Mol Biol Rep (2009) 36:631–639
DOI 10.1007/s11033-008-9223-6

Epidermal growth factor receptors: function modulation by phosphorylation and glycosylation interplay

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Received: 31 December 2007 / Accepted: 4 March 2008 / Published online: 14 March 2008

Abstract Post-translational modifications (PTMs) of proteins induce structural and functional changes that are most often transitory and difficult to follow and investigate in vivo. In silico prediction procedures for PTMs are very valuable to foresee and define such transitory changes responsible for the multifunctionality of proteins. Epidermal growth factor receptor (EGFR) is such a multifunctional transmembrane protein with intrinsic tyrosine kinase activity that is regulated primarily by ligand-stimulated transphosphorylation of dimerized receptors. In human EGFR, potential phosphorylation sites on Ser, Thr and Tyr residues including five autophosphorylation sites on Tyr were investigated using in silico procedures. In addition to phosphorylation, *O*-GlcNAc modifications and interplay between these two modifications was also predicted. The interplay of phosphorylation and *O*-GlcNAc modification on same or neighboring Ser/Thr residues is termed as Yin Yang hypothesis and the interplay sites are named as Yin Yang sites. Amongst these modification sites, one residue is localized in the juxtamembrane (Thr 654) and two are found in the catalytic domain (Ser 1046/1047) of the EGFR. We propose that, when EGFR is *O*-GlcNAc modified on Thr 654, EGFR may be transferred from early to late endosomes, whereas when EGFR is *O*-GlcNAc modified on Ser 1046/1047 desensitization of the receptor may be prevented. These findings suggest a complex interplay between phosphorylation and *O*-GlcNAc modification resulting in modulation of EGFR's functionality.

Keywords Epidermal growth factor receptor · Phosphorylation · *O*-GlcNAc modification · Lysosomal targeting · Desensitization

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Introduction

Different PTMs, such as phosphorylation and *O*-GlcNAc modification are major regulators of protein function. When receptor tyrosine kinases (RTKs) become active, they phosphorylate a series of cytoplasmic substrate proteins that in turn become activated. Receptor kinases are activated by physiological receptor agonists and overexpression or mutations causing constitutive activation of the receptor can lead to uncontrolled RTK signaling and

subsequent development and progression of certain types of cancers [1, 2].

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that regulates fundamental processes of cell growth and differentiation [3]. EGFR is a transmembrane protein that binds to its cognate ligand such as epidermal growth factor (EGF) or transforming growth factor-α (TGF-α). Consequently, the EGFR undergoes dimerization that stimulates its intrinsic Tyrosine kinase activity in its cytoplasmic tail [3]. Activation of RTK results in autophosphorylation of EGFR's *C*-terminal, which contains 3 major autophosphorylation sites (Tyr 1068, 1148 and 1173) [4] and 2 minor autophosphorylation sites (Tyr 992 [5] and Tyr 1086 [6]). Autophosphorylation of the EGFR provides docking sites for signal/adaptor proteins, like phospholipase Cγ, growth factor receptor binding protein 2 (Grb2) and the adaptor protein Shc [3]. Binding of these signal/adaptor proteins to the EGFR starts the signal transduction cascade, which eventually leads to activation of transcription in the nucleus [3].

Cellular activation via the EGFR increases cytosolic calcium [7]. This rise in calcium inhibits EGFR's intrinsic RTK activity. The activation of the calcium/calmodulin dependent Ser/Thr protein kinase-II (CaM-II) inhibits RTK by phosphorylating EGFR on Ser 1046/1047 in EGFR's catalytic domain [7]. Phosphorylation of EGFR on Ser 1046/1047 by CaM-II leads to desensitization of EGFR, lowering the affinity of the receptor for its ligand, in EGF simulated cells [8], and contributes to transactivation of the EGFR [7]. The protein calmodulin (CaM) binds to EGFR in the juxtamembrane portion and inhibits its RTK activity [7]. When CaM binds to EGFR, it prevents protein kinase C (PKC) from phosphorylating EGFR [9, 10]. The phosphorylated EGFR on Thr 654 by PKC in the juxtamembrane domain promotes recycling of endocytosed receptors back to the cellular surface, thereby maintaining EGFR signaling in cells [11], whereas, the EGFR bound to CaM is prevented from recycling after endocytosis [9, 10]. Furthermore, phosphorylation of EGFR by mitogen activated protein kinase (MAPK) and protein kinase A (PKA) transmodulates EGFR by decreasing the activity of its intrinsic Tyrosine kinase [12–14]. MAPK phosphorylates EGFR on Thr 669 [13, 15, 16] and is activated either through the Ras/MAPK signaling pathway [3] or through transactivation of the EGFR by PKC [17]. It thus appears that several kinases work together to ensure non-constitutive signaling by EGFR.

