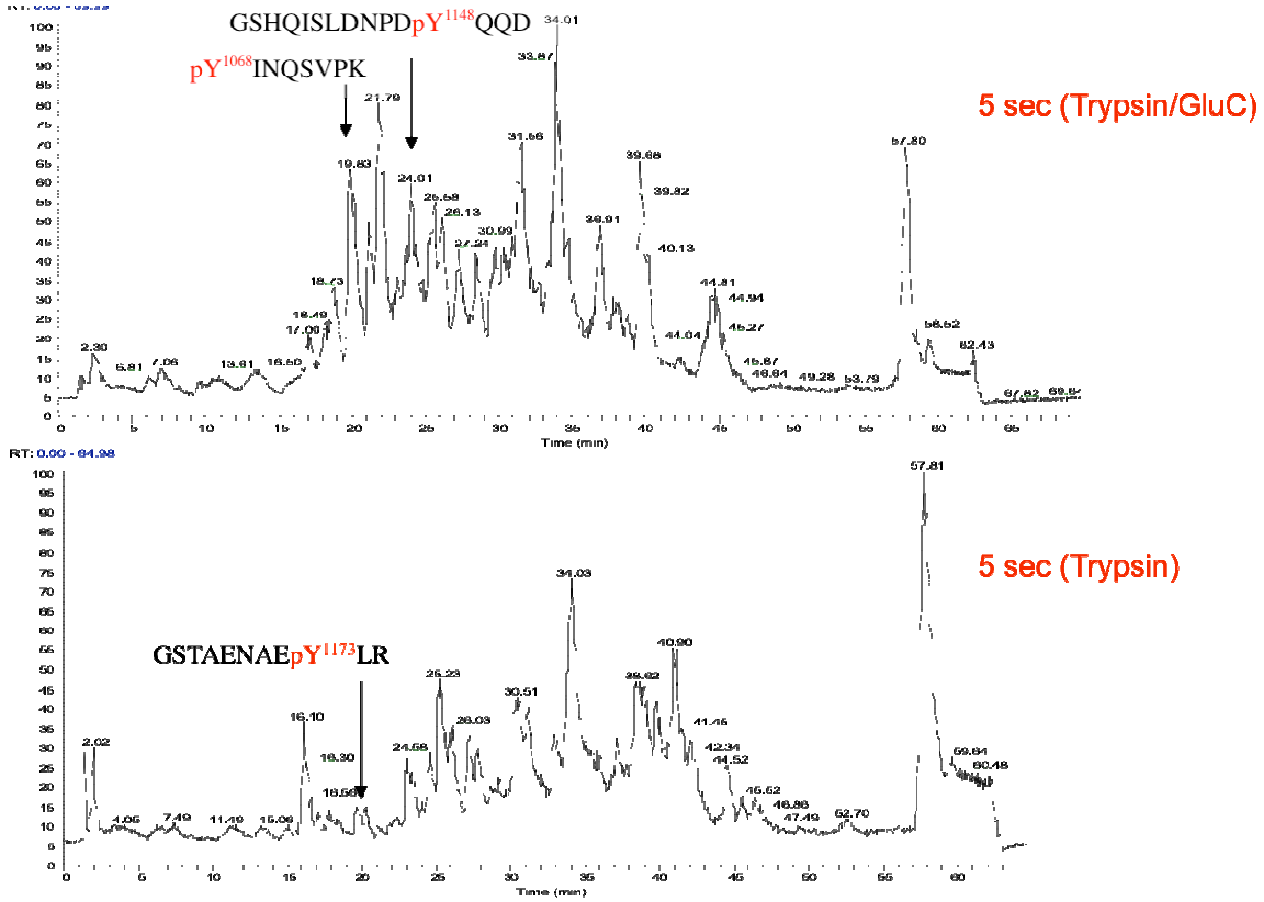


Fig. S4B.

