

Molecular Basis of Distinct Interactions Between Dok1 PTB Domain and Tyrosine-phosphorylated EGF Receptor

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Phosphotyrosine binding (PTB) domains of the adaptor proteins Doks (downstream of tyrosine kinases) play an important role in regulating signal transduction of cell-surface receptors in cell growth, proliferation and differentiation; however, ligand specificity of the Dok PTB domains has until now remained elusive. In this study, we have investigated the molecular basis of specific association between the Dok1 PTB domain and the tyrosine-phosphorylated EGFR. Using yeast two-hybrid and biochemical binding assays, we show that only the PTB domain from Dok1 but not Dok4 or Dok5 can selectively bind to two known tyrosine phosphorylation sites at Y1086 and Y1148 in EGFR. Our structure-based mutational analyses define the molecular determinants for the two distinct Dok1 PTB domain/EGFR interactions and provide the structural understanding of the specific interactions between EGFR and PTB domains in the divergent Dok homologues.

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The Dok proteins (downstream of tyrosine kinases) represent a family of adaptor proteins that play an important role in regulating signal transduction in cell growth, proliferation and differentiation.^{1–3} Five Dok proteins with distinct functions have been identified thus far.³ All Dok proteins consist of an N-terminal pleckstrin homology (PH) domain followed by a central phosphotyrosine binding (PTB) domain and a C-terminal domain that contains multiple potential tyrosine phosphorylation sites and proline-rich sequences.³ While the C-terminal region may serve as docking sites for SH2 and SH3-containing proteins, the PH domain is necessary for targeting Dok to binding phospholipids in the cell membrane.⁴ The conserved PTB domains in Dok proteins are important for protein–protein interactions not only in cell spreading and migration but also in cell growth and Ras activity in B16F10 cells.⁵ For instance, Dok1 (also known as p62^{Dok}) associates with SHIP1

through its PTB domain. The complex is required for the SHIP1-dependent regulation of cell migration.^{6,7} Dok1 and Dok2 can bind through their PTB domains to the Abelson tyrosine kinase,⁸ and an intact PTB domain of Dok2 is critical for its association with EGF and Tek receptors.^{9,10} Finally, Dok4 and Dok5 PTB domains bind to tyrosine-phosphorylated RET at tyrosine 1062, which results in tyrosine phosphorylation of the Dok proteins.³

The structurally conserved PTB domains found in many cytoplasmic proteins are important for numerous cellular processes including signaling of cell-surface receptors and protein trafficking.^{11,12} The discovery of the PTB domains in the signaling protein Shc and insulin receptor substrate 1 (IRS-1)¹³ attributes to their recognition of phosphotyrosine in the context of NPXpY sequences (where pY is phosphotyrosine and X is any amino acid) with hydrophobic residues N-terminal to this sequence^{14–16} — a function distinct from that of the classical SH2 domain. Recent studies show that the PTB domain can bind proteins independent of tyrosine phosphorylation or even the canonical NPXY motif. In addition, the PTB domains of Shc¹⁷ and mammalian protein Disabled¹⁸ interact with phospholipids. The functional versatility of

Abbreviations used: PTB, phosphotyrosine; Doks, downstream of tyrosine kinases; PH, pleckstrin homology; IRS-1, insulin receptor substrate 1.

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the PTB domain family is further highlighted by membrane-anchored adaptor proteins FRS2 α / β (FGFR substrate 2 α / β ; known as SNT-1/2, *suc1*-associated neurotrophic factor target).^{19–21} FRS2 α / β PTB domains are capable of interacting with unrelated sequences in two different receptors, i.e., a tyrosine-phosphorylated NPXpY motif in Trk receptor tyrosine kinase^{22,23} and a non-phosphorylated segment in FGF receptors containing no tyrosine or asparagine residues.^{24–26}

Despite the functional significance of Dok proteins, ligand specificity of Dok PTB domains was, until now, poorly understood. To understand molecular mechanisms of Dok proteins in cell signaling, we use the Dok1 PTB domain and EGFR association as a model system to investigate the specificity of Dok PTB domains.