An equally important and dynamic PTM, the *O*-GlcNAc modification, is detectable in nearly all higher eukaryotic organisms [18]. It is an ubiquitous modification that is regulated by *O*-GlcNAc transferase (OGT) (adds *O*-GlcNAc to protein backbone) and *O*-GlcNAcase (OGN) (removes *O*-GlcNAc from protein backbone). Compared to other *O*-linked glycosylation processes such as *O*-GalNAc modification, *O*-GlcNAc modification involves a single sugar residue modification. Complex interplay between phosphorylation and *O*-GlcNAc modification on the same or neighboring residues, the yin-yang sites, has been observed in several nuclear and cytoplasmic proteins [19], and evidence of *O*-GlcNAc modification in EGFR type III has been experimentally verified [20]. This alternative modification of Ser/Thr residues by *O*-GlcNAc and phosphate often results in functional switches of a protein.

Computer-assisted methods are useful in assessing the modification potential of a given protein [21]. Prediction of modification and experimental authentification is only possible where the half-life and availability of protein allows it. The computer assisted procedures are necessary where condition of limited availability and instability of proteins prevail for transitorily-modified proteins to perform diverse functions. Computational procedures have played an important role in understanding genomics, proteomics and defining the contribution of phosphorylation, sulfation and glycosylation in various contexts of functional protein regulation. Programs based on artificial neural networks have been applied to predict glycosylation and phosphorylation sites in proteins with reliable accuracy [21]. The interplay between phosphorylation and *O*-GlcNAc modification results in temporary structure–function changes. Assessing these temporary structural changes induced by modifications, in vivo, is not possible with the existing methodology. Protein functional information can be deduced from their 3-D structures. However, determination of the 3-D structure in vivo is difficult because of constant intra- and inter-molecular interactions occurring between proteins in body fluids or in the cell. Most of the structural data available in protein databases is only partially relevant to the dynamic behavior of proteins in vivo. Defining protein functions in physiological environments remains a daunting task due to the presence of innumerable other molecules that constantly interact with the test protein.

Evolutionarily-conserved residues have been found to be functionally important [22]. Interplay of phosphorylation and *O*-GlcNAc modification on conserved Ser/Thr residues in histone H3 [23] and other proteins [24, 25] has been documented.

In this study we have identified Yin Yang sites in human EGFR utilizing in silico procedures. We propose that an interplay between phosphorylation and *O*-GlcNAc modification on Ser and Thr residues in the juxtamembrane and in the *C*-terminal region of the EGFR occurs, suggesting an important role of OGT in the functional regulation of human EGFR.

Materials and methods

Sequence data

The sequence data, for prediction of phosphorylation and *O*-GlcNAc modification sites in EGFR (*Homo sapiens*), was retrieved from Swiss-Prot sequence database, with the entry name EGFR_HUMAN and primary accession no. P00533 [26]. BLAST search was performed using the NCBI database for all known organisms' sequences [27]. A total of 1561 hits were obtained and the sequences with the highest bits score and zero expect values were selected to locate conserved Ser/Thr and Tyr residues of the EGFR. The chosen sequences, including that of human EGFR, were multiply aligned using ClustalW [28], a multiple sequence alignment program for DNA or proteins. The five sequences selected were from *Mus musculus* (RefSeq. AAG24386.1), *Rattus norvegicus* (RefSeq. NP_113695.1), *Sus scrofa* (RefSeq. NP_999172.1), *Danio rerio* (RefSeq. NP_919405.1) and *Xiphophorus xiphidium* (RefSeq. AAP55673.1).

PTMs prediction methods

Artificial neural network based prediction methods are generally used in biological sequence analysis of proteins. Neural networks are composed of a large number of highly interconnected processing elements (simulated neurones) that work in parallel to solve a complex problem. These networks are trained by sequence patterns of modified and non-modified proteins so that they become able to recognize and predict a pattern in a new protein for their potential of modification. Artificial neural networks receive many inputs and give one output as a result. NetPhos 2.0 [29] was developed by training the neural networks with phosphorylation data from Phosphobase 2.0 [30]. A threshold value of 0.5 is used by Netphos 2.0 to determine possible potential for phosphorylation. Netphos 2.0 (http://www.cbs.dtu.dk/services/Netphos/) was utilized to predict potential phosphorylation sites in human EGFR.

YinOYang 1.2 employs the sequence data to train a jury of neural networks on 40 experimentally determined *O*-GlcNAc acceptor sites for recognizing the sequence context and surface accessibility. The method has the capability to predict the sites known as Yin Yang sites that can be *O*-GlcNAc modified and alternatively phosphorylated. The threshold value used by YinOYang 1.2 varies depending upon surface accessibility of the different amino acid residues. To predict potential of *O*-GlcNAc modification and Yin Yang sites in human EGFR YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) (unpublished) was used.

NetphosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) [31] was used to predict the potential of kinases most likely to phosphorylate Ser, Thr and Tyr in human EGFR.

Comparison of human OGT's sequence with EGFR's autophosphorylation sites

The comparison of the sequence environment in human OGT with the sequence environment of EGFR's autophosphorylation sites was performed. OGT's sequence was retrieved from the Swiss-Prot sequence database [26] with the entry name OGT1_HUMAN and primary accession no. O15294. The protein sequence of OGT in rat was aligned with that of human OGT to determine the position of Tyr 979 of rat OGT to human OGT.

Results

Phosphorylation and *O*-GlcNAc modification in human EGFR

Prediction results of human EGFR by Netphos 2.0 of Ser, Thr and Tyr are given in Fig. 1a and in Table 1. These results show a high phosphorylation potential in human EGFR. Prediction of possible *O*-GlcNAc modification in human EGFR by YinOYang 1.2 is given in Table 1 and in Fig. 1b.

Yin Yang sites in EGFR

The prediction results for Yin Yang sites in EGFR by YinOYang 1.2 are illustrated in Fig. 1c and given in Table 2. In several cases Ser and Thr residues show a very high potential for either *O*-GlcNAc modification or phosphorylation or show a potential very close to the specific threshold value predicted by the existing methods. When a conserved residue shows a very high potential for phosphorylation and a potential for *O*-GlcNAc modification very close to the threshold value it appears to be a false negative Yin Yang site as on these sites OGT and kinases may have an equal accessibility to modify a specific site. The in silico predicted Yin Yang sites, Thr 1005 and Ser 1006, and the false negative Yin Yang site, Thr 654, were fully conserved in mammals and in pisces (Fig. 2). The other predicted Yin Yang sites, Ser 1046/1047 and Ser 1180, were fully conserved in mammals (Fig. 2).

Autophosphorylation of the EGFR

All potential predicted Tyr autophosphorylation sites (Table 3) were found to be conserved in mammals and pisces, except Tyr 1086 which has been mutated to Gly in pisces (Fig. 2). The predicted autophosphorylated Tyr