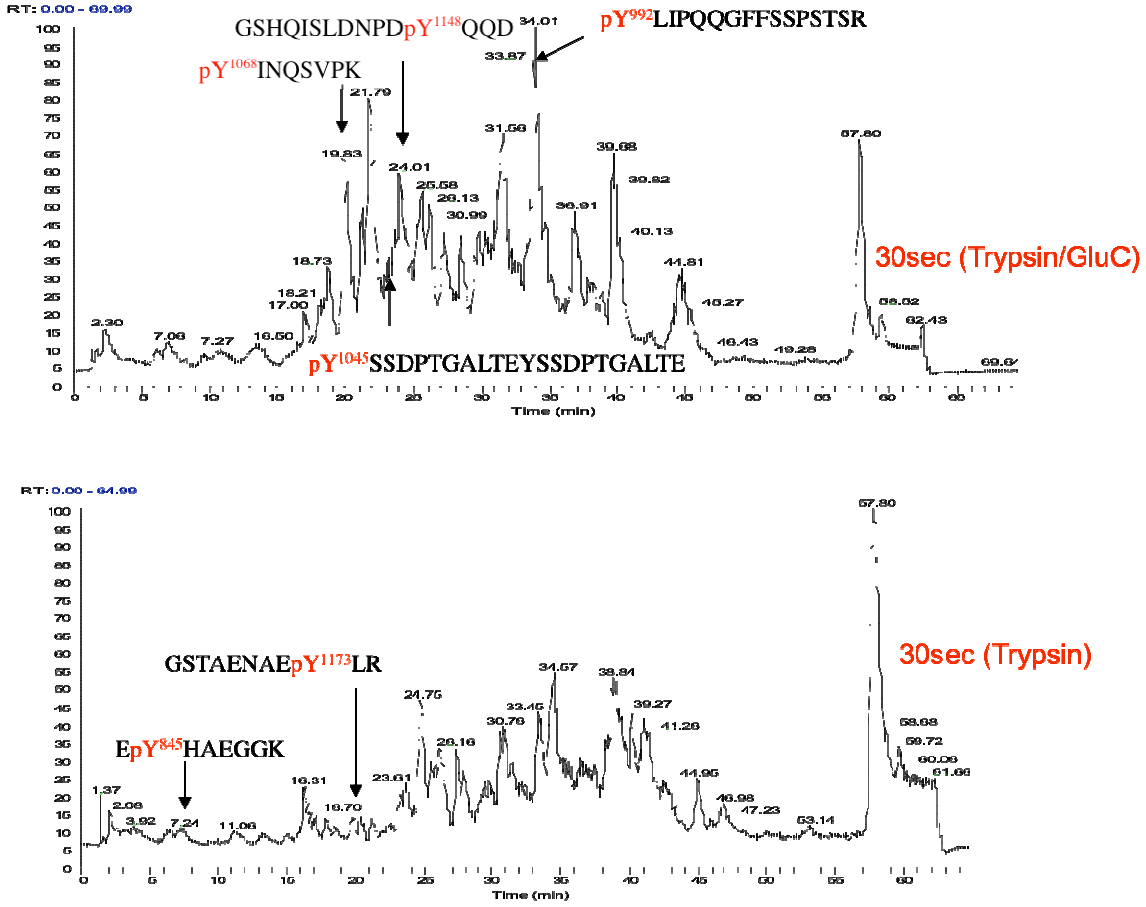

RT: 0.00 - 69.99
GSHQISLDNPDpY1148QQD
pY992LIPQQGFFSSPSTSR
pY1068INQSVPK
pY1045SSDPTGALTEYSSDPTGALTE
30sec (Trypsin/GluC)
Time (min)
RT: 0.00 - 64.99
GSTAENAEpY1173LR
EpY845HAEGGK
30sec (Trypsin)
Time (min)

Fig. S4C.

