

EGFR kinase possesses a broad specificity for ErbB phosphorylation sites, and ligand increases catalytic-centre activity without affecting substrate binding affinity

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We previously found that EGF (epidermal growth factor) increases the EGFR (EGF receptor) kinase-binding affinity towards the major tyrosine phosphorylation sites in downstream adaptor proteins such as Gab1 (Grb2-associated binding protein 1) and Shc [Src homology 2 (SH2) domain and collagen containing protein], but not that towards EGFR autophosphorylation sites [Fan, Wong, Deb and Johnson (2004) *J. Biol. Chem.* **279**, 38143–38150]. EGFR activation can also result in transphosphorylation of tyrosine residues in the C-terminal region of the related receptors ErbB2, ErbB3 and ErbB4 in heterodimers which are formed upon ligand stimulation. In the present study, we investigated the specificity of EGFR kinase by comparing the steady state kinetic parameters for peptides derived from all four ErbBs in the absence or presence of EGF. Our results demonstrated that (i) EGFR kinase can efficiently phosphorylate a broad range of diverse peptide sequences representing ErbB sites; (ii) certain ErbB2, ErbB3 and ErbB4 sites had higher specificity constants than any

EGFR sequence and (iii) EGF stimulation consistently increases the k_{cat} approx. 5-fold, but does not significantly alter the K_m for any ErbB peptides. Furthermore, peptides containing lysine at position –2 or –3 N-terminal to the target tyrosine were found to be poor EGFR kinase substrates, and substitution of these lysines with glutamine decreased the K_m and increased the k_{cat} for these substrates. We conclude that EGFR kinase-mediated ErbB transphosphorylations are mostly controlled at the level of oligomerization, and not by a preference of the EGFR kinase for phosphorylation sites in any particular ErbB. The results also demonstrated that, unlike phosphorylation sites in select downstream targets, EGF does not regulate the recognition of phosphorylation sites in the C-terminal region of any of the ErbBs.

Key words: autophosphorylation, epidermal growth factor receptor (EGFR), ErbB receptor, Michaelis–Menten, receptor tyrosine kinase (RTK) specificity, binding affinity.

INTRODUCTION

EGFR (epidermal-growth-factor receptor, ErbB1, HER1) is the prototypical member of the ErbB family of RTKs (receptor tyrosine kinases), which also includes ErbB2/HER2/*neu*, ErbB3/HER3 and ErbB4/HER4. ErbBs consist of an extracellular ligand-binding domain, a transmembrane spanning segment, an intracellular tyrosine kinase domain and a C-terminal region that contains multiple tyrosines. Among the ErbB receptor family, ErbB2 has no identified ligand and ErbB3 does not possess kinase activity. The ErbBs mediate signalling by a large number of growth factors that are structurally related to EGF such as transforming growth factor- α and amphiregulin. This family of receptor kinases function in the proliferation, migration, survival and differentiation of mammalian cells [1,2], and dysregulation of signalling by ErbBs has been implicated in cell transformation and cancer [3,4]. Engagement of the ligand induces the formation of receptor–receptor homo- and hetero-dimerization and activation of the intrinsic kinase. The heterodimer formation diversifies ErbB-mediated signalling, and almost every possible dimeric combination of ErbBs has been observed [5,6]. The kinase activation results in phosphorylation of specific tyrosine residues in their C-terminal tails (autophosphorylation), in other ErbBs and in cytosolic downstream targets such as Gab1 (Grb2-associated binding protein 1) [7], Shc [Src homology 2 (SH2) domain and collagen containing protein] [8], and PLC γ -1 (phospholipase C γ -1) [9,10].

Receptor kinase specificity towards various targets is believed to be critical for selective EGFR cellular signalling. The use of synthetic peptides in detailed studies investigating protein kinase specificity and catalysis is widely recognized and supported by crystallographic observations that peptide bound to the active site appears to exist in an extended conformation [11,12]. By using a degenerate-peptide library it has been shown previously that each tyrosine kinase has its own optimal peptide substrate, demonstrating that specificity is predominantly based on the residues immediately surrounding the phosphoacceptor tyrosine. The identified optimal peptide for EGFR kinase was EEEEEY-FELV [13]. The validity of using synthetic peptides in detailed investigations of kinase specificity and catalysis has also been supported by our previous study, which examined the phosphorylation of specific sites in full-length proteins relative to phosphorylation of these sites in peptides derived from the proteins [14]. This work evaluated the effects of EGF on the steady state kinetic parameters of EGFR-catalysed phosphorylation of peptides derived from its C-terminal region and on cellular targets. We found that EGF-stimulation consistently increases k_{cat} values for all tested peptides, but selectively increases the binding affinities for the major phosphorylation sites in Gab1 (Y627) and Shc (Y317) in peptides, and the full-length proteins. The results indicated that the ligand alters EGFR kinase specificity towards physiological targets [14].