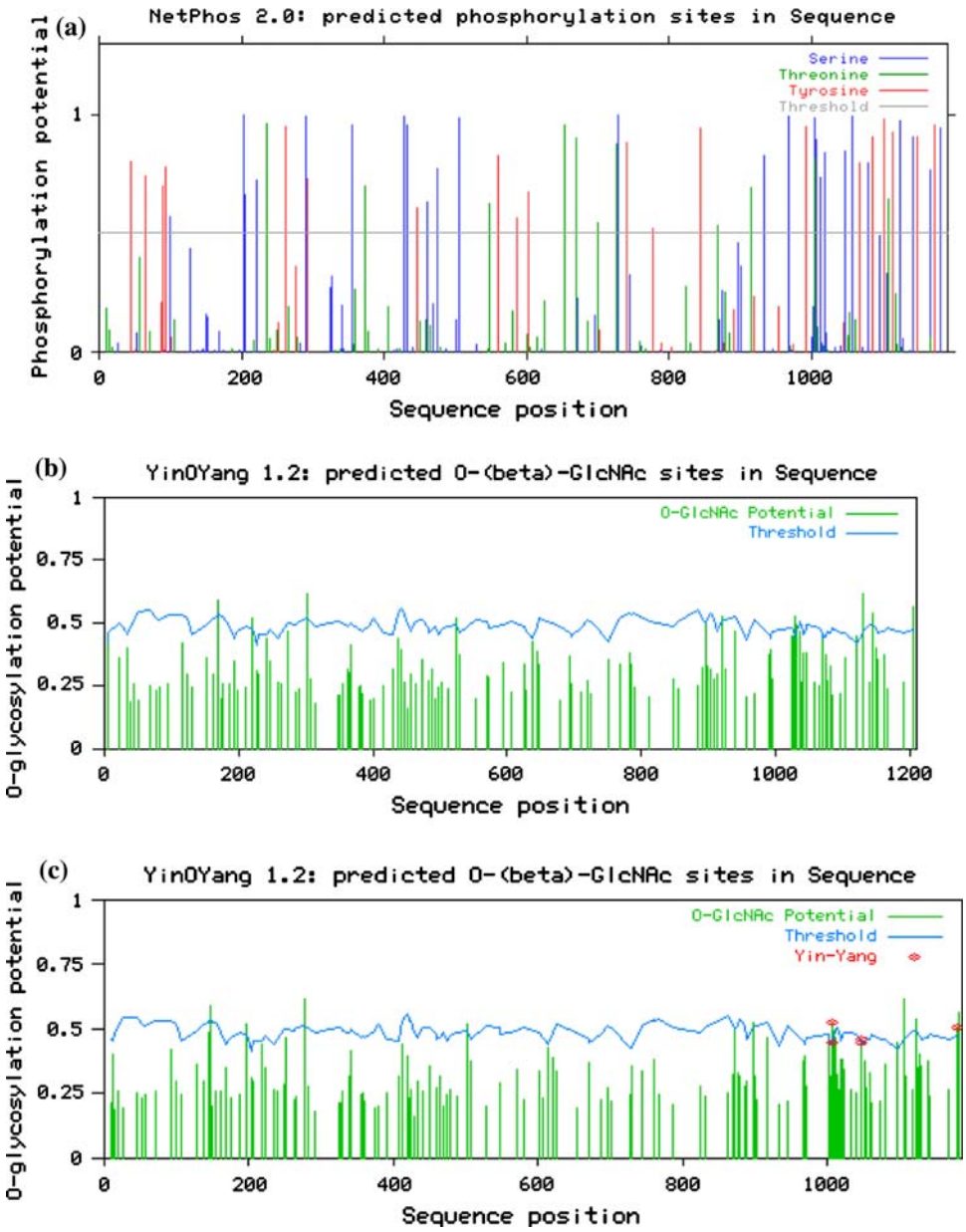

Fig. 1 (**a**) Graphic presentation of the potential phosphate modification on Ser and Thr residues in human EGFR. The blue vertical lines show the potential phosphorylated Ser residues; the green lines show the potential phosphorylated Thr residues; the red line show the potential phosphorylated Tyr residues. The light grey horizontal line shows threshold for modification potential. (**b**) Graphic representation of potential for *O*-GlcNAc modification of Ser and Thr residues in human EGFR. The green vertical lines show the *O*-GlcNAc potential of Ser/Thr residue and light blue horizontal wavy line shows threshold for modification potential. (**c**) Graphic representation of potential Yin Yang sites in human EGFR. The green vertical lines show the *O*-GlcNAc potential of Ser/Thr residue and light blue horizontal wavy line shows threshold for modification potential. The red asterisk shows the potential Yin Yang sites in human EGFR

Table 1 In silico predicted phosphorylation- and *O*-GlcNAc modification sites in human EGFR

Predicted phosphorylation sites	Ser 99, 203, 205, 222, 262, 291, 356, 428, 433, 460, 474, 506, 728, 744, 933, 967, 1002, 1004, 1006, 1013, 1018, 1046, 1047, 1057, 1080, 1125, 1142, 1166, 1180
	Thr 235, 373, 548, 654, 669, 701, 727, 868, 916, 1005, 1107
	Tyr 45, 64, 89, 93, 261, 292, 447, 561, 586, 602, 740, 777, 845, 992, 1068, 1086, 1101, 1114, 1148, 1173
Predicted glycosylation sites	Ser 146, 196, 501, 897, 1006, 1046, 1047, 1096, 1106, 1180, 1181
	Thr 278, 1005, 1008, 1121

residues (Tyr 992, 1068, 1086, 1148 and 1173) are located in the cytoplasmic tail of EGFR, which is downstream the RTK domain, and is in the catalytic domain of the EGFR. The results of NetphosK 1.0, for predicting the potential of kinases involved in the EGFR's autophosphorylation sites, show that EGFR is the only potential kinase to modify these sites (Table 3, Fig. 3).

Table 2 In silico predicted Yin Yang and false-negative sites in human EGFR

False-negative site	Thr 654
Yin Yang sites	Thr 1005
	Ser 1006
	Ser 1046
	Ser 1047
	Ser 1180

Comparison of OGT's sequence with EGFR's autophosphorylation sites

When the sequence of OGT in rat was aligned with the sequence of OGT in human, it was found that Tyr 979 in rat OGT corresponds to Tyr 989 in human OGT. The sequence motif of the predicted autophosphorylation sites in EGFR was compared with the sequence motif of Tyr 989 in human of OGT. These results showed that three out of five EGFR autophosphorylation sites have a similar sequence to that of OGT around Tyr 989 (Fig. 4).

Table 3 In silico predicted autophosphorylation Tyr sites in human EGFR

Residue	NetphosK 1.0 prediction
992, 1068, 1086, 1148, 1173	EGFR

Discussion

Phosphorylation of EGFR is the primary mechanism in the regulation of its RTK activity, and contributes significantly to the process of the signal transduction. A complex interplay between phosphorylation and *O*-GlcNAc modification leads to functional changes of the EGFR that either diverts EGFR toward degradation or altogether prevents it.