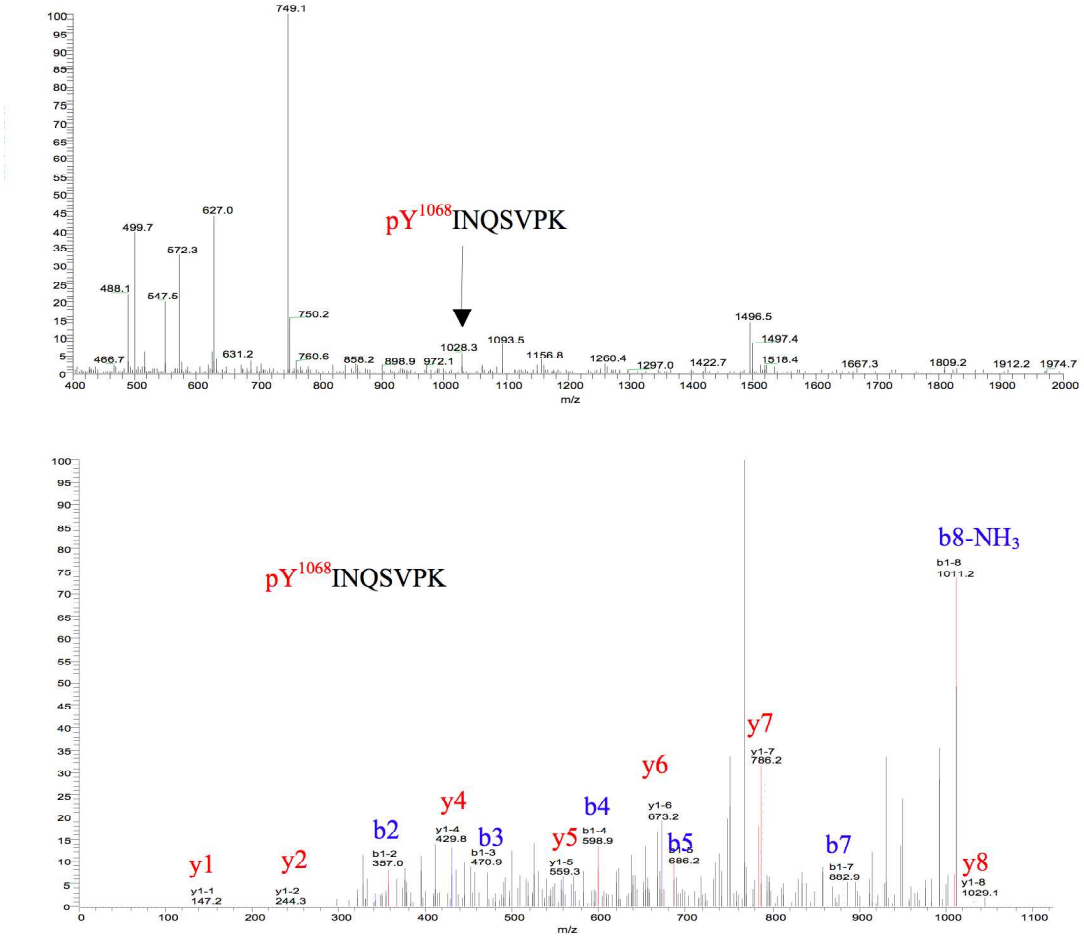

pY1068INQSVPK
749.1
627.0
499.7
572.3
488.1
647.6
750.2
631.2
1028.3
1496.5
1497.4
pY1068INQSVPK
b8-NH3
b1-8
1011.2
y7
y1-7
786.2
y6
y1-6
073.2
y4
y1-4
429.8
b2
b1-2
357.0
b3
b1-3
470.9
b4
b1-4
598.9
y5
y1-5
559.3
b5
686.2
b7
b1-7
902.9
y1
y1-1
147.2
y2
y1-2
244.3
y8
y1-8
1029.1
m/z

Fig. S4D.

