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A highly efficient peptide substrate for EGFR activates the kinase by inducing aggregation

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Synopsis

Formation of an asymmetric dimer by the epidermal growth factor receptor (EGFR) kinase domains results in allosteric activation. Since this dimer does not readily form in solution, the EGFR kinase domain phosphorylates most peptide substrates with a relatively low catalytic efficiency. Peptide C is a synthetic peptide substrate of EGFR developed by others that is phosphorylated with a significantly higher catalytic efficiency, and we sought to understand the basis for this. Peptide C was found to increase EGFR kinase activity by promoting formation of the EGFR kinase domain asymmetric dimer. Activation of the kinase domain by Peptide C also enhances phosphorylation of other substrates. Aggregation of the EGFR kinase domain by Peptide C likely underlies activation, and Peptide C precipitates several other proteins. Peptide C was found to form fibrils independent of the presence of EGFR, and these fibrils may facilitate aggregation and activation of the kinase domain. These results establish that a peptide substrate of EGFR may increase catalytic activity by promoting kinase domain dimerization by an aggregation-mediated mechanism.

Keywords

EGFR; dimer; kinetics; aggregation; activation; fibril

Introduction

The kinase domain of the epidermal growth factor receptor (EGFR; also known as ErbB1) is activated by an allosteric mechanism upon receptor dimerization. The carboxy-terminal lobe

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(C-lobe) of one kinase subunit (the activator) interacts with the amino-terminal lobe (N-lobe) of a second kinase subunit (the receiver) and results in the allosteric activation of the receiver through the formation of an asymmetric dimer of the kinase domains [1]. The recombinant kinase domain of EGFR is predominantly monomeric *in vitro* and thus does not readily undergo allosteric activation, resulting in very low catalytic activity. A construct of EGFR that includes the cytoplasmic juxtamembrane segment and the kinase domain forms the asymmetric dimer much more readily, and the catalytic efficiency is at least 70-fold higher than for the isolated kinase domain [2,3]. The catalytic efficiency of the isolated kinase domain can also be increased by tethering it to small unilamellar vesicles, thereby increasing its effective concentration [1].

The most important substrates of EGFR are tyrosine residues located in the long carboxy-terminal (C-terminal) tail of the receptor. When phosphorylated, these tyrosines serve as docking sites for effector proteins that relay the signal onward through the cell [4]. Short peptides containing these tyrosine residues ("tail peptides") are poor substrates for the EGFR kinase domain, presumably because efficient phosphorylation of the tail occurs within an EGFR dimer or higher order oligomer [5-7]. For example, a 15-residue peptide containing Tyr-1173 from the tail of human EGFR, which we refer to as Tail Peptide A, has an estimated value of 1 mM for the Michaelis constant, K_M , when phosphorylated by the EGFR kinase domain. Note that the numbering system we use for EGFR does not include the 24 residues of the amino-terminal (N-terminal) signal sequence.

Researchers at GlaxoSmithKline screened several peptide libraries for EGFR activity and identified a peptide substrate that is phosphorylated with a much higher efficiency than tail peptides (referred to as Peptide C in their work; we retain this name in this paper) [8]. The value of the catalytic efficiency, k_{cat}/K_M , for this peptide was reported to be ~26-fold higher than that for Tail Peptide A [9]. Additional studies with peptides derived from various proteins that are EGFR substrates or from synthetic peptide libraries have identified other peptide substrates for EGFR. Notably, the primary sequence of Peptide C is more hydrophobic than the other peptide substrates, which are predominantly acidic [10,11].

Owing to the increased catalytic efficiency of the EGFR kinase domain when Peptide C is used as a substrate, we sought to use Peptide C to develop robust biochemical assays for EGFR. The reported value of K_M of 128 μ M for Peptide C permits EGFR activity to be measured at saturating peptide concentrations, which is not feasible using tail peptides for which the values of K_M are in the low millimolar range [5,9]. Several other studies have used Peptide C as an EGFR substrate peptide due to these favorable catalytic parameters [12-14].