Autophosphorylation sites of the human EGFR were predicted to be Tyr 992, 1068, 1086, 1148 and 1173 (Table 3), which is in agreement with experimentally verified data [4–6]. These sites were also correctly

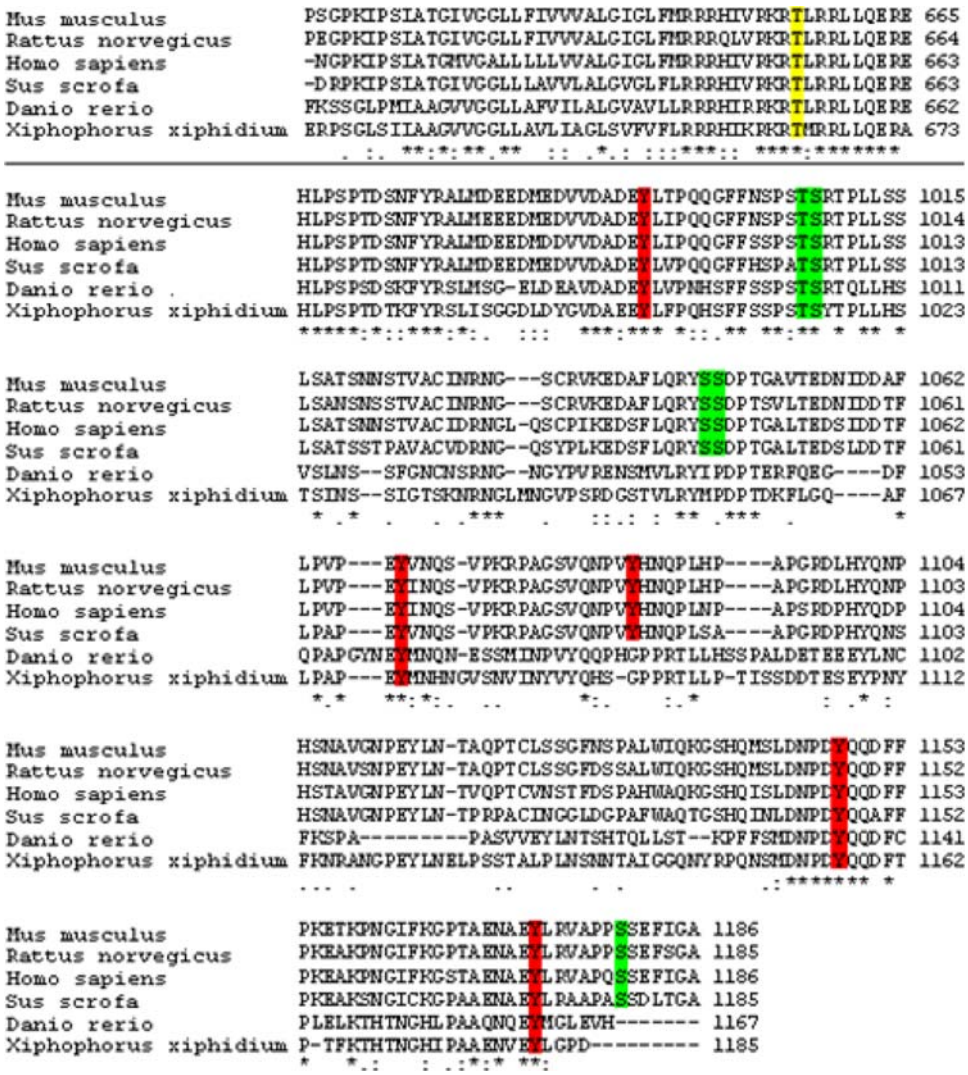

Fig. 2 Multiple alignments of EGFR sequences of four mammals (*Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Sus scrofa*) and two pisces (*Danio rerio* and *Xiphophorus xiphidium*). The consensus sequence is marked by an asterisk, conserved substitution by a double dot, semiconserved substitution by a single dot and deletion gaps by a small single line. The different sequences are ordered as in aligned results from ClustalW. The positively predicted Yin Yang sites are highlighted in green, and the negatively predicted Yin Yang site is highlighted in yellow. Furthermore the predicted autophosphorylation sites are highlighted in red