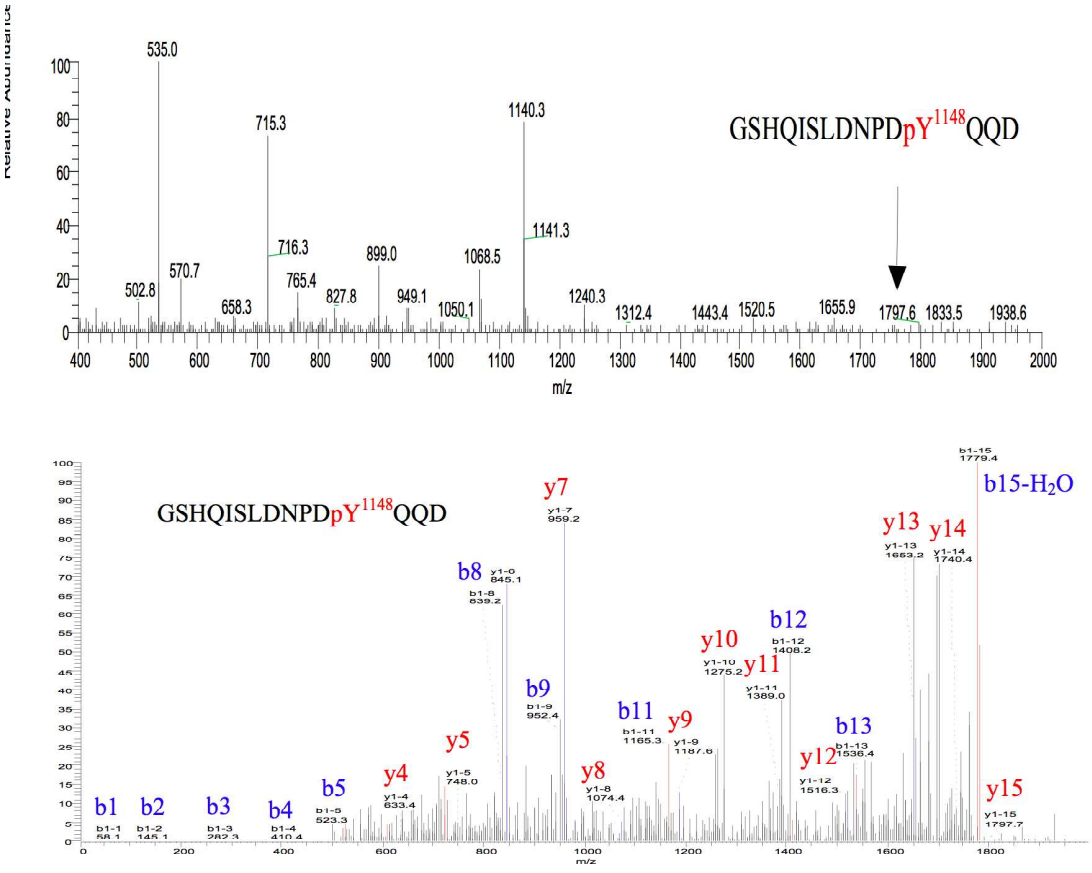

Relative Abundance
GSHQISLDNPDpY1148QQD
535.0
715.3
1140.3
1141.3
716.3
899.0
1068.5
570.7
765.4
502.8
658.3
827.8
949.1
1050.1
1240.3
1312.4
1443.4
1520.5
1655.9
1797.6
1833.5
1938.6
m/z
GSHQISLDNPDpY1148QQD
y7
b8
b9
b11
y9
y10
b12
y11
y13
y14
b15-H2O
b13
y12
y15
y5
y4
y8
b5
b1
b2
b3
b4
m/z

Fig. S4E.

