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Research paper

# Molecular basis of the binding of YAP transcriptional regulator to the ErbB4 receptor tyrosine kinase

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ABSTRACT

The newly discovered transactivation function of ErbB4 receptor tyrosine kinase is believed to be mediated by virtue of the ability of its proteolytically-cleaved intracellular domain (ICD) to physically associate with YAP2 transcriptional regulator. In an effort to unearth the molecular basis of YAP2–ErbB4 interaction, we have conducted a detailed biophysical analysis of the binding of WW domains of YAP2 to PPXY motifs located within the ICD of ErbB4. Our data show that the WW1 domain of YAP2 binds to PPXY motifs within the ICD in a differential manner and that this behavior is by and large replicated by the WW2 domain. Remarkably, while both WW domains absolutely require the integrity of the PPXY consensus sequence, non-consensus residues within and flanking this motif do not appear to be critical for binding. In spite of this shared mode of binding, the WW domains of YAP2 display distinct conformational dynamics in complex with PPXY motifs derived from ErbB4. Collectively, our study lends new insights into the molecular basis of a key protein–protein interaction involved in a diverse array of cellular processes.



## 1. Introduction

A key member of the receptor tyrosine kinase (RTK) family, ErbB4 is comprised of a central single-helical transmembrane (TM) domain flanked between an N-terminal extracellular domain (ECD) and a C-terminal intracellular domain (ICD) (Fig. 1a). Upon stimulation with its extracellular ligand heregulin or in response to TPA (12-*O*-tetradecanoylphorbol-13-acetate)-induced activation of protein kinase C, the ErbB4 receptor tyrosine kinase undergoes intracellular proteolytic cleavage by γ-secretase [1,2]. This coupled event culminates in the release of ICD and marks the initiation of ErbB4 intracellular signaling. Notably, the ICD of ErbB4 harbors putative PPXY motifs (designated PY1, PY2 and PY3)—the chemical baits that serve as recognition sites for the recruitment of WW-containing proteins such as YAP (YES-associated protein) transcriptional regulator [3,4], WWOX (WW-containing oxidoreductase) tumor suppressor [5], and ITCH ubiquitin ligase [6]. The physical association between YAP and ICD facilitates translocation of the latter to the nucleus [3], where it is believed to regulate the transcription of hitherto unidentified target genes involved in key cellular processes including embryonic development [7]. While YAP acts as transcriptional co-activator of ErbB4, interaction with WWOX not only results in the cytoplasmic sequestration of ICD but also suppresses its transcriptional co-activation by YAP [5]. On the other hand, binding to ITCH promotes polyubiquitination and degradation of ErbB4, thereby regulating its stability and the availability of ICD for subsequent transcriptional regulation in the nucleus [6]. The concerted action of WWOX and ITCH thus antagonizes the co-activation function of YAP by virtue of their ability to bind to the ICD of ErbB4 in a competitive manner.

Originally identified as a binding partner of YES tyrosine kinase [8], YAP is comprised of two major isoforms termed YAP1 and YAP2, also referred to as YAP1-1 and YAP1-2 on the basis of new nomenclature [9]. While YAP2 contains a tandem copy of WW domains (termed WW1 and WW2) located N-terminal to the transactivation (TA) domain (Fig. 1b), WW2 domain is deleted in YAP1 through RNA splicing [10]. In addition to its key role in

*Abbreviations:* ErbB4, erythroblastic (Erb) leukemia viral oncogene homolog B4; ITC, isothermal titration calorimetry; ITCH, ubiquitin ligase itchy homolog; LIC, ligation-independent cloning; MM, molecular modeling; PPII, polyproline type II (helix); RTK, receptor tyrosine kinase; SEC, size-exclusion chromatography; SH3, Src homology 3; SLS, static light scattering; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; YAP, YES-associated protein; WWOX, WW-containing oxidoreductase.

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