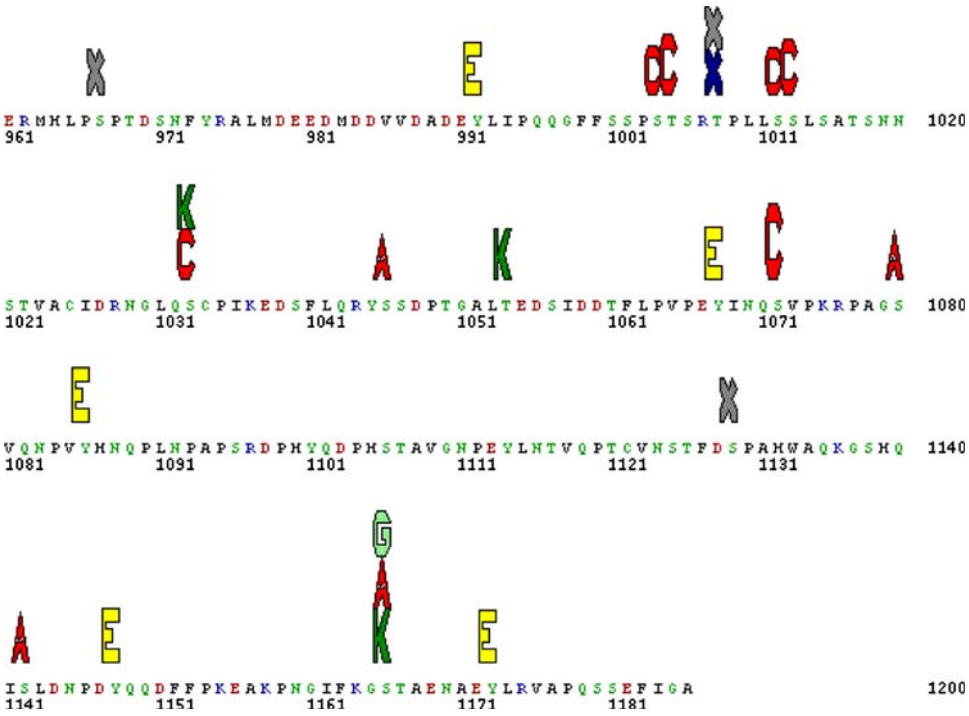

Fig. 3 Kinase landscape prediction for human EGFR sequence. The E shows where EGFR's intrinsic Tyr kinase phosphorylates EGFR in its catalytic domain. Is it observed that only EGFR's intrinsic Tyr kinase phosphorylate EGFR on its autophosphorylation sites. (A: PKA, C: PKC, G: PKG, K: CKII, E: EGFR, X: GSK3, X: p38MAPK)

The sequence of human OGT

IAVKLGTDLEYLKKIRGKVW 998

The sequences of human EGFR's auto phosphorylation sites

DMDDVVDADEYLIPQQGFFSS 1002
IDDTFLPVPEYINQSVPKRPA 1078
RPAGSVQNPVYMNQPLNPAPS 1096
SMQISLDNPDYQQDEFFPKEA 1158
FKGSTAENAEYLRVAPQSSEF 1183

Fig. 4 Comparison of human OGT sequence around Tyr 989 with EGFR's autophosphorylation sites. The yellow highlighted Y shows the residue in OGT, where phosphorylation occurs. In human EGFR the highlighted Y's show the receptors five autophosphorylation sites. The blue and red residues in human EGFR show the identical or similar residues as in the human OGT sequence

predicted by the NetphosK 1.0 [31] and suggest their phosphorylation only by EGFR's intrinsic RTK (Fig. 3). Other phosphorylated Tyr residues were predicted to be potentially phosphorylated by Src protein tyrosine kinase, which is consistent with Stover et al. [32], who reported that Src phosphorylates EGFR on several Tyr residues in its catalytic domain, and thereby provides additional docking sites for SH2-domain containing proteins.

Normal endocytosis leads to gradual attenuation or desensitization of receptor signaling or finally to degradation of the receptor [33]. Activated EGFR is internalized in coated pits, sorted through early endosomes, where the ligand is released from the receptor [34]. Finally unoccupied and ligand free EGFR is either recycled back to the cell surface or ultimately degraded in lysosomes (receptor termination) [34].

Endosomal sorting of the EGFR depends somewhat of its intrinsic Tyr kinase activity [35], but in some instances when RTK's activity is partially inhibited, ubiquitination of the receptor can mediate its internalization.

In this in silico study, we have predicted a false-negative Yin Yang site in the juxtamembrane of the EGFR, Thr 654 (substrate of PKC) (Table 2). Threonine 654 is fully conserved in mammals, and pisces (Table 2, Fig. 2). When EGFR is phosphorylated on Thr 654, it prevents transfer of activated EGFRs from early to late endosomes, and diverts receptors to recycling via endosomes back to the cellular surface [11]. c-Cbl is a multi-adaptor protein with E3-ubiquitin ligase activity that ubiquinates EGFR on several Lys residues [36], and is essential for lysosomal targeting of the receptor [37]. The protein c-Cbl binds to the catalytic domain of the EGFR on phosphorylated Tyr 1045, which in turn transphosphorylates the c-Cbl protein in its RING finger domain [35]. The ubiquitin ligase c-Cbl can also bind indirectly to EGFR by binding to Grb2 via its *C*-terminal, but direct binding of c-Cbl to the EGFR is favored [38]. When EGFR becomes phosphorylated on Thr 654 by PKC, it is no longer able to engage c-Cbl [11]. Furthermore, phosphorylated Thr 654 also prevents binding of CaM to EGFR's juxtamembrane domain [9, 10], suggesting that PKC, CaM and c-Cbl work together to regulate the activity of EGFR. PKC and PKA are inversely related to OGT in cerebellar neurons [39]. We suggest, when Thr 654 is *O*-GlcNAc modified, EGFR cannot be