The PTB Domain of Dok1 Specifically Interacts with EGFR

It has been reported that Dok1 is tyrosine phosphorylated upon activation of EGFR.^{27,28} To confirm whether the tyrosine phosphorylation of Dok1 requires its direct interaction with EGFR and whether the Dok1/EGFR interaction involves the PTB domain of Dok1, we employed the yeast two-hybrid binding assay. Yeast cells co-transfected with the intracellular domain of EGFR (EGFR-IC) and Dok1 or just the Dok1 PTB domain grow on selective medium, whereas no growth was observed for yeast cells co-transfected with EGFR-IC and Dok1 with a deleted PTB domain (Figure 1(a)). In contrast, yeast cells co-transfected with EGFR-IC and full-length Dok4 or Dok5 or their PTB domains did not grow on selective medium, suggesting that EGFR is specifically associated with the PTB domain of Dok1 but not Dok4 or Dok5. We have also tried to use the PTB domains as baits to do reciprocal tests. Unfortunately, they were testified to be auto-activated and not suitable for further study. This specific interaction between Dok1 PTB domain and EGFR was further demonstrated by GST pull-down assay *in vitro*. As shown in Figure 1(b), the Dok1 PTB domain-GST fusion protein specifically recognizes EGFR expressed in COS7 cells, which were activated by EGF treatment. The PTB domain of Dok4 or Dok5 shows little, if any, visible interaction with the tyrosine-phosphorylated EGFR under the same assay conditions. Together, these results demonstrate that Dok1 forms a specific complex with the tyrosine-phosphorylated EGFR through its PTB domain.

Dok1 PTB Domain Selectively Recognizes pY1086 and pY1148 on EGFR

To determine which tyrosine residues in EGFR mediate the receptor interaction with the Dok1 PTB domain, we performed a GST pull-down assay of

Dok1 PTB domain and EGFR binding in the presence of a series of synthetic, tyrosine-phosphorylated peptides derived from the known major phosphorylation sites in EGFR (Figure 2(a)). While peptides containing pY992, pY1114 or pY1173 did not reduce Dok1 PTB domain binding to EGFR, incubation of peptides containing pY1086 or pY1148 at 30 μ M in the binding assay buffer resulted in almost complete loss of Dok1 PTB domain binding to EGFR (Figure 2(b)). These results demonstrate that Dok1 PTB domain specifically interacts with activated EGFR *via* both pY1086 and pY1148.

To confirm their importance for the interaction between Dok1 PTB domain and EGFR, we mutated Y1086 and Y1148 in EGFR individually to Phe. Yeast two-hybrid assays with these two mutants (Y1086F and Y1148F) showed an eight or twofold reduction in binding activity, respectively, when compared to the wild-type receptor (Figure 2(c)). Moreover, a double Y1086F/Y1148F mutant almost completely eliminated the receptor interaction with the PTB domain. These data also indicate that pY1086 is the major site and pY1148 is a minor site in EGFR for Dok1 PTB domain binding.

Identification of Key Dok1 PTB Domain Residues Critical for Interactions with EGFR

To identify the functionally important amino acid residues for ligand specificity of the Dok1 PTB domain, we first compared and analyzed structure-based sequences between the PTB domains of Dok1 and IRS-1, and then performed structural homology modeling using the IRS-1 PTB domain/IL-4R peptide complex^{12,29} as a template (data not shown). Based on the modeling, we carried out site-directed mutagenesis of the protein residues that appear involved in direct interactions with the EGFR peptides. In an effort to dissect the PTB domain interactions with two different phosphorylation sites in EGFR, we also generated EGFR constructs that carry either a single or double mutation by substituting Y1086 and/or Y1148 with phenylalanine.

As shown in Figure 3(a), change of T204, R223, Q252 or K253 to alanine in the protein does not affect PTB domain interaction with EGFR at pY1086, whereas mutation of Y203, L206, R207, Y209, R222 or I249 to alanine results in a nearly complete loss of the EGFR binding. These mutagenesis data together with the model structure provide an explanation for the possible ligand-binding contributions of the residues at the ligand-binding site: (1) R207 and R222 are important for phosphotyrosine binding; (2) Y203 is important for hydrophobic recognition of Val(pY–1) (pY–1 represents the position 1 amino acid residue N-terminal to the phosphotyrosine, whereas pY+1 means one amino acid residue C-terminal to the phosphotyrosine); (3) L206 and I249 likely interact with hydrophobic moieties of the side-chains of Asn(pY–3) and Pro(pY–2) of the