Our studies confirm the remarkable activity of EGFR with Peptide C but also reveal unexpected properties that are likely to be undesirable in studies of EGFR enzymatic activity. We find that the value of k_{cat}/K_M for Peptide C in our assays is over 250-fold increased when directly compared to Tail Peptide A. We confirm that the high activity with Peptide C requires formation of the asymmetric dimer of EGFR kinase domains. Furthermore, the activity of EGFR with Peptide C as a substrate shows a steep dependence on the concentration of Peptide C, with a Hill coefficient of nearly 3. This behavior

suggested to us initially that the peptide could be binding at an allosteric site on EGFR and thereby promoting kinase dimerization and increased activity. However, a detailed analysis of Peptide C found that it most likely stimulates EGFR activity by promoting aggregation of the kinase domain. Previous reports have described the ability of other molecules to aggregate and activate EGFR, and Peptide C represents the first peptide substrate of EGFR shown to have this effect [15-17].

Methods

Protein expression and purification

The EGFR kinase domain construct (residues 672-998) was expressed and purified as described previously [1]. Mutations were introduced by the Quikchange mutagenesis kit (Stratagene) and confirmed by sequencing. All constructs assayed included the N-terminal His₆ tags.

Peptide substrates

The peptide substrates were produced by solid phase synthesis and purified by HPLC to a purity of at least 90% as analyzed by mass spectrometry. The sequence of Peptide C is RAHEEIYHFFFAKKK [8]. The sequence of Tail Peptide A is RRKGSTAENAEYLRV and is derived from the Tyr-1173 autophosphorylation site of EGFR [18]. The sequence of Tyrsb is EELEDDYEDDMEE and is derived from the protein human erythrocyte Band 3 [10].

Enzyme-coupled kinase assays

The enzyme-coupled kinase assay was performed as described previously [19]. EGFR kinase domain concentration was either 2 μ M for assays with Peptide C or 8 μ M for assays with Tail Peptide A or Tyrsb. In experiments in which pairs of different kinase domain dimerization mutants were assayed, each kinase domain was assayed at 1 μ M for 2 μ M total kinase domain concentration. Peptide concentrations were varied as indicated. The ATP concentration was 500 μ M. Buffer conditions were 10 mM MgCl₂ and 20 mM Tris, pH 7.5. Assays were performed at room temperature. Values for the kinetic parameters k_{cat} and K_M for Tail Peptide A and Tyrsb were derived from fits to the Michaelis-Menten equation using Prism, version 5.0c (Graphpad). Because the data for Peptide C fit better to a sigmoidal curve, the Top and EC₅₀ values that were derived in the Hill analysis, which is described in the subsequent section, were used as the values for the k_{cat} and K_M , respectively, for EGFR catalysis with Peptide C.

Hill analysis

Hill coefficients were derived from fitting data to the Log(Agonist) vs. Response, Variable Slope equation defined as $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope})})$ using Prism, version 5.0c (Graphpad), in which Y is the specific rate of catalysis, Top and Bottom are maximum and minimum specific rates, respectively, EC₅₀ is the concentration of peptide at which half of the maximum specific rate is attained, and X is the base-10 logarithm of the substrate concentration. Because the Tail Peptide A and Tyrsb analysis did

not reach saturating concentrations, better fits for the Hill analysis were obtained when the Top (k_{cat}) values were constrained to those derived from the Michaelis-Menten fits.

Immunoblot analysis

Autophosphorylation reactions were performed with 800 nM EGFR kinase domain, 500 μ M ATP, 10 mM $MgCl_2$, 20 mM Tris, pH 7.5, 400 μ M NaF, 1 mM Na_3VO_4 , and Peptide C at the indicated concentrations in 30 μ l at room temperature. Reactions were terminated after 1 minute by addition of 20 mM EDTA. Samples were run on SDS-PAGE gels and subjected to immunoblot analysis. Levels of EGFR and autophosphorylation of EGFR were measured with primary antibodies specific to either EGFR (sc-03, Santa Cruz) or each of the phosphotyrosines at Tyr-845 (Cell Signaling), Tyr-974 (Cell Signaling), or Tyr-992 (Cell Signaling). The secondary antibody was goat anti-rabbit coupled to horseradish peroxidase (Cell Signaling). Band intensities were quantified using ImageJ and normalized to the level of EGFR for each sample [20].