phosphorylated, readily interacts with CaM in the juxtamembrane portion of the receptor and allows c-Cbl to interact with the EGFR. This suggests that OGT plays a role in the lysosomal targeting of EGFR.

The kinase CaM-II phosphorylates Ser 1046/1047 in the catalytic tail of the EGFR [39]. These sites are of functional importance, because HER2/c-erbB2 (isoform of EGFR) that does not contain CaM-II phosphorylation sites in its catalytic domain has been shown to be highly oncogenic [40]. Phosphorylation on Ser 1046/1047 by CaM-II inhibits RTK and desensitizes the EGFR [8, 40]. The Ser 1046/1047 residues of human EGFR are positively predicted Yin Yang sites (Table 2) and OGT may equally have access to *O*-glycosylate these residues. Ser 1046/1047 are conserved in mammals (Fig. 2). Increase in intracellular calcium levels activates calcium-dependent kinase, which reduces *O*-GlcNAc levels in neurons [39] and cardiomyocytes [41]. This suggests that inverse relationship between *O*-GlcNAc levels and calcium may exist. Furthermore *O*-GlcNAc modification of Synapsin I interferes with phosphorylation of Synapsin I by CaM-II [42], suggesting that OGT and CaM-II may work together to regulate the function of different proteins. Thus, when calcium levels are low in cells, OGT may add *O*-GlcNAc on Ser 1046/1047, and prevent CaM-II from phosphorylating EGFR. We propose that a possible interplay between OGT and CaM-II may either desensitize EGFR when CaM-II is active or activate EGFR when *O*-GlcNAc acts on EGFR (Fig. 5). At the same time PKC may also be modified by *O*-GlcNAc, which has been known to become inactivated by increased intracellular *O*-GlcNAc incorporation levels [39, 43] and thus at a time PKC or CaM-II is available to phosphorylate EGFR.

It is interesting to observe that all positively predicted Yin Yang sites were in the catalytic domain of the EGFR. In this domain, second messenger proteins containing either a SH2-domain or a PTB-domain interact with the receptor, suggesting that OGT also participates in signal transduction. It is known that OGT contains multiple tetratricopeptide repeats that mediate the enzyme's trimerization, substrate recognition, and protein–protein interactions [44]. Furthermore, the enzyme is modified by Tyr phosphorylation as well as *O*-GlcNAc modification [45]. The activity of OGT can be regulated by its Tyr phosphorylation [39] on Tyr 989 in human (corresponding to 979 in rat) possibly via the Tyr kinase signal