Upon the formation of a heterodimer, EGFR kinase phosphorylates tyrosine sites in other ErbB partner *in trans*. Each ErbB

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; Gab1, Grb2-associated binding protein 1; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight; PLC, phospholipase C; PI3K, phosphoinositide 3-kinase; RP-HPLC, reverse-phase HPLC; RTK, receptor tyrosine kinase; Shc, Src homology 2 (SH2) domain and collagen containing protein.

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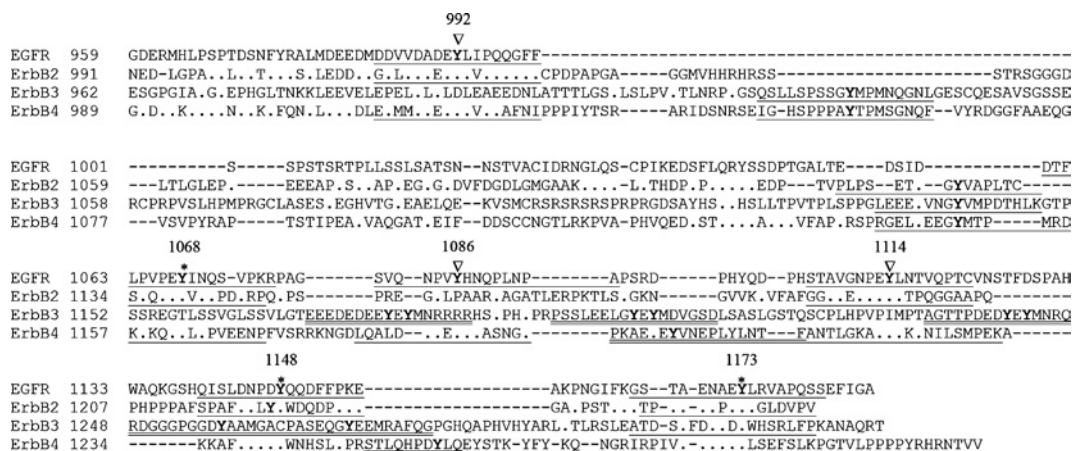


Figure 1 C-terminal sequence alignment of human ErbB family receptors

ErbB amino acid residue numbers are presented on left. Identical residues are denoted by dots and gaps which were introduced are represented by hyphens. The major and minor phosphorylation of tyrosine residues in EGFR are marked by asterisks and triangles respectively, with position number above. The tyrosine residues are highlighted in bold and the surrounding sequences included in synthetic peptides containing conserved tyrosines or non-conserved phosphorylation sites are underlined with single or double lines respectively.

receptor possesses a distinct pattern of tyrosine phosphorylation sites in its C-terminus. It has been demonstrated that phosphorylation of the ErbB receptors upon growth factor stimulation is dependent on the dimerization partner, and the differential receptor phosphorylation is suggested to be the basis for the differences in the cellular signalling elicited by homodimerization and heterodimerization [15]. To investigate EGFR kinase specificity for tyrosine sites from various ErbBs, and whether the relative specificity is altered upon EGF-stimulation, in the present study we have compared the phosphorylation of peptides derived from ErbBs by EGFR kinase. The results indicated that EGFR kinase efficiently phosphorylates ErbB peptides with a broad diversity of sequences. For all ErbB sites tested, EGF-stimulation increased the k_{cat} approx. 5-fold without significant effects on the K_m .

MATERIALS AND METHODS

Expression and purification of EGFR

Full-length human EGFR with a 25-amino-acid sequence containing Myc and hexahistidine epitope sequences at the C-terminus was expressed in a stable 32D cell line and the tagged EGFR was purified by Co^{2+} -based immobilized metal affinity chromatography as described previously [14]. Briefly, cells were grown in 2 l stirring flasks to a density of approx. 1.5×10^6 cells/ml, harvested by centrifugation at 2000 g and pellets were extracted for 20 min at 4°C with lysis buffer [20 mM Hepes (pH 7.4), 1% Triton X-100, 10% glycerol, 200 mM NaCl, 5 mM β -glycerophosphate, 0.1 mM PMSF, 0.2 μ g/ml pepstatin A, 0.2 μ g/ml aprotinin, 0.2 μ g/ml leupeptin, 1 mM sodium orthovanadate and 5 mM imidazole]. After centrifugation at 15000 g for 40 min, the supernatant was incubated with Talon metal affinity resin (Clontech) at 4°C for 1 h. EGFR was eluted with lysis buffer containing 50 mM imidazole. EGFR concentration was measured by silver staining and Western blotting of SDS/PAGE gels using BSA and Myc-GST (glutathione S-transferase)-Grb2 as standards respectively.