Radiometric kinase assays

Reaction mixtures of 500 nM EGFR kinase domain, 750 μ M Tail Peptide A, 25 μ M ATP, 8.325 μ Ci/ml [γ - ^{32}P]ATP, 15 mM Tris, pH 7.5, 0.02% v/v polysorbate 20, and 10 mM $MgCl_2$ in a final volume of 60 μ l were incubated for 10 minutes at room temperature. A variant of Peptide C with the sequence RAHEEIAHFFFAKKK was included at the indicated concentrations. Reactions were terminated by addition of 60 μ l of 0.5% v/v phosphoric acid, and 55 μ l of the quenched reaction was spotted on each of two P81 phosphocellulose filter discs (Whatman). The discs were filtered and washed three times with 0.5% v/v phosphoric acid using a vacuum manifold (Hoefer Scientific Instruments). Five ml of UniverSol scintillation cocktail (MP Biomedicals) were added to the dried discs in scintillation vials, and the radiation was measured by liquid scintillation counting (Beckman Coulter).

Precipitation analysis

Samples of Peptide C at indicated concentrations and 2 μ M WT EGFR kinase domain, I682Q EGFR kinase domain, or BSA (Sigma Aldrich) were incubated in 10 mM Tris, pH 7.5, 20 mM NaCl, and 0.02% v/v polysorbate 20 in 75 μ l total volume for about 20 minutes at room temperature. The samples were centrifuged at $15800 \times g$ for 10 minutes at 4° C. Supernatants were removed, and pelleted materials were resuspended and resolved by SDS-PAGE. The bands were quantified by densitometry in ImageJ [20].

Dynamic light scattering measurements

Samples of 1 mM Peptide C in water, 20 mM NaCl, or assay buffer (15 mM Tris, pH 7.5, 0.02% v/v polysorbate 20, and 20 mM NaCl) were analyzed by DLS at 25° C using a DynaPro Titan (Wyatt). Measurements were made with the 60 mW laser at 15% power and at an angle of 90°. Buffer components were filtered prior to measurements. The filter retained Peptide C and as a result the peptide could not be filtered.

Transmission electron microscopy

Samples of Peptide C at indicated concentrations with and without 2 μ M wild type or I682Q EGFR kinase domain were prepared in 10 mM Tris, pH 7.5, 20 mM NaCl, and 0.02% v/v polysorbate 20 and incubated for approximately ten minutes at room temperature. Six μ l of the samples were adsorbed on Formvar carbon-coated 400 mesh copper grids (Electron Microscopy Sciences), and the grids were negatively stained with three applications of 6 μ l 1% w/v uranyl acetate. The grids were viewed with a JEOL 1200 EX transmission electron microscope.

Results and Discussion

Analysis of catalytic parameters and cooperativity for EGFR peptide substrates

We used a spectrophotometric enzyme-coupled kinase assay to characterize the kinetic parameters for Peptide C phosphorylation by EGFR in comparison to two other peptide substrates, Tail Peptide A and Tyrsb, a 13-residue peptide based on a sequence from the protein human erythrocyte Band 3 [10,19]. We were unable to reach saturating levels with Tail Peptide A and Tyrsb, and thus their catalytic parameters are estimates based on fits to the Michaelis-Menten equation (Supplemental Figure 1). Peptide C does indeed stimulate EGFR kinase activity, with a catalytic rate constant, k_{cat} , of 0.160 s^{-1} that is roughly eight-fold higher than that for Tail Peptide A (0.021 s^{-1}) and Tyrsb (0.019 s^{-1}) (Table 1). More dramatically, the value of the Michaelis constant, K_M , for Peptide C (30 μ M) is greatly decreased relative to the low millimolar values obtained for the peptides with natural sequences. Taken together, the catalytic efficiency (k_{cat}/K_M) for Peptide C is approximately 250- and 760-fold higher than those for Tail Peptide A and Tyrsb, respectively, confirming that Peptide C is a very efficient substrate for EGFR.