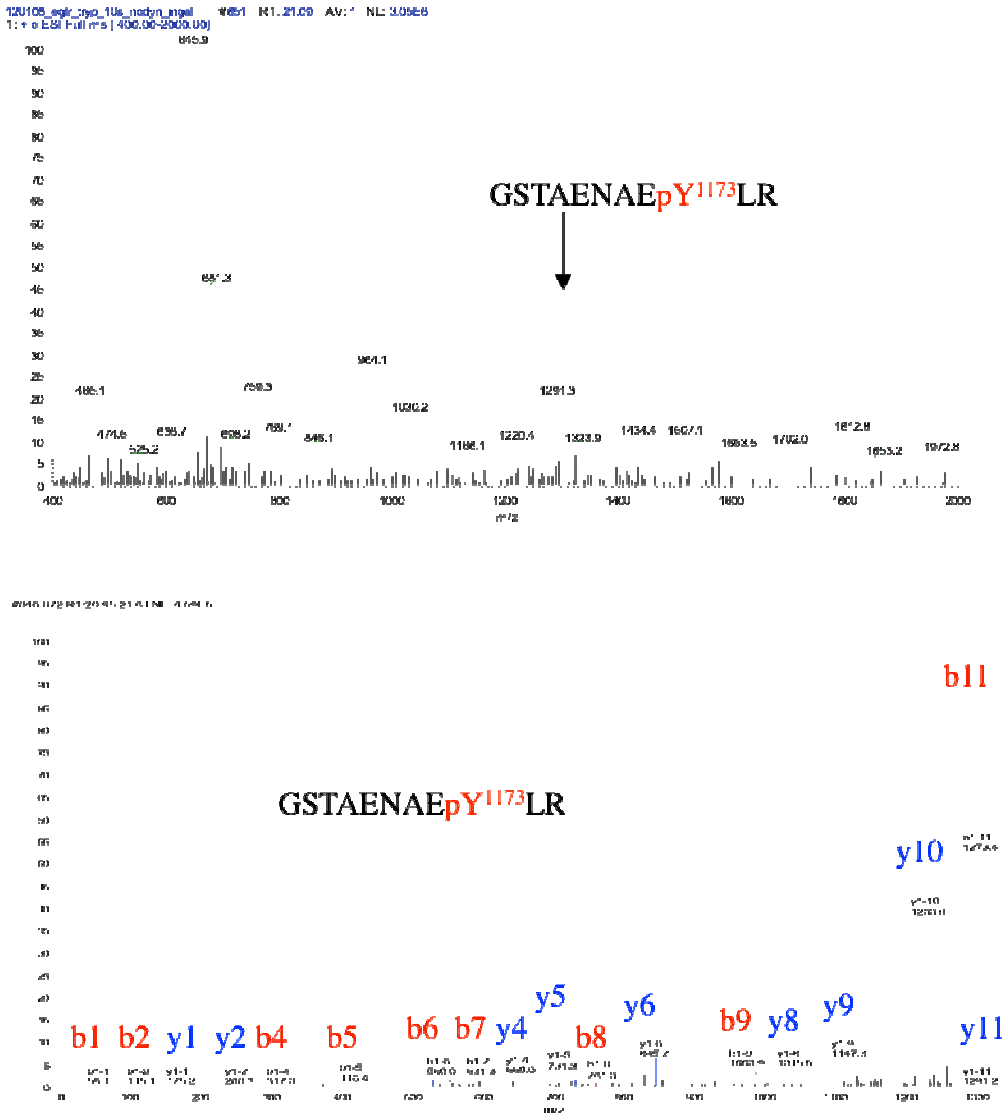

GSTAENAEpY1173LR
b11
y10
b1 b2 y1 y2 b4 b5 b6 b7 y4 y5 b8 y6 b9 y8 y9 y11

Fig. S4F.