Preparation of peptides

Synthetic peptides were prepared by solid-phase synthesis using standard Fmoc (*N*-9-fluorenyl methoxycarbonyl) chemistry on an Applied Biosystems 432A peptide synthesizer and purified using

preparative RP (reverse-phase)-HPLC. The identity and purity of peptides were confirmed by MALDI-TOF (matrix-assisted laser desorption ionization-time-of-flight)-MS and analytical RP-HPLC.

Tyrosine kinase assay

All kinase reactions were performed in 25 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 50 μ M sodium orthovanadate, 0.5 mM dithiothreitol, 0.2% Triton X-100, 40 μ g/ml BSA, 25 μ M ATP with 62.5 μ Ci/ml [γ - ^{32}P]ATP (Amersham) and 9 nM EGFR in a total volume of 40 μ l at 25°C. EGFR was pre-incubated with or without 1 μ M EGF for 10 min at 25°C. Reactions were initiated by addition of peptide and were stopped using 8.5% H_3PO_4 . Terminated reaction mixtures were transferred to P30 Filtermats (Wallac), washed 3 \times with 0.85% H_3PO_4 , 3 \times with water and bound radioactivity was quantified with a Trilux Macrobeta scintillation counter (Perkin Elmer). Initial rates were determined from the linear portion of the reaction. Replicates (4) were measured in all experiments and kinetic parameters were calculated using the Enzyme Kinetic Module of Sigma Plot (Jandel Scientific).

RESULTS

Steady-state enzyme kinetic studies provide measurements of relative substrate-binding affinity (K_m) and the catalytic-centre activity (k_{cat} , 'turnover number'). The combined parameter, k_{cat}/K_m , which is referred to as the specificity constant, is the best measurement for comparison of catalytic efficiencies towards different substrates. EGFR possesses three major and two minor tyrosine autophosphorylation sites located at Y1068, Y1148, Y1173, and at Y992 and Y1086 respectively. In addition, EGFR Y1114 is preceded by glutamic acid (Figure 1), which should be preferred by the EGFR kinase as indicated in previous work [16]. Our previous study demonstrated that human EGFR peptides representing these sites exhibit specificity constants $> 50 \text{ min}^{-1} \cdot \text{mM}^{-1}$ in the presence of EGF, with the exception of the Y1086 peptide ($12 \text{ min}^{-1} \cdot \text{mM}^{-1}$). EGF activation does not alter the affinity binding of EGFR peptides to the kinase, but consistently increases the k_{cat} and specificity constants approx. 5-fold for the EGFR peptides [14].

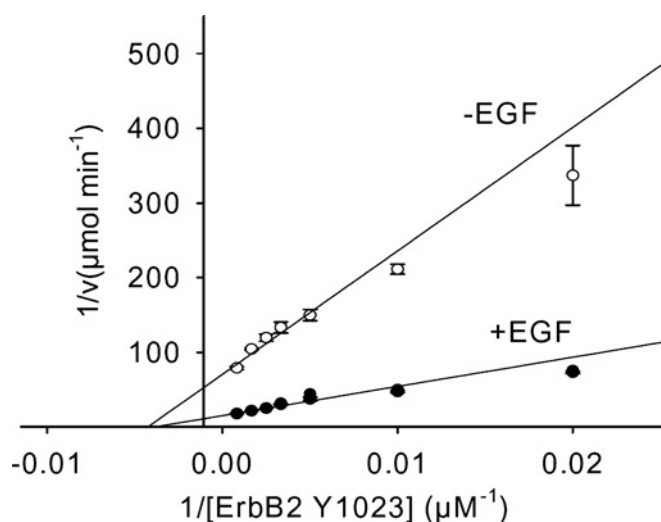


Figure 2 Lineweaver-Burk plots of ErbB2-Y1023 peptide phosphorylation by EGFR

Purified EGFR (7 nM) was pre-incubated with or without 1 μ M EGF for 10 min at 25 °C followed by the addition of kinase reaction buffer containing 25 μ M radiolabelled ATP. The reactions were initiated by the addition of various concentrations of peptide and terminated using 8.5% phosphoric acid. The results represent the means \pm S.E.M. for four replicates.

The C-terminal domain is the most variable region among the ErbB family receptors. As aligned in Figure 1, most of the identified phosphorylation tyrosines in this region are conserved in human EGFR, ErbB2 and ErbB4. However, the short sequences surrounding these tyrosines are not identical. The C-terminal domain of ErbB3 has much less consensus relative to other ErbB members and has only one tyrosine (Y1309) which is conserved relative to the other three ErbBs. In addition, Y1035 in ErbB3 is conserved in ErbB4, and Y1140 of ErbB3 is conserved in ErbB2 and ErbB4 (Figure 1).