The rate of EGFR-catalyzed phosphorylation rises steeply in response to increased Peptide C concentration, and fitting of the data to the Hill equation confirms positive cooperativity with a Hill coefficient of ~ 2.8 (Figure 1A). Conversely, Tail Peptide A and Tyrsb exhibit little or no cooperativity (Figure 1B,C). The observed cooperativity with Peptide C suggested that the peptide might achieve its high catalytic efficiency by a mechanism not employed by the other peptide substrates.

Peptide C stimulates phosphorylation of other substrates

Although the activation of the EGFR kinase domain relies principally on formation of the asymmetric dimer, phosphorylation of Tyr-845 in the activation loop of the kinase domain is also expected to increase activity [21]. We wondered whether Peptide C could increase the catalytic activity of EGFR by increasing phosphorylation of Tyr-845. Immunoblotting with a phosphotyrosine-845 specific antibody confirmed that Tyr-845 phosphorylation increases in a dose-dependent manner with addition of Peptide C (Figure 2A,B). Surprisingly, the same dose-dependent increase in phosphorylation is observed for the tyrosines in the truncated C-terminal tail that is within the kinase domain construct, Tyr-974 and Tyr-992, indicating that Peptide C generally increases the rate of EGFR kinase domain autophosphorylation (Figure 2A,B). Phosphorylation of EGFR at Tyr-845 only results in a slight increase in *in vitro* catalytic activity, and so autophosphorylation at Tyr-845 does not

account for the marked increase in activity observed with Peptide C (Supplemental Figure 2).

The increase in autophosphorylation in the presence of Peptide C suggested that phosphorylation of other peptide substrates may also be promoted. To test this, we measured the phosphorylation of Tail Peptide A in a radiometric assay in the presence of a Peptide C variant in which the tyrosine was replaced with an alanine. This variant peptide cannot be phosphorylated by EGFR. Phosphorylation of Tail Peptide A by EGFR increases in response to the concentration of the Peptide C variant (Figure 2C). Interestingly, the activity profile with the Peptide C variant resembles that of Peptide C itself; the EC_{50} is 45 μ M and a rough fit to the Hill equation yields a Hill coefficient of ~ 2.4 (Supplemental Figure 3). Thus, the ability of Peptide C to stimulate EGFR activity is apparently independent of its role as a substrate.

Increased EGFR activity with Peptide C requires formation of the asymmetric dimer

We wondered whether Peptide C promotes the formation of the asymmetric dimer as a mechanism to increase the catalytic activity of EGFR. Such a mechanism could also underlie the observed positive cooperativity. To test whether asymmetric dimer formation is required, we utilized EGFR kinase domain constructs bearing mutations that block formation of the asymmetric dimer. One mutation, I682Q, is located on the N-lobe of the kinase domain and restricts the mutant kinase domain to serve only as an activator. The second mutation, V924R, is on the C-lobe and restricts the mutant kinase domain to being a receiver. When assayed independently, neither of these mutants alone is sufficient to produce high activity. However, combining the two mutants at high effective concentrations permits reconstitution of the asymmetric dimer [1].

The activating properties of Peptide C are entirely abrogated when assayed with either the I682Q or V924R mutants alone. However, when the two mutant EGFR kinase domains are combined, catalysis is regained to about 60% of that of the wild type kinase domain (Figure 3A). Therefore, the ability to form the asymmetric dimer is required for activation by Peptide C. In the EGFR asymmetric dimer only the receiver is activated, and this might explain why lower activity is observed with the combination of the V924R and I682Q kinase domains than for the wild type protein.