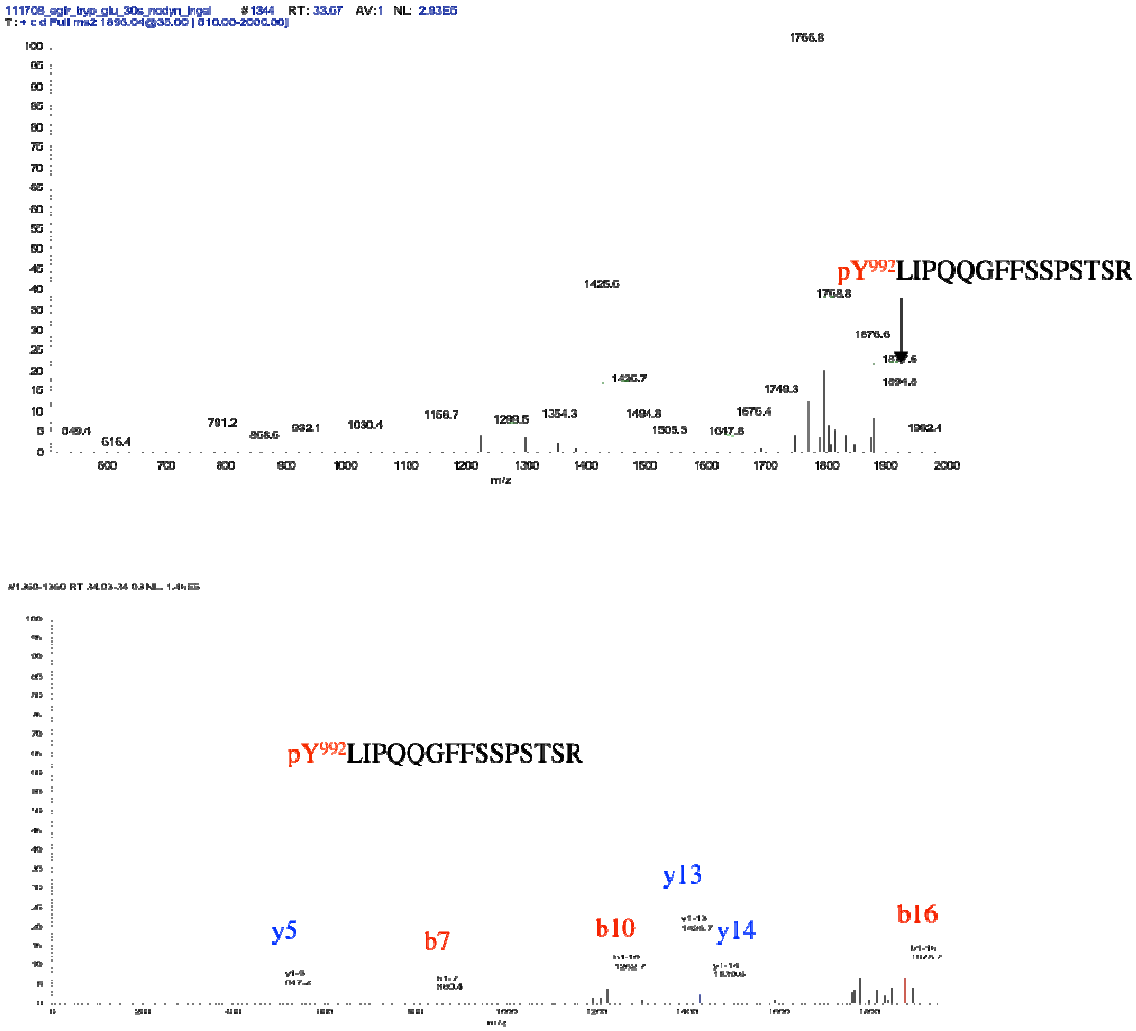
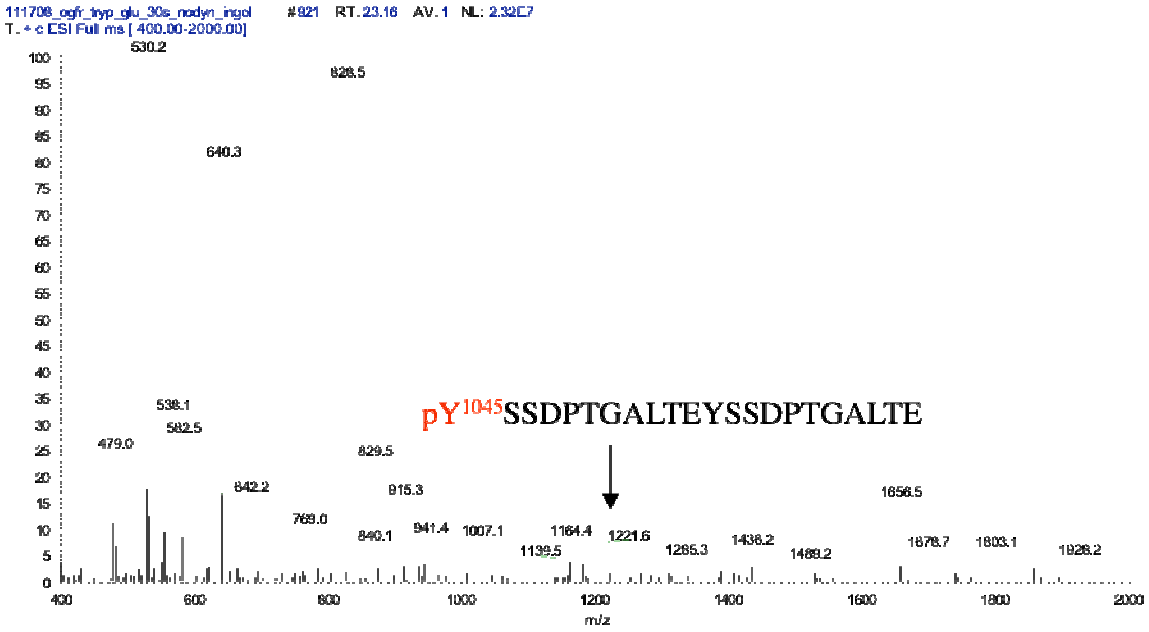

111708_sglr_tryp_glu_30s_modyn_lrgal #1344 RT: 33.67 AV: 1 NL: 2.93E6
pY992LIPQQGFFSSPSTSR
pY992LIPQQGFFSSPSTSR
y13
y5
b7
b10
y14
b16
m/z

Fig. S4G.