To explore EGFR substrate selectivity towards identified or potential phosphorylation sites contained within the C-terminal regions of other human ErbBs, and whether ligand affects this selectivity, we synthesized and carried out phosphorylation kinetic analyses of the peptides representing these sites. The synthetic 20 mer peptides used in the present study contain sequences identical to 17 amino acids surrounding the phosphoacceptor tyrosine and were named by origin + tyrosine position (i.e. ErbB2-Y1023). The peptides also contained two or one lysine residues at the N- and C-termini respectively, allowing them to bind strongly to phosphocellulose filters upon acidification [17].

Although no ligand has been identified for ErbB2, it appears to be the preferred heterodimerization partner for all other ErbBs

[18]. Five conserved tyrosines in ErbB2 compared with EGFR have been identified as autophosphorylation sites [19,20] and peptides were prepared representing these ErbB2 tyrosines. Additionally, the peptide corresponding to ErbB2-Y1127 was prepared and studied because this site is conserved in ErbB3 and ErbB4 (Figure 1). Figure 2 contains representative Lineweaver-Burk plots of ErbB2-Y1023 phosphorylation at a fixed ATP concentration of 25 μ M in the presence or absence of EGF. The double reciprocal plots in the presence and absence of EGF intersected with the abscissa at the same point, indicating that EGF did not influence the K_m . However, EGF increased the k_{cat} value approx. 4.7-fold as evidenced by distinct intersections of the plots with ordinate. As summarized in Table 1, the ErbB2 peptides exhibited specificity constants ≥ 40 $\text{min}^{-1} \cdot \text{mM}^{-1}$ in the presence of EGF with the exception of the ErbB2-Y1127 peptide (10.2 $\text{min}^{-1} \cdot \text{mM}^{-1}$). Clearly peptides representing the five identified phosphorylation tyrosines were phosphorylated much more efficiently by the EGFR kinase than was the Y1127 peptide. Surprisingly, the peptide ErbB2-Y1221/Y1222 that contains tandem tyrosines, but does not have an acidic residue at position -1 N-terminal to the target tyrosine, possessed the highest specificity constant among the ErbB2 peptides (159 $\text{min}^{-1} \cdot \text{mM}^{-1}$ in the presence of EGF). This specificity constant was at least 2-fold greater than that previously determined for any of the EGFR autophosphorylation site peptides [14]. EGF had no significant (<40%) effects on the K_m values for any of the ErbB2 peptides, but enhanced the k_{cat} values 3.8–4.8-fold.

Using an EGFR/ErbB3 chimaera, tyrosine binding sites in ErbB3 for the p85 subunit of PI3 kinase and Shc binding have been identified by phosphopeptide competition [21]. Whereas Y1039 of ErbB3 is responsible for Shc binding, 6 YXXM motifs are found to interact with the p85 subunit of PI3K. Of the ErbB3 peptides, three corresponding to Y1178/Y1180, Y1203/Y1205 and Y1241/Y1243 contain two tyrosine residues (Table 2). Peptides representing these sites were synthesized and their phosphorylation by EGFR kinase was analysed. ErbB3-Y1140 was also included because this tyrosine is conserved in ErbB2 and ErbB4. The results are summarized in Table 2. As was observed for ErbB2-Y1221/Y1222, the three ErbB3 peptides containing dual tyrosines (Y1178/Y1180, Y1203/Y1205 and Y1241/Y1243) were found to be highly preferred by the EGFR kinase with specificity constants > 100 $\text{min}^{-1} \cdot \text{mM}^{-1}$ in the presence of EGF. Peptides ErbB3-Y1140, -1257 and -1309 were also effectively phosphorylated by EGFR kinase with specificity constants ≥ 20 $\text{min}^{-1} \cdot \text{mM}^{-1}$. ErbB3-Y1035 and -Y1270 exhibited relatively low specificity constants (<10 $\text{min}^{-1} \cdot \text{mM}^{-1}$) probably due to the lack of acidic residue in position -1. By contrast, the ErbB3-Y1140 peptide does not contain an acidic residue at position -1, but had a high specificity constant of approx. 92 $\text{min}^{-1} \cdot \text{mM}^{-1}$. As was found for EGFR peptides [14], EGF had little demonstrable

Table 1 Steady-state kinetic constants of EGFR tyrosine kinase with peptide substrates derived from ErbB2

Results represent means \pm S.E.M.