The requirements for catalytic activity of the activator and receiver kinase domains were tested by assaying kinase domains in which a catalytic residue, Asp-813, was mutated to asparagine (D813N), a mutation commonly made to inactivate kinases [22]. The combination of the inactive mutant activator I682Q/D813N and competent receiver V924R exhibits nearly identical activity with Peptide C to the combination of I682Q and V924R (Figure 3B). Alternatively, inactive mutant receiver D813N/V924R paired with competent activator I682Q has no catalytic activity, as expected from the asymmetric dimer model. Taken together, these results indicate that the mechanism by which Peptide C stimulates EGFR catalysis is consistent with formation of the asymmetric dimer; both activator and receiver kinase domains are required for activation, but only the receiver undergoes activation [1].

The dimerization mutants exhibit similar cooperativity with Peptide C to wild type EGFR

The increases in specific rate in response to titration of Peptide C for the pairs of EGFR kinase domain dimerization mutants occurs with high cooperativity, similar to what was observed with the wild type EGFR kinase domain. The Hill coefficients derived from fitting the data to the Hill equation are ~3.0 and ~2.5 for the V924R kinase domain assayed with the I682Q and I682Q/D813N kinase domains, respectively, and are similar to the Hill coefficient of ~2.8 for the wild type kinase domain (Figure 3C). This result is unexpected. We expected that the high cooperativity of the wild type kinase domain with Peptide C might result from the ability of the kinase domain to oligomerize as a chain through the asymmetric dimer interface as seen in crystal structures [1,23]. The I682Q and V924R kinase domain mutants should not, however, form chains when combined, and so the high values of the Hill coefficients observed with these combinations could not be rationalized.

Aggregation may also give rise to steep dose response curves, and in this case high Hill coefficients are often transferable between assays and enzymes [24]. Previous work has shown the abilities of several molecules to aggregate EGFR, and aggregation of EGFR correlates with increased kinase activity [15-17]. We hypothesized that, likewise, Peptide C could increase catalytic efficiency by functioning as an aggregator of the EGFR kinase domain.

Peptide C precipitates the EGFR kinase domain

To test the capacity of Peptide C to aggregate EGFR, we incubated the kinase domain with Peptide C, pelleted the insoluble materials, and resolved the samples by SDS-PAGE. The kinase domain precipitates as a function of Peptide C concentration, and the Peptide C concentration dependence of precipitation resembles that for kinase activity (Figure 4A,D). The correlation of aggregation and activation of EGFR in the presence of Peptide C suggests two possibilities. Peptide C could promote the active conformation and consequently the aggregation of the kinase domain. EGFR kinase domain constructs that are more catalytically active also have a higher propensity to aggregate [25]. Alternatively, Peptide C may aggregate EGFR, and the asymmetric dimer is stabilized as a byproduct of aggregation.

Peptide C precipitates proteins and forms aggregates on its own

To clarify the connection between activity and aggregation, we tested the ability of Peptide C to precipitate additional proteins. Peptide C precipitates the I682Q dimerization mutant EGFR kinase domain similarly to the wild type kinase domain (Figure 4B,D). This suggests that kinase activity is not required for precipitation and rather that activation occurs as result of aggregation. Furthermore, Peptide C appears to precipitate other proteins. BSA precipitates in the presence of Peptide C over the same concentration range of the peptide, although with a higher EC₅₀ (Figure 4C,D). These data suggest that Peptide C precipitates proteins over a narrow concentration range and that this precipitation appears linked to the dimerization-mediated activation of the EGFR kinase domain.