111708_egfr_tryp_glu_30s_nodyn_ingol #921 RT: 23.16 AV: 1 NL: 2.32E7
T: + c ESI Full ms [400.00-2000.00]
pY1045SSDPTGALTEYSSDPTGALTE
m/z

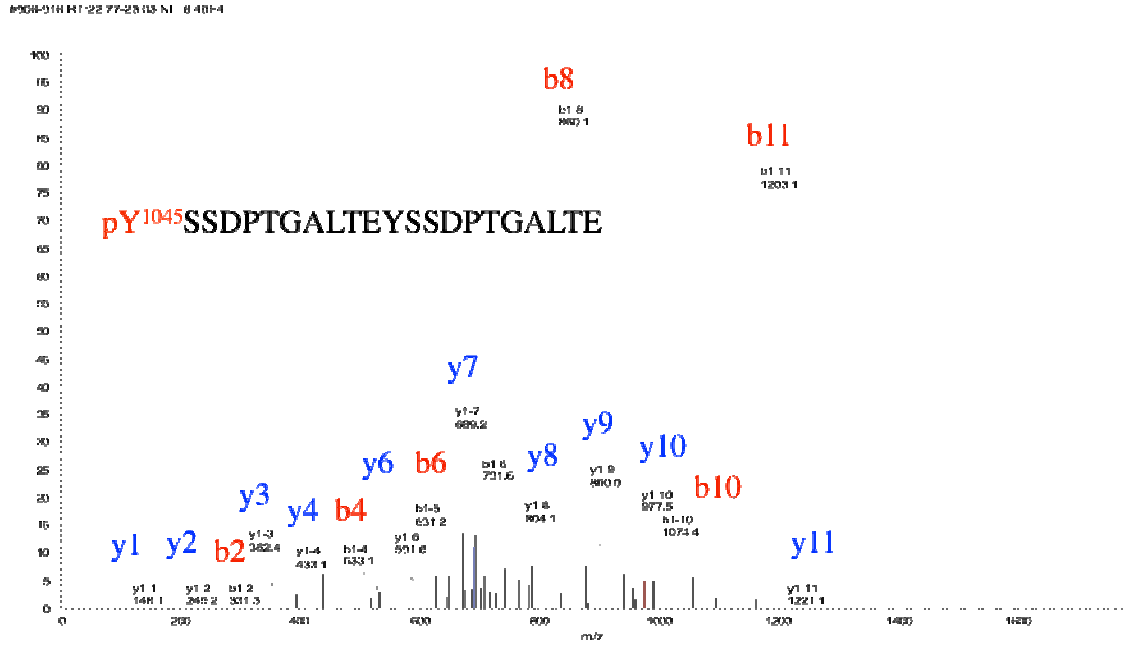
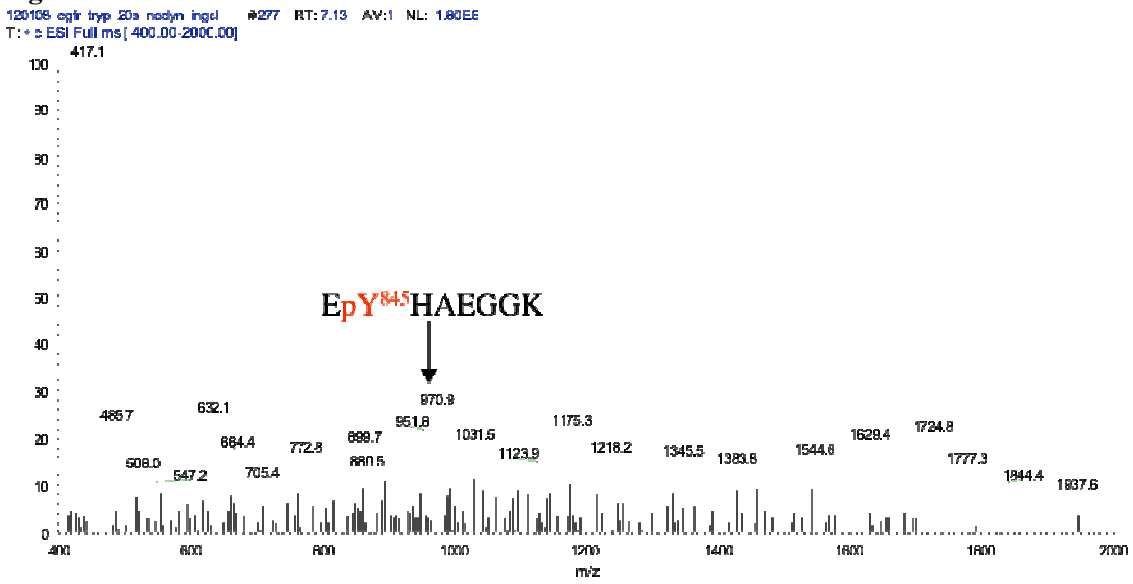