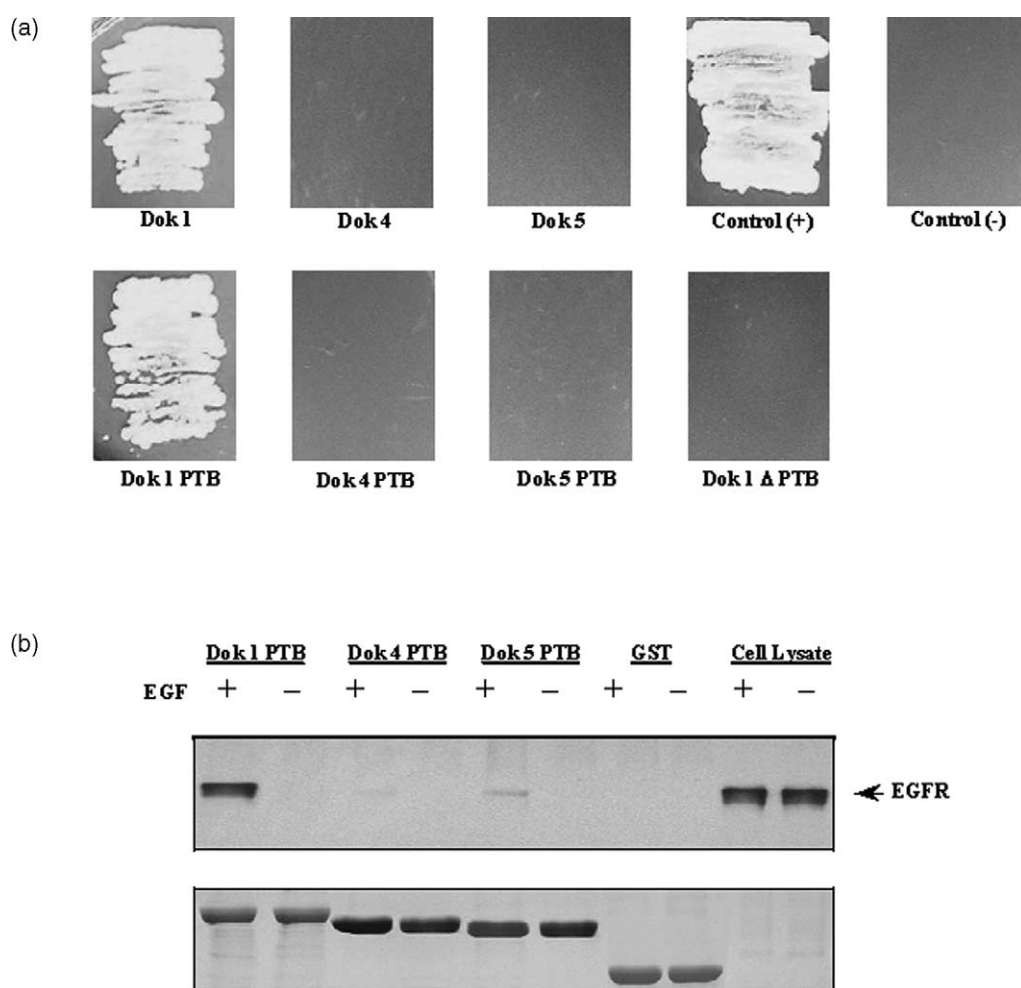


Figure 1. The Dok1 PTB domain specifically interacts with EGFR. (a) Interaction of Dok proteins or their PTB domains with EGFR in the yeast two-hybrid assay. The yeast strain EGY48 and MatchMaker LexA system were from Clontech. The co-transformants were plated on SD/gal/-ura-his-trp-leu drop-out galactose and only those co-transformants in which the hybrid proteins interact will form colonies. Yeast co-transfected with pGilda and pB42AD vector served as negative control, or with pLexA-53 and pB42AD-T as positive control. (b) GST pull-down assay assessing the Dok PTB domains' interaction with EGFR present in lysates from unstimulated or EGF-stimulated COS7 cells. EGFR bound to the immobilized GST Dok proteins was detected by immunoblotting with anti-EGFR antibodies (upper panel). A SDS-PAGE gel demonstrates relative equal amount of the GST fusion proteins used in the each experiments (lower panel). COS7 cells were stimulated for ten minutes at 37 °C with 100 ng/ml EGF (Shanghai SIBC) in DMEM supplemented with 5 mM HEPES (pH 7.4) and 0.1% bovine serum albumin. Purified GST fusion proteins (10 µg) were incubated with 100 µl COS7 cell lysates and glutathione-Sepharose 4B beads for three hours at 4 °C with gentle rotation. After washing three times with lysis buffer, the bound proteins were subjected to SDS-PAGE. GST fusion proteins were visualized by Coomassie blue staining, while EGFR was detected by immunoblotting using anti-EGFR(sc-03) antibody (Santa Cruz) and visualized with ECL (Pierce).

peptide; and (4) Y209 and I249 located in the hydrophobic pocket form extensive intermolecular interactions with the bulky hydrophobic Val(pY-5).