ErbB2 tyrosine position	Sequence	K_m (μ M)		k_{cat} (min^{-1})		k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)	
		EGF. . . -	+	-	+	-	+
1023	GDLVDAEEYLVPPQGGFF	215 \pm 28	228 \pm 22	2.5 \pm 0.1	11.7 \pm 0.2	11.7 \pm 1.7	52.2 \pm 5.0
1127	PLPSETDGYVAPLTCSP	516 \pm 51	450 \pm 44	1.1 \pm 0.1	4.6 \pm 0.1	2.1 \pm 0.5	10.2 \pm 1.8
1139	LTCSPQPEYVNPDPVVRP	220 \pm 20	149 \pm 12	4.3 \pm 0.1	18.8 \pm 0.2	19.5 \pm 3.8	126 \pm 13
1196	GGAVENPEYLTPOGGAA	280 \pm 31	168 \pm 13	2.1 \pm 0.1	8.4 \pm 0.1	7.6 \pm 1.0	49.8 \pm 5.1
1221/1222	SPAFDNLYYWDQDPPER	140 \pm 14	104 \pm 11	4.4 \pm 0.1	16.5 \pm 0.1	31.8 \pm 3.7	159 \pm 18
1248	TPTVAENPEYLGLDVPV	197 \pm 15	122 \pm 12	1.6 \pm 0.1	6.5 \pm 0.1	8.1 \pm 1.2	53.3 \pm 6.7

Table 2 Steady-state kinetic constants of EGFR tyrosine kinase with peptide substrates derived from ErbB3Results represent means \pm S.E.M.

ErbB3 tyrosine position	Sequence	K_m (μ M)		k_{cat} (min^{-1})		k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)	
		EGF... -	+	-	+	-	+
1035	SLSPSSGYMPMNQGNL	528 \pm 91	841 \pm 87	0.1 \pm 0.1	0.5 \pm 0.1	0.2 \pm 0.1	0.6 \pm 0.1
1140	LEEEDVNGYVMPDTHLK	152 \pm 15	176 \pm 13	2.5 \pm 0.1	16.2 \pm 0.4	16.1 \pm 2.7	92.1 \pm 10
1178/1180	EEEDDEEYVYMNRRRR	129 \pm 10	114 \pm 8	3.0 \pm 0.1	14.5 \pm 0.3	23.1 \pm 2.6	128 \pm 12
1203/1205	PSSLEELGYEYMDVGS	111 \pm 11	153 \pm 10	3.1 \pm 0.1	15.8 \pm 0.3	28.3 \pm 3.5	103 \pm 9
1241/1243	AGTTPDEDYVYMNRRQRD	118 \pm 13	109 \pm 6	3.2 \pm 0.1	19.4 \pm 0.3	27.5 \pm 3.9	179 \pm 13
1257	DGGGPGDYAAMGACPA	343 \pm 29	460 \pm 20	1.4 \pm 0.1	9.2 \pm 0.4	4.1 \pm 0.7	20.0 \pm 1.8
1270	ACPASEQYVYEMRAFQ	931 \pm 99	836 \pm 64	1.2 \pm 0.1	7.6 \pm 0.3	1.3 \pm 0.23	9.1 \pm 1.1
1309	DSAFDNDPYWHSRLFPK	245 \pm 16	232 \pm 10	2.8 \pm 0.1	15.7 \pm 0.2	11.3 \pm 1.1	67.8 \pm 4.0

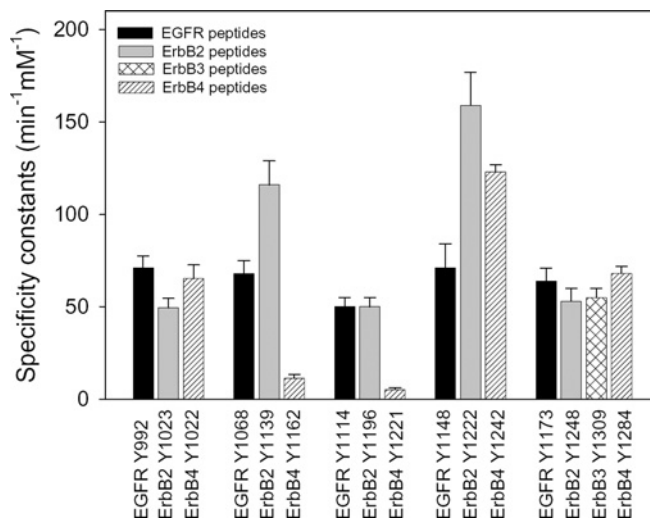
Table 3 Steady-state kinetic constants of EGFR tyrosine kinase with peptide substrates derived from ErbB4Results represent means \pm S.E.M.