pY1045SSDPTGALTEYSSDPTGALTE
b8
b11
y7
y9
y10
y6
b6
y8
b10
y3
y4
b4
y1
y2
b2
y11
m/z

Fig. S4H.


120108 cgjr tryp 20a nodyn ingcl #277 RT: 7.13 AV:1 NL: 1.80E6
T: + c ESI Full ms [400.00-2000.00]
417.1
EpY845HAEGGK
970.9
951.8
1031.5
1175.3
1123.9
1218.2
1345.5
1383.8
1544.6
1628.4
1724.8
1777.3
1844.4
1937.6
632.1
664.4
772.8
699.7
880.5
705.4
547.2
508.0
485.7
m/z

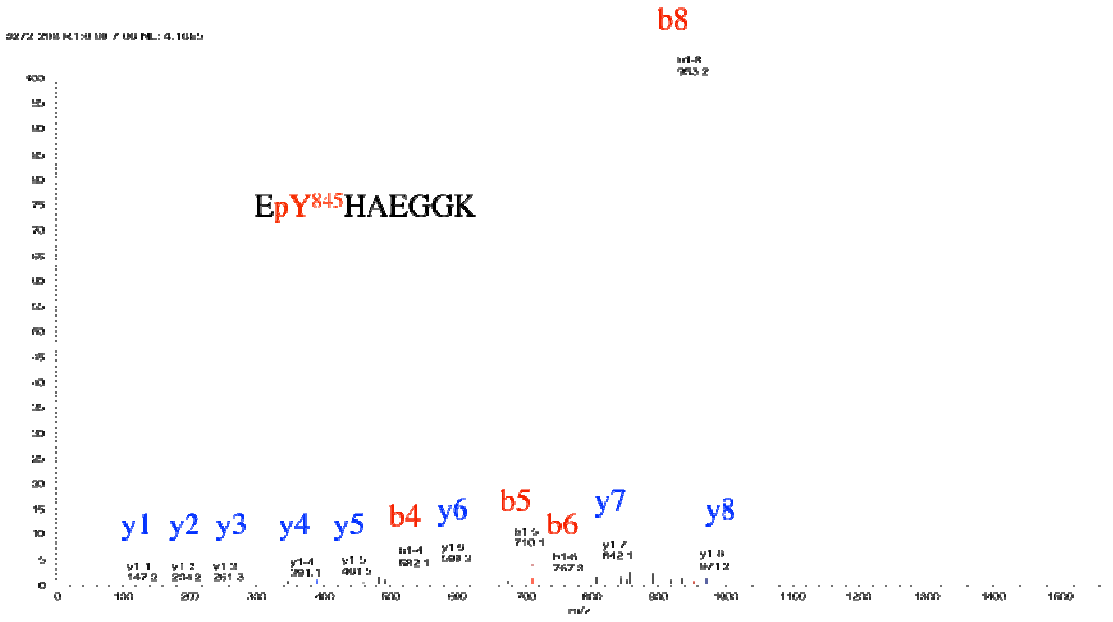

b8
EpY845HAEGGK
y1
y2
y3
y4
y5
b4
y6
b5
b6
y7
y8

Figure S5 A-B. Gefitinib effect on autophosphorylation of wild type (A) and L834R (B) EGFR. Autophosphorylation reaction of wild type and L834R EGFR was performed in the presence of increasing gefitinib concentration. Twenty five μM wild type and L834R EGFR was incubated with increasing concentration of gefitinib for 5 min at 25 °C and was phosphorylated in the presence of 0.5 mM ATP and 10 mM $MnCl_2$ for 2 min. Phosphorylation of EGFR were analyzed by SDS-PAGE and probed with phosphospecific antibodies.