As shown in Figure 3(b), change of Q252 or K253 to alanine in the protein does not affect the interaction of PTB domain with EGFR at pY1148, while mutation of Y203, L206, R207, Y209, or I249 to alanine results in a nearly complete loss of the EGFR binding. These results suggested that these residues might play a similar role for the PTB domain recognizing the EGFR pY1148 site and pY1086 site. However, an alanine substitution at T204 and R223, which has shown no effect on PTB domain binding

to EGFR at pY1086 site, resulted in a loss of binding to EGFR at pY1148 site. The functional contributions of T204 and R223 in binding to pY1148 but not pY1086 are further confirmed in the wild-type EGFR binding, as a point mutation of T204 or R223 to Ala results in an affinity reduction comparable to that observed in Dok1/EGFR Y1148F association (Figure 3(a) and (c), lanes 2 and 7). Finally, as expected, the double mutant Y1086F/Y1148F failed to interact with the Dok1 PTB domain or its mutants almost completely (Figure 3(d)).

Interestingly, while amino acid sequences flanking pY1086 and pY1148 in EGFR contain the same

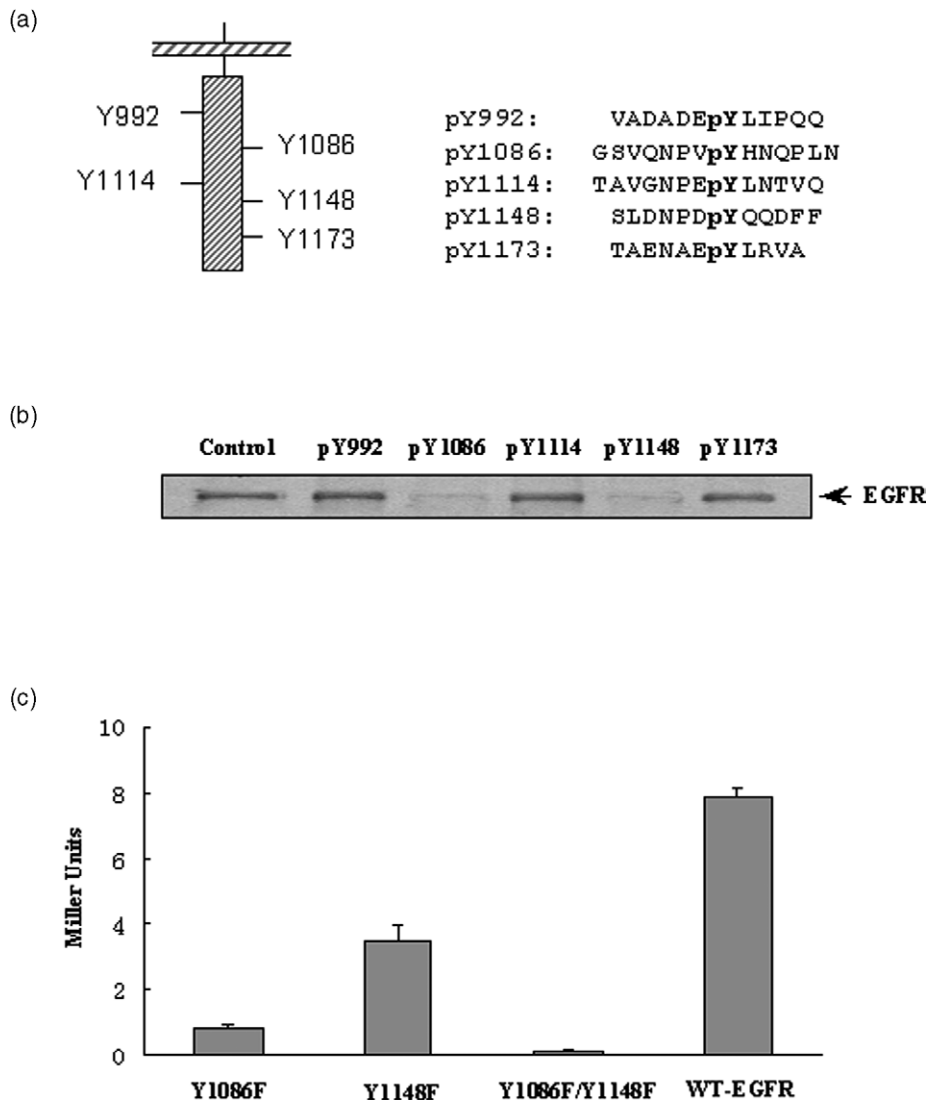


Figure 2. Dok1 PTB domain recognition of EGFR at pY1086 and pY1148. (a) Schematic diagram showing the intracellular domain of EGFR (left) and the amino acid sequences of the synthetic peptides derived from the known tyrosine phosphorylation sites in EGFR (right). (b) Competition of EGFR peptides against EGFR binding to the Dok1 PTB domain. In the peptide inhibition assays, the immobilized GST Dok1 PTB domain was incubated with the lysates from EGF-treated cells with or without (control) one of the tyrosine-phosphorylated peptides (30 μ M). EGFR bound to the immobilized GST-Dok1 PTB domain was detected by immunoblotting using anti-EGFR antibodies. (c) Binding