ErbB3 tyrosine position	Sequence	K_m (μ M)		k_{cat} (min^{-1})		k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)	
		EGF... -	+	-	+	-	+
1022	EDMMDAEEYLVQAFNI	279 \pm 13	260 \pm 20	3.1 \pm 0.1	17.0 \pm 0.4	11.1 \pm 0.9	65.3 \pm 7.4
1056	IGHSPPPAYTPMSGNQF	1072 \pm 120	1752 \pm 234	0.7 \pm 0.1	3.0 \pm 0.2	0.7 \pm 0.1	1.7 \pm 0.4
1150	RGELDEEGYMTMRDKP	865 \pm 44	579 \pm 32	4.1 \pm 0.1	15.5 \pm 0.4	4.7 \pm 0.4	26.8 \pm 2.3
1162	MRDKPKQYLNVPVEENP	1100 \pm 154	1093 \pm 81	2.0 \pm 0.1	12.5 \pm 0.5	1.9 \pm 0.4	11.4 \pm 1.4
1188	LQALDNPEYHNASNGPP	291 \pm 23	286 \pm 16	2.4 \pm 0.2	15.3 \pm 0.3	8.2 \pm 0.9	53.5 \pm 3.0
1202	ASNGPPKAEDEYVNEPL	284 \pm 19	194 \pm 10	2.9 \pm 0.1	14.8 \pm 0.2	10.1 \pm 1.1	76.3 \pm 4.9
1221	ANTLGKAEYLNKNNILSM	1805 \pm 365	1567 \pm 198	1.4 \pm 0.2	8.1 \pm 0.3	0.8 \pm 0.4	5.2 \pm 0.7
1242	KKAFDNDPYWNHSLPPR	276 \pm 18	172 \pm 16	4.4 \pm 0.2	21.1 \pm 0.6	15.9 \pm 2.0	122.7 \pm 3.4
1258	HSLPPRSTLQHPDYLQ	377 \pm 19	271 \pm 14	3.5 \pm 0.2	19.5 \pm 0.3	9.3 \pm 0.1	72.0 \pm 2.8
1284	PIVAENPEYVSEFSLKP	335 \pm 36	287 \pm 25	2.7 \pm 0.1	15.7 \pm 0.3	8.1 \pm 0.8	54.7 \pm 4.6

effect on the K_m values for ErbB3 peptides but consistently increased the k_{cat} values approx. 5-fold.

ErbB4 displays the highest sequence homology with EGFR and has 6 conserved tyrosines (Figure 1). In addition, Y1056 of ErbB4 was identified as a binding site of PI3K [22]. Peptides representing these tyrosines were prepared and enzymatically analysed as EGFR kinase substrates. Peptides corresponding to Y1202 and Y1258 were also studied because of the presence of a -1 acidic residue. The results are summarized in Table 3. In general, results obtained for peptides derived from ErbB4 were similar to those of other ErbB peptides. Most of the peptides were efficiently phosphorylated by EGF-activated EGFR, whereas some, such as ErbB4-Y1056, -Y1162 and -Y1221, exhibited relatively low specificity constants ($< 20 \text{ min}^{-1} \cdot \text{mM}^{-1}$). EGF did not significantly influence the K_m values for ErbB4 peptides but consistently increased the k_{cat} values approx. 5-fold.

The specificity constants in the presence of EGF for peptides representing the conserved tyrosines in each of the ErbBs are compared in Figure 3. Peptides containing tyrosine conserved relative to EGFR-Y992 or -Y1173 have similar specificity constants, whereas ErbB2-Y1139, ErbB2-Y1221/Y1222 and ErbB4-Y1242 have higher specificity constants than their EGFR homologues, EGFR-Y1068 and -Y1148 respectively. ErbB4-Y1162 and -Y1221 peptides, which are conserved relative to EGFR-Y1068 and -Y1114 respectively, and contain glutamic acid residues at position -1, had relatively low specificity constants. This was mainly due to weak binding affinities for EGFR kinase (K_m values of approx. 1 and 1.6 mM for ErbB4-Y1162 and -Y1221 respectively). Taken together, these results demonstrated that the EGFR kinase was incapable of efficiently phosphorylating certain tyro-

**Figure 3 Comparison of EGFR kinase specificity constants for peptide substrates derived from conserved regions of the ErbBs**

The specificity constants were determined in the presence of EGF. The values for EGFR peptides were taken from [14].

sine-containing peptides, but exhibited a broad catalytic specificity towards substrate primary sequences. In addition, certain ErbB2, ErbB3 and ErbB4 peptides had higher specificity constants than their EGFR counterparts.

Table 4 Effects of amino acid substitution on enzymatic parameters for EGFR-Y845 and ErbB4-Y1221 peptides

The substituted residues are shown in *italic bold*. Results represent means \pm S.E.M.