We monitored the aggregation of Peptide C by itself in solution using dynamic light scattering (DLS) and found that the peptide alone is sufficient to form aggregates. A 1 mM solution of the peptide in water exhibited limited aggregation, and the hydrodynamic radius

of the sample was almost entirely described by a polydisperse distribution of 1-10 nm. Preparation of 1 mM Peptide C in an assay buffer (15 mM Tris, pH 7.5, 0.02% v/v polysorbate 20, and 20 mM sodium chloride) resulted in a highly aggregated sample with very low mobility, which was indicated by a spike in laser intensity and minimal decay within the intensity autocorrelation decay function (Supplemental Figure 4). A 1 mM Peptide C sample in 20 mM sodium chloride showed aggregation to an intermediate degree between the aqueous solution and the buffered samples. Thus Peptide C has a propensity to form large aggregates in solution, and aggregation of Peptide C appears to be increased by the addition of other solutes.

Electron microscopy shows that Peptide C forms fibrils

We examined samples of Peptide C in the absence and presence of EGFR kinase domain by transmission electron microscopy (TEM) to gain insight into the structures formed during aggregation. Samples of Peptide C at concentrations of 20, 40, and 80 μ M all deposit as clumps of flexible fibrils that are several hundreds of nanometers in length (Figure 5A-C). The overall morphology of the fibrils appears to be independent of Peptide C concentration. Globular aggregates closely associated with the fibrils are also observed (Figure 5A-C, arrows). The TEM images show that Peptide C forms fibrils at concentrations relevant to activation of EGFR. Additionally, the globular aggregates formed by Peptide C and any monomeric, well-behaved Peptide C, which cannot be visualized by TEM, may also be important for activation of EGFR with Peptide C. The predominance and homogeneity of the fibrils suggests that if Peptide C is present in a monomeric form, the transition of Peptide C to the fibrillar state is cooperative.

We also examined samples of Peptide C in the presence of the EGFR kinase domain to try to establish a link between Peptide C and EGFR kinase domain aggregation and activation. Fibrils similar to those formed by Peptide C alone as well as globular aggregates localized near the fibrils are observed in all samples containing Peptide C (Figure 5E,F). The addition of the EGFR kinase domain causes the edges of the fibrils to appear less crisp, suggesting that EGFR might associate with the surface of the fibrils. EGFR in samples without Peptide C forms numerous small aggregates on the grid, and these are also observed in the sample with 20 μ M Peptide C (Figure 5D,E). These small, dispersed aggregates are not observed in samples of EGFR kinase domain with 80 μ M Peptide C, which suggests that free EGFR becomes depleted from solution in the presence of increasing amounts of Peptide C (Figure 5F). This observation is consistent with our studies of EGFR precipitation as a function of Peptide C concentration.

Additionally, we examined samples of the I682Q EGFR kinase domain with different concentrations of Peptide C, and the samples appear indistinguishable from wild type (Supplemental Figure 5A-C). This indicates the ability of EGFR to dimerize and activate does not grossly affect its interactions with Peptide C. The nature of the interaction between the EGFR kinase domain and Peptide C that underlies activation of the kinase domain remains unknown.

Conclusions

In this study, we have shown that Peptide C, a peptide selected for enhanced phosphorylation by EGFR, serves as a highly efficient substrate by aggregating EGFR. Aggregation appears to occur non-specifically, but because the activity of EGFR depends on the local concentration of the kinase domain, aggregation of EGFR by Peptide C results in formation of the asymmetric dimer and EGFR kinase domain activation. It has been shown previously that poly-lysine, poly-arginine, sphingosine, and protamine can aggregate and activate EGFR in an apparently cooperative manner, although it is not known if these molecules form macromolecular assemblies like those formed by Peptide C [16,17]. Peptide C appears to be the first such molecule, though, that serves as an EGFR substrate in addition to increasing kinase activity by an aggregation-mediated mechanism. All of these previously studied aggregators of EGFR are positively charged, and Peptide C contains an N-terminal arginine and three C-terminal lysines, suggesting that positive charge within the aggregating molecule may be required to aggregate EGFR.