of the Dok1 PTB domain to the wild-type or mutant EGFR proteins as determined by a β -galactosidase liquid assay using the yeast two-hybrid system. All of the mutants were made by QuikChange site-directed mutagenesis kit (Stratagene). Liquid culture assay using *o*-nitrophenyl-D-galactoside (ONPG) was performed following the Clontech Yeast Protocols Handbook. One Miller unit of β -galactosidase is defined as the amount that hydrolyzes 1 mM of ONPG to *o*-nitrophenol and D-galactose per minute per cell. The β -galactosidase values represent three independent assays, each of which is an average of triplicate determinations.

canonical Φ XNPXpY motif (where Φ is a bulky hydrophobic amino acid) for PTB domain recognition, they bind to the Dok1 PTB domain with very different binding affinity (Figures 2(c), 3(a) and (b)). Change of Val(pY-1) at the pY1086 site to a negatively charged Asp at the pY1148 site would result in a loss of its hydrophobic-aromatic interaction with Y203 of the protein (Figure 3(b)); this difference could possibly create an electrostatic repulsion between Asp(pY-1) and E179 and E181 in the β 2/ β 3 loop of the protein (data not shown). A similar change at the pY1114 site, which otherwise

contains the canonical Φ XNPXpY sequence, likely prevents an interaction with PTB domain completely (Figure 2(a) and (b)). Surprisingly, this change at the pY1148 site did not abolish the PTB domain interaction, but caused only an \sim threefold reduction in binding affinity (Figure 2(c)). The model structure suggests that R223 is likely to interact directly with Asp(pY+3) in the pY1148 site (data not shown), thus compensating the unfavorable interactions with the pY-1 residue.

To test the relative contributions to the interaction for the pY-1, pY+3 sites, we performed further