Peptide	Sequence	K_m (μ M)		k_{cat} (min^{-1})		k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)	
		EGF... -	+	-	+	-	+
EGFR-Y845	LLGAE EE KEYHAEGGKVP	931 \pm 56	943 \pm 42	1.4 \pm 0.1	5.6 \pm 0.1	1.5 \pm 0.1	5.9 \pm 0.7
EGFR-Y845/K843Q	LLGAE EE Q Y HAEGGKVP	266 \pm 18	213 \pm 10	2.1 \pm 0.1	11.2 \pm 0.2	7.1 \pm 0.9	52.6 \pm 3.6
ErbB4-Y1221	ANTLGK AE YLKNNILSM	1805 \pm 365	1567 \pm 198	1.4 \pm 0.2	8.1 \pm 0.3	0.8 \pm 0.4	5.2 \pm 0.7
ErbB4-Y1221/K1218Q	ANTLG Q AEYLKNNILSM	567 \pm 63	521 \pm 40	1.8 \pm 0.2	11.3 \pm 0.3	3.2 \pm 0.2	21.7 \pm 1.0

As described above, the relatively low specificity constants for the peptides corresponding to ErbB4-Y1162 and -Y1221 were unexpected considering the presence of a glutamic acid at position -1. This is similar to the scenario found for a peptide derived from the EGFR activation loop (Y845) in our previous work [14]. Even this target tyrosine is preceded by glutamate residues at positions -1, -3 and -4, which have been determined to be optimal for the EGFR substrate [13]. This peptide has a relatively low specificity constant ($\sim 6 \text{ min}^{-1} \cdot \text{mM}^{-1}$ in the presence of EGF) due to a high K_m ($\sim 1 \text{ mM}$) and a modest k_{cat} ($\sim 6 \text{ min}^{-1}$) (Table 4). We proposed that the lysine residue in position -2 or -3 in these peptides may exert negative effects and be unfavourable. To test this hypothesis we replaced the lysine residue of EGFR-Y845 at position -2 and that of ErbB4-Y1221 at position -3 with a glutamine. Consistent with our proposal, the substitution in EGFR-Y845 peptide resulted in an approx. 4-fold reduction in the K_m and an approx. 2-fold increase in the k_{cat} , and the substitution in ErbB4-Y1221 decreased the K_m approx. 3-fold and increased the k_{cat} approx. 1.4-fold. Furthermore, the k_{cat} values for both peptides containing lysine to glutamine substitutions were increased approx. 5-fold upon EGF stimulation without obvious change in the K_m values, indicating that the lysine substitution did not alter the ligand regulation of the kinase activity towards these peptides.

DISCUSSION

We have previously compared the EGFR kinase-catalysed phosphorylation of peptides representing its autophosphorylation sites with that of peptides derived from other cellular substrates [14]. We found that EGF consistently enhances the k_{cat} for all peptides approx. 5-fold, but selectively increases the binding affinity for peptides representing the major EGFR-mediated phosphorylation sites of adaptor proteins, such as Gab1 Y627 and Shc Y317, and the EGFR optimal peptide substrate which contains the EEEE-YFELV sequence. In the present study, we investigated EGFR kinase-catalysed phosphorylation of peptides derived from ErbB2, ErbB3 and ErbB4 receptors. Our result indicated that the EGFR kinase was able to efficiently phosphorylate a broad range of diverse ErbB peptide sequences. EGF-stimulation resulted in approx. 5-fold increases in the k_{cat} values with no obvious change in K_m values. This is similar to the results found for EGFR peptides, but different from those for peptides derived from Gab1 and Shc [14]. It seems striking that of the many physiologically relevant substrate sequences tested to date, including all of the ErbBs, we have only observed ligand-induced increased binding affinity (i.e. recognition) in sequences derived from downstream cellular targets.

Whereas the EGFR kinase is able to efficiently phosphorylate peptide substrates with a broad diversity of sequences, the broad range of specificity constants indicates the preference of the

kinase for certain peptides. Interestingly, the ErbB2-Y1139 and -Y1221/Y1222 peptides had higher specificity constants than their homologous counterparts in other ErbBs (Figure 3). This is consistent with the previous observation that EGFR kinase phosphorylates an EGFR/ErbB2 C-terminal domain chimaera to a higher level than EGFR autophosphorylation both *in vivo* and *in vitro* [23]. It has been recognized for a long time that ErbB2 is the preferred dimerization partner for the ErbB receptors (for reviews see [5,6]). Our findings in the present study suggest that ErbB2 possesses preferred phosphorylation target sites for EGFR kinase which may partially account for the enhanced transforming activity in cells coexpressing ErbB2 and EGFR. It would be of interest to determine whether these ErbB2 sites represent preferred substrates for the ErbB2 and ErbB4 kinases.