The fibrils formed by Peptide C might provide a surface on which the EGFR can dimerize and activate. A similar mechanism has been observed for the allosteric activation of procaspase-3 upon binding to the surface of fibrils formed by a small molecule [26]. Our TEM data show that the edges of the fibrils are less clearly defined when the EGFR kinase domain is present, suggesting that the kinase domain may be associated with the surface of the fibrils.

Peptide C activates EGFR via formation of the asymmetric dimer. In one possible scenario, the fibrils formed by Peptide C might serve as a surface on which the EGFR kinase domain may bind to undergo aggregation and activation. This strategy resembles activation at the cell membrane, in which ligand binding to the full length receptor causes dimerization and activation [4]. This work studying Peptide C joins the growing body of evidence that recognizes the importance of EGFR asymmetric dimer formation in a concentration dependent manner as the mechanism underlying kinase activation [1,27,28].

The phosphorylation of Peptide C by EGFR occurs with a desirable catalytic efficiency, and as a result other studies have utilized Peptide C as a substrate for EGFR [12-14]. Despite the apparent excellent catalytic parameters of the peptide, Peptide C must be employed as a substrate with caution to avoid misinterpreting data as reflecting EGFR mechanism instead of the unusual properties of Peptide C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

C-lobe	carboxy-terminal lobe
C-terminal	carboxy-terminal
DLS	dynamic light scattering
EGFR	epidermal growth factor receptor
n_H	Hill coefficient
N-lobe	amino-terminal lobe
N-terminal	amino-terminal
TEM	transmission electron microscopy

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Figure 1. Hill coefficient measurements for EGFR kinase domain catalysis with EGFR peptide substrates

Hill coefficients for EGFR kinase domain catalysis with EGFR kinase peptide substrates (A) Peptide C, (B) Tail Peptide A, and (C) Tyrsusb were derived by fitting the specific rates of catalysis at Log_{10} (peptide concentration) to the Hill equation using Prism software by GraphPad. Because the Tail Peptide A and Tyrsusb measurements did not attain saturating concentrations, better fits for the Hill analysis were obtained when the Top (k_{cat}) values were constrained to those derived from the Michaelis-Menten fits (Table 1 and Supplemental Figure 1). Specific rates for each of the peptides were measured using the enzyme-coupled kinase assay with either 2 or 8 μM EGFR kinase domain for Peptide C or Tail Peptide A and Tyrsusb, respectively. Data points represent the means $\pm$ standard errors of mean from a minimum of three replicates, and n_H values are $\pm$ standard errors of mean.

Figure 2. The effects of Peptide C on EGFR kinase domain phosphorylation of other substrates Autophosphorylation of the EGFR kinase domain on the activation loop at Tyr-845 and the C-terminal tail at Tyr-974 and Tyr-992 in the presence of 0-60 μM Peptide C was measured by immunoblot and is shown by (A) representative immunoblots and (B) quantified band densities. Autophosphorylation reactions were performed with 800 nM EGFR kinase domain for one minute and terminated by addition of EDTA prior to immunoblotting with antibodies specific to EGFR and each phosphotyrosine. Band densities were quantified using ImageJ and normalized to EGFR kinase domain levels [20]. Values are relative to the maximum levels of phosphorylation for each phosphotyrosine. Data points represent the averages $\pm$ standard errors of mean from a minimum of three replicates. (C) Phosphorylation of Tail Peptide A ($\gamma\text{-P}$ incorporated) was measured in a radiometric kinase assay in the presence of a Peptide C variant in which the substrate tyrosine was replaced by alanine and could not undergo phosphorylation. Reactions were performed for ten minutes with 500 nM EGFR kinase domain, 750 μM Tail Peptide A, Peptide C variant at indicated concentrations, and 25 μM ATP labeled at 8.325 $\mu\text{Ci/ml}$ [$\gamma\text{-}^{32}\text{P}$]ATP and terminated by addition of 0.5% v/v phosphoric acid. Reactions were spotted onto filters, and the radioactivity was quantified by liquid scintillation counting. Data points represent the means $\pm$ standard errors of mean from four replicate filters.