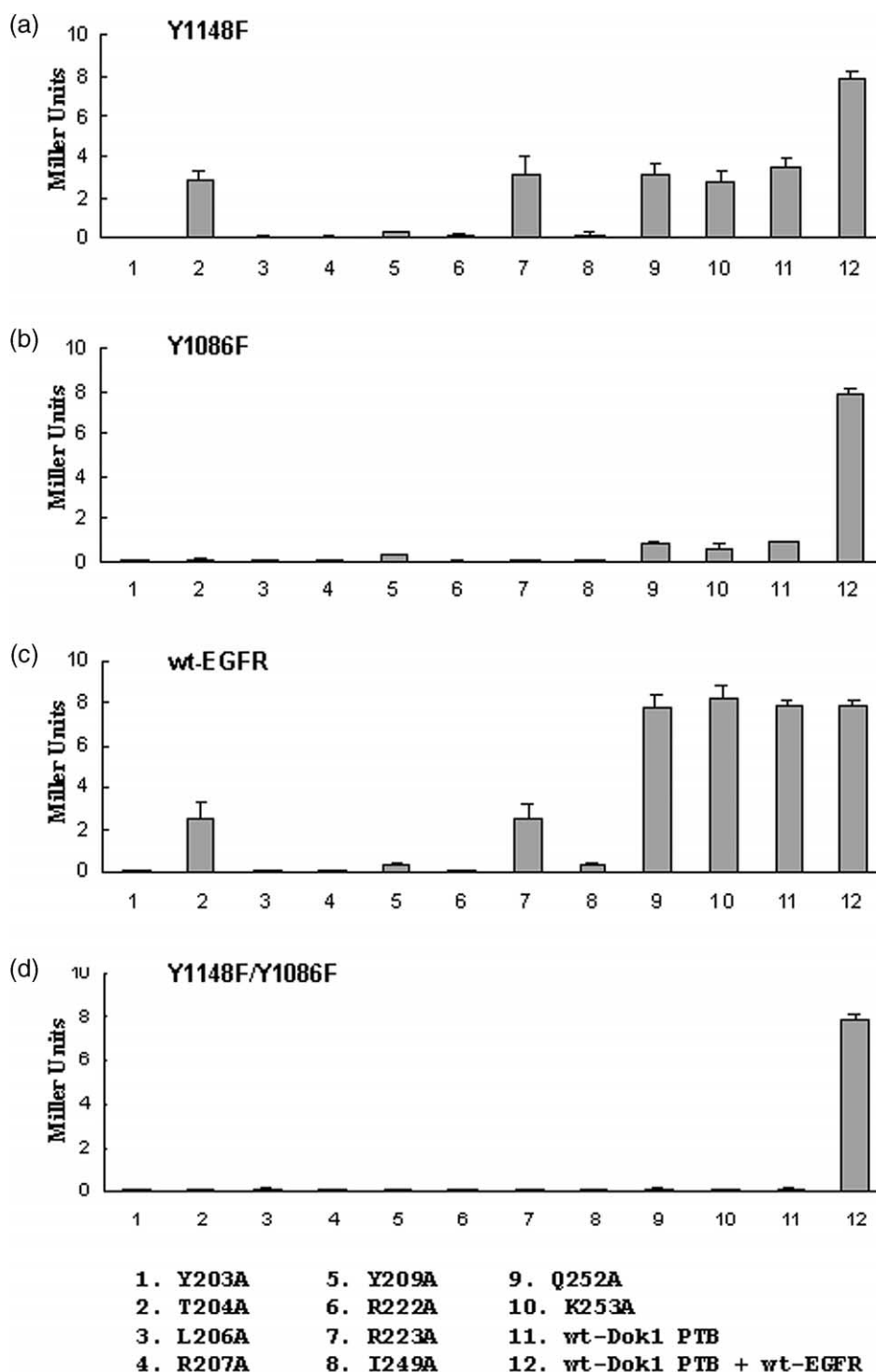


Figure 3. Mutational analysis of Dok1 PTB domain and EGFR interactions. (a–d) Effects of a single mutation of Y1148F or Y1086F or a double mutation of Y1148F/Y1086F on EGFR binding to the wild-type or various mutants of the Dok1 PTB domain were assessed by a β -galactosidase liquid assay using the yeast two-hybrid system as described above. The β -galactosidase values represent three independent assays, each of which is an average of triplicate measurements.

mutagenesis analysis. Val(pY–1) at the pY1086 site was changed to Asp, and Asp(pY–1) at the pY1148 site was changed to Val. As shown in the second column of Figure 4(a), change of Val(pY–1) at the pY1086 site to a negatively charged Asp in EGFR results in a significant decreased interaction.

Change of Asp(pY–1) at the pY1048 site to Val, as shown in the second column of Figure 4(b), results in a significant increased interaction. These results indicated that the negatively charged residue at pY–1 site in EGFR would be unfavorable for the interaction with Dok1 PTB domain. The