In previous investigations, Y1035 in ErbB3 and Y1056 in ErbB4 have been identified as PI3K binding sites via SH2 interactions and are important in signalling mediated by these receptors [21,22,24]. However, we found that peptides representing these sites had the lowest specificity constants among all the peptides tested with EGFR kinase. This suggests that these sites are not efficiently phosphorylated by EGFR in oligomeric complexes and that ErbB2 or ErbB4 kinases may mediate direct phosphorylation of the ErbB3 Y1035 or ErbB4 Y1056 sites. Another possibility is that these sites are phosphorylated by other cytosolic protein tyrosine kinases such as Src and Abl, which can be activated upon EGF stimulation. We believe that this latter possibility may be more plausible because the EGFR, ErbB2 and ErbB4 kinases have similar substrate preferences [25].

Relative selectivity for each specific position in peptide substrates has been assigned for many tyrosine kinases using a degenerate-peptide library [13]. We calculated an additive score for each peptide tested in our previous [14] and present studies using the published relative EGFR position selectivity. We found no correlation between these predicted peptide scores and either the k_{cat} , K_m or k_{cat}/K_m values (results not shown), suggesting a complicated synergy of amino acids flanking the target tyrosine with respect to peptide-binding and catalysis. The optimal EGFR peptide had a specificity constant of approx. $2500 \text{ min}^{-1} \cdot \text{mM}^{-1}$ in the presence of EGF, which is much higher than those of all the physiologically relevant substrates tested, mainly due to its very low K_m (K_m of $5.6 \mu\text{M}$ in the presence of EGF) [14]. However, many peptides tested had significantly higher k_{cat} values than the optimal EGFR peptide.

Early studies indicated that EGFR kinase prefers to phosphorylate substrates with an acidic residue at position -1 relative to the target tyrosine [16,26]. However, we found that some peptides such as EGFR-Y845, ErbB4-Y1162 and ErbB4-Y1221 contain a glutamic acid at position -1 but were relatively poor substrates for the EGFR kinase, mainly due to the low binding affinities (Tables 3 and 4). Our amino acid substitution experiments indicated that a lysine at position -2 or -3 exerted a dominant

negative effect. On the other hand, a -1 acidic residue in the peptide was not a requisite for efficient phosphorylation by EGFR. Among the 6 peptides which lack a glutamic or aspartic acid at position -1, ErbB2-Y1221/Y1222 and ErbB3-Y1140 were efficient receptor kinase substrates (Tables 1 and 2). However, these peptides contain one or more acidic residues at positions -2, -3 or -4, which may play a role in EGFR recognition and binding of peptides in the absence of a -1 acidic side chain [27].

The broad specificity of EGFR kinase may be essential to the phosphorylation of diverse *in vivo* substrates and activation of multiple signalling pathways. This diversity has been exemplified by our finding that the EGFR kinase was able to efficiently phosphorylate a broad diversity of peptides representing ErbB phosphorylation sites. However, high fidelity signal transduction also requires phosphorylation of specific signalling proteins, implying that EGFR specificity should be controlled by other mechanisms in addition to the primary target sequence. Numerous studies have demonstrated that EGFR-mediated phosphorylation of ErbB receptors is dictated by dimerization partners, which in turn is determined by ligands [6]. Furthermore, for individual ErbB dimers, different ligands induce differential receptor phosphorylation patterns and therefore elicit differential cell signalling [28]. Among the ErbBs, only EGFR and ErbB4 have both ligands and kinase activity. Previous investigations have demonstrated that EGFR can be a dimerization partner of and be phosphorylated by ErbB4 upon neuregulin1- β stimulation. However, there is no evidence for EGF-stimulated EGFR/ErbB4 dimers or ErbB4 tyrosine phosphorylation [22,29]. Thus ErbB4 is unlikely to be phosphorylated by EGFR in cells even though several ErbB4 peptides are phosphorylated by EGFR efficiently *in vitro*. However, it is possible that other ErbB ligands, such as betacellulin, may induce EGFR kinase-mediated phosphorylation of ErbB4 [30,31]. Ligand binding and subsequent dimerization may induce conformational changes in the kinase domain. Slight conformational adjustments within the active site can result in dramatic changes in enzymatic activity and specificity of an enzyme [32,33]. In addition, it has been suggested that secondary and tertiary structures are important for target recognition [34]. We previously found that EGFR-Y1114 peptide was phosphorylated efficiently by EGFR with a specificity constant of $\sim 50 \text{ min}^{-1} \cdot \text{mM}^{-1}$ [14], but this site in the intact EGFR was not found to be phosphorylated either in autophosphorylation reactions *in vitro* or in cells after EGF stimulation [35]. Access of these tyrosine residues to the kinase catalytic site may be prohibited by the secondary or tertiary structure. Taken together, we conclude that whereas the primary sequences near the target tyrosine residues play an important role in receptor kinase specificity, but EGFR kinase-mediated ErbB transphosphorylation is mostly controlled at the level of oligomerization.

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