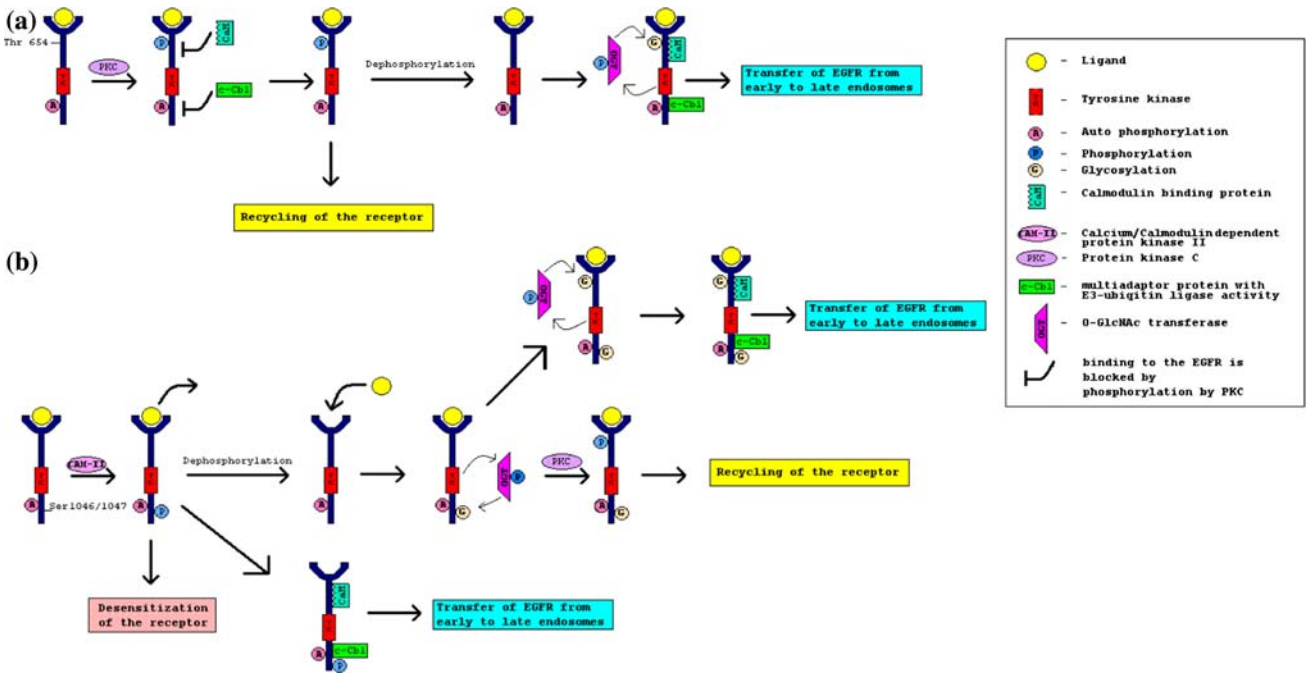

Fig. 5 (**a**) Upon ligand binding, EGFR's intrinsic Tyr kinase is activated, and starts phosphorylation in EGFR's cytoplasmic tail. Autophosphorylation of the EGFR triggers the signal transduction. When EGFR is phosphorylated on Thr 654 by PKC, it prevents binding of CaM and c-Cbl to the EGFR. Phosphorylated EGFR is transferred to recycling endosomes from where they return to the cell surface and maintain cell signal transduction. When Thr 654 is dephosphorylated, OGT might become phosphorylated by EGFR's Tyr kinase. Furthermore OGT *O*-GlcNAc modifies Thr 654, which might prevent its recycling and promote transfer of the receptor to late endosomes. (**b**) When EGFR is phosphorylated in the cytoplasmic tail on Ser 1046/1047 by CAM-II, it leads to desensitization of the receptor, and inhibits its intrinsic Tyr kinase activity. The ligand unoccupied EGFR becomes target of CaM and c-Cbl. If EGFR is dephosphorylated on Ser 1046/1047, EGFR is transactivated, and the ligand binds to the EGFR on the cell surface. PKC phosphorylates the EGFR and cell signaling continues. If OGT *O*-GlcNAc modifies Ser 1046/1047 and Thr 654, EGFR interacts with CaM and c-Cbl and might move into late endosomes

transduction [45]. When the sequence environment around Tyr 989 in human OGT was explored, it was found to be similar to the sequence of three of EGFR's autophosphorylation sites (Fig. 4). This indicates that EGFR's intrinsic Tyr kinase might be able to phosphorylate OGT. We suggest that phosphorylation of OGT on Tyr by EGFR may activate it to modify EGFR by *O*-GlcNAc in its catalytic domain (Fig. 5).

Calcium levels play a significant role in the regulation of EGFR's multifunctionality. Calcium can activate PKC and binding of calcium to CaM activates CaM kinases [3]. An elevation of calcium activates PKC, which is able to phosphorylate activated EGFR. Phosphorylated EGFR is then translocated to recycling endosomes from where signaling continues. Elevated calcium also binds to CaM and activates CaM-II. Activation of CaM-II phosphorylates EGFR in its cytoplasmic tail and desensitizes the receptor. Depending on the signaling and the required conditions EGFR signaling continues or is temporarily stopped. In another scenario where calcium levels are low or limited, OGT is activated. EGFR's intrinsic Tyr kinase may be able to phosphorylate OGT. Tyrosine phosphorylated OGT thus modify EGFR by *O*-GlcNAc on Thr 654 and/or Ser 1046/1047. It appears that OGT contribute to lysosomal targeting of activated receptors in either recycling endosomes or to late endosomes (Fig. 5).

These in silico investigations illustrate the role of the complex interplay between phosphorylation and *O*-GlcNAc modification of EGFR is modulated by signaling.

Our results suggest that OGT plays a dual role in the functional regulation of the EGFR, which could provide a mechanism for achieving proper balance between the activation and repression of EGFR signaling in cells. We have highlighted the importance of PKC and CaM-II and their interplay with OGT in the differential control of EGFR's signaling. We have predicted in silico the number and location of Yin Yang sites within important structural domains of the EGFR. An important goal for future research will be to fully understand the structural and functional aspects of these residues, and to elucidate the different roles of PTM of the EGFR.

Acknowledgement Nasir-ud-Din acknowledges support from Pakistan Academy of Sciences for this work.

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