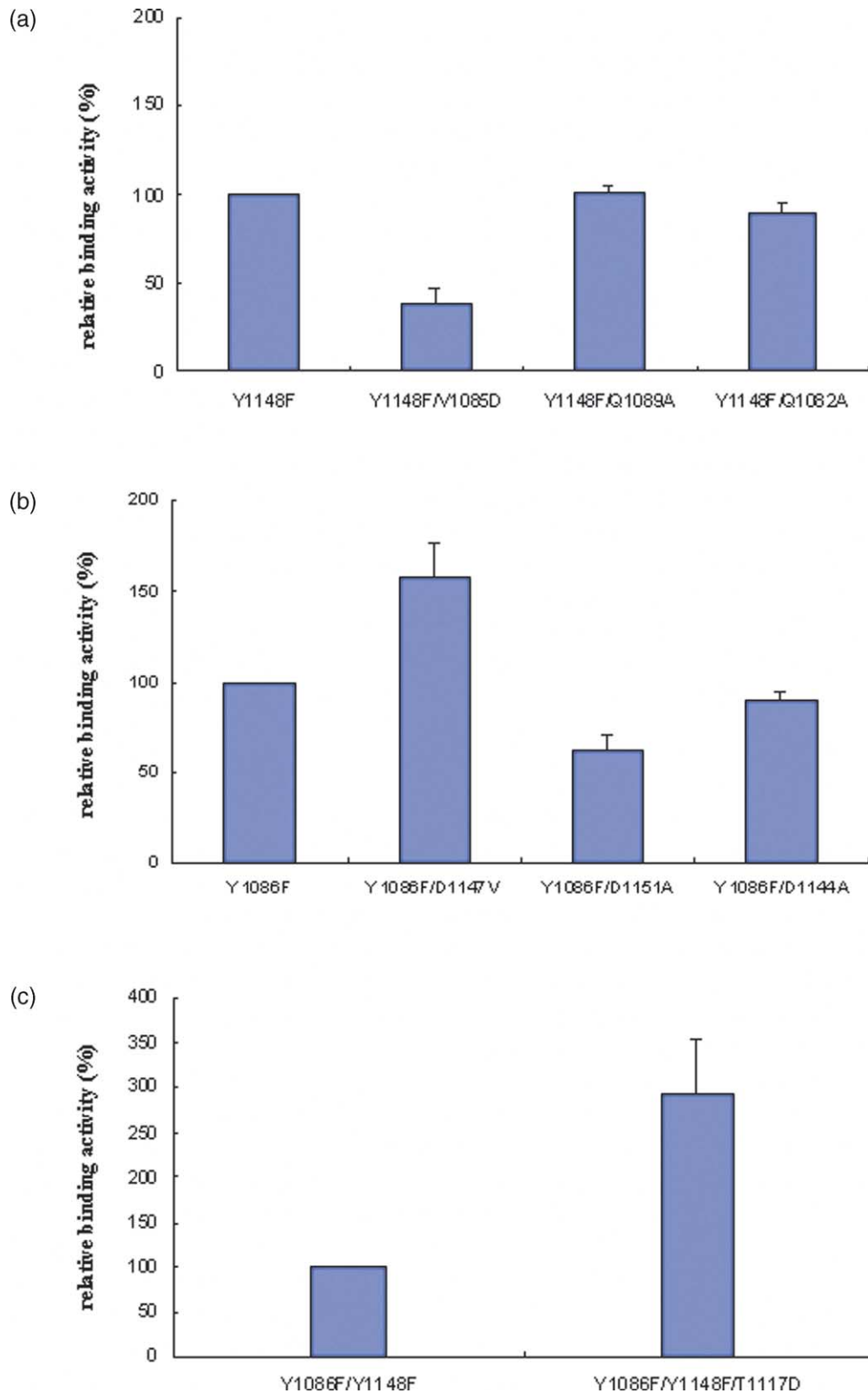


Figure 4. Mutational analysis of the relative contribution of residues pY-1, pY-4 and pY+3 sites in EGFR for interaction with wild-type Dok1 PTB domain. Binding activity of the EGFR mutants to Dok1 PTB domain was determined by a β -galactosidase liquid assay using the yeast two-hybrid system as described above. The normalized data shown represent at least three independent assays.

Asp(pY+3) residue at pY1148 site is critical for the interaction with Dok1 PTB domain, because the mutation of D1151 to alanine results in a significant reduction of interaction as shown in the third column of Figure 4(b). The functional contributions of Asp(pY+3) in binding to Dok1 PTB domain is further confirmed in pY1114 site, as a point mutation of T1117 to Asp results in an increased binding activity as shown in Figure 4(c). In contrast, change of Q1089 to alanine at pY1086 site does not affect the interaction with Dok1 PTB domain as shown in the third column of Figure 4(a).

As there is also an Asp at pY-4 in pY1148 site, Ala substitutions were made also at this position in both pY1148 and pY1086 site. As shown in Figure 4(a) and (b), Y1086F/D1144A displayed a similar activity with Y1086F, while Y1148F/Q1082A presented no significant difference with Y1148F. These results suggested that pY-4 is unlikely important in either 1086 or 1148 site in EGFR for the interaction with Dok1 PTB domain.