Figure 3. Catalytic activities of EGFR kinase domain dimerization mutants with Peptide C
 (A) The specific rates of catalysis of EGFR kinase domain dimerization mutants I682Q, which is on the N-lobe, and V924R, which is on the C-lobe, either alone or combined were measured over a titration of Peptide C. (B) The kinase inactivating mutation D813N was introduced to each of the I682Q and V924R dimerization mutants, and specific rates for each of the kinase domain pairs I682Q/D813N with V924R or I682Q with D813N/V924R over a titration of Peptide C were measured. (C) Hill coefficients for the pairs of I682Q and V924R or I682Q/D813N and V924R were derived by fitting the specific catalytic rates from (A) and (B) at $\log_{10}$ (peptide concentration) to the Hill equation using Prism software by GraphPad. Specific rates at indicated Peptide C concentrations were measured using the enzyme-coupled kinase assay. All measurements were made using 2 μM of total kinase domain, and 1 μM of each of the mutant kinase domains when measured as a pair. Data points represent the means $\pm$ standard errors of mean from a minimum of three replicates, and n_H values are $\pm$ standard errors of mean.

Figure 4. Precipitation of proteins by Peptide C

Co-sedimentation of (A) wild type EGFR kinase domain, (B) I682Q EGFR kinase domain, and (C) BSA in the presence of Peptide C is visualized by representative SDS-PAGE gels, and (D) band densities of the precipitated proteins are quantified. Each protein (2 μ M) was incubated with 0–175 μ M Peptide C for ten minutes and centrifuged, and the resuspended pellets were resolved by SDS-PAGE. Band densities were quantified using ImageJ [20]. Data points represent the means $\pm$ standard errors of mean from a minimum of three replicates.

Figure 5. TEM images of Peptide C with and without EGFR kinase domain

Representative TEM images of (A) 20 μ M, (B) 40 μ M, and (C) 80 μ M samples of Peptide C show Peptide C forms fibrils as well as globular aggregates, which are indicated by the arrows. Representative TEM images upon addition of 2 μ M EGFR kinase domain to (D) 0 μ M, (E) 20 μ M, and (F) 80 μ M Peptide C indicate that Peptide C forms fibrils in the presence of EGFR. There appears to be lower levels of EGFR in solution at high Peptide C concentration, and the fibril edges are less defined. Samples were incubated for about 10 minutes prior to being deposited on Formvar carbon grids. The grids were negatively stained with 1% uranyl acetate and viewed using a JEOL 1200 EX transmission electron microscope. Scale bars are 100 nm.

Table 1

Kinetic parameters for EGFR kinase domain activity with EGFR peptide substrates. The specific rates of EGFR kinase domain catalysis as a function of peptide concentration were fit to the Michaelis-Menten equation to derive the catalytic rate constant k_{cat} and Michaelis constant K_M for Tail Peptide A and Tyrsb. Because the curve generated for specific rate as a function of peptide concentration for Peptide C is sigmoidal, the values of k_{cat} and K_M are instead derived from fitting the data to the Hill equation (Figure 1A). Specific rates for each of the peptides were measured in at least triplicate using the enzyme-coupled kinase assay with either 2 or 8 μM EGFR kinase domain for Peptide C or Tail Peptide A and Tyrsb, respectively. Peptide sequences are: Peptide C – RAHEEIYHFFAKKK, Tail Peptide A – RRKGSTAENAEYLRV, and Tyrsb – EELEDDYEDDMEE. The values are $\pm$ standard errors of mean and generated from data points measured in at least triplicate.

Peptide Substrate	k_{cat} (s^{-1})	K_M (μM)	$10^{-3} \times k_{\text{cat}}/K_M$ ($\text{s}^{-1}\text{M}^{-1}$)
Peptide C	0.160 ± 0.003	30 ± 1	5.3
Tail Peptide A	0.021 ± 0.001	980 ± 80	0.021
Tyrsb	0.019 ± 0.001	2900 ± 300	0.007