Concluding Remarks

Unlike the classical SH2 domains, the conserved PTB domains exhibit broad ligand binding specificity.^{14,16} Some single PTB domains are capable of recognizing multiple, structurally diverse ligands.^{11,12} The diverse functionality of PTB domains does not necessarily compromise their highly distinctive ligand selectivity. The strategy for achieving such high selectivity is well illustrated in the molecular mechanisms by which the Dok1 PTB domain interacts with EGFR at pY1086 and pY1148 sites. Like many other PTB domains,¹⁴⁻¹⁶ the Dok1 PTB domain utilizes a conserved mechanism to recognize the three core elements of the canonical Φ XNPXpY motif at both tyrosine phosphorylation sites. Particularly, the PTB domain uses structurally conserved R207 and R222 residues to recognize pY. The corresponding Arg residues in the PTB domains of Shc, IRS-1 and FRS2 α have been shown to be important for pY binding.^{13,19-21} The NPXpY motif of the peptide, bound in a cleft formed between β 5 and the C terminus of α 1 adopts a β -turn conformation, projecting the pY into its binding pocket to coordinate with the two conserved Arg residues. L206 and I249 in the PTB domain likely interact with hydrophobic moieties of the side-chains of Asn(pY-3) and Pro(pY-2) of the peptide. Finally, the bulky, hydrophobic residue Val or Leu at pY-5 forms extensive interactions with Y209 and I249 in a hydrophobic pocket lined between β 5 and α 1.

Our new knowledge of the mechanisms underlying Dok1 PTB domain/EGFR association explains why Dok4 or Dok5 PTB domains do not recognize the tyrosine-phosphorylated EGFR. The reason is clear in the structure-based sequence alignment of the Dok PTB domains. Corresponding to the functionally important residues of Y203, T204, R223 and I249 in Dok1 are Leu, Ser, Met and Ala

in Dok4 and Leu, Cys, Met and Thr in Dok5, respectively. Besides possible additional differences in structures and/or sequences of the PTB domains, these differences of key amino acid residues at the ligand-binding site alone could result in loss of EGFR association by the Dok4 or Dok5 PTB domain. By the same reason, we predict that the Dok2 PTB domain may possess ligand selectivity similar to that of the Dok1 PTB domain. On the other hand, the ligand specificity of the Dok3 PTB domain remains unclear, as it has an Asp at the position corresponding to I249 in Dok1.

The influence on the affinity of Dok1 PTB domain/EGFR association by varying the residue at pY-1 of the ligand sequence is reminiscent of the IRS-1 PTB domain/receptor interaction.²⁹ Change of this Ala(pY-1) to Glu at the known IRS-1 binding site in IR at pY960 (LYASSNPEpYLS) generates unfavorable interactions, resulting in a 48-fold decrease in affinity.²⁹ In this study, a hydrophobic residue at pY-1 such as Val in the pY1086 site, which interacts with Y203 of the protein, may be essential for the tight association of the Dok1 PTB domain and EGFR, as mutation of Val to a negatively charged Asp results in a significantly decreased interaction. Substitution of Val to Glu at pY-1 as in the pY1114 sequence, which otherwise contains a complete canonical Φ XNPXpY motif, could lead to an electrostatic repulsion between Glu(pY-1) and E179 and E181 of the PTB domain, thus resulting in a complete loss of the complex. However, this impairment of the complex formation due to the unfavorable interactions at pY-1 can be compensated to some extent by the creation of new interactions between the PTB domain and other amino acid residues in the ligand sequence. Particularly, for the pY1148 site, which contains Asp at pY-1 and the canonical Φ XNPXpY motif, the PTB domain is likely to recognize Asp(pY+3) through specific interactions with T204 and R223. These additional interactions do not completely overcome the unfavorable repulsive force at pY-1, but allow a stable association between the PTB domain and the ligand.

While this work was being reviewed, a three-dimensional crystal structure of the Dok1 PTB domain in complex with a tyrosine-phosphorylated peptide (STWIENKLpYGMDSGGK) derived from RET has been reported.³⁰ While the coordinates of this new structure are not available at this time, this structure, as described here confirms our predicted structural model of molecular interactions of this PTB domain for hydrophobic amino acid residues at pY-1 and pY-5 positions in EGFR at pY1086, which are indeed similar to those seen in the IRS1 PTB domain interactions with IL-4R at pY960.²⁹

In summary, the systematic structural and biochemical analyses of the Dok1 PTB domain reported in this study provide the structural and molecular basis as to why the Dok1 PTB domain recognizes pY1086 and pY1148 sites but not other Φ XNPXpY sites in the activated, tyrosine-phosphorylated EGFR, how PTB domain binding

affinity for the pY1086 site is significantly higher than that for the pY1148 site, and why the structurally homologous Dok4 and Dok5 PTB domains do not bind to the tyrosine-phosphorylated EGFR. The mechanisms underlying Dok1 PTB domain/EGFR association may benefit the further structural understanding of the specific interactions between receptor tyrosine kinase and PTB domains of the divergent Dok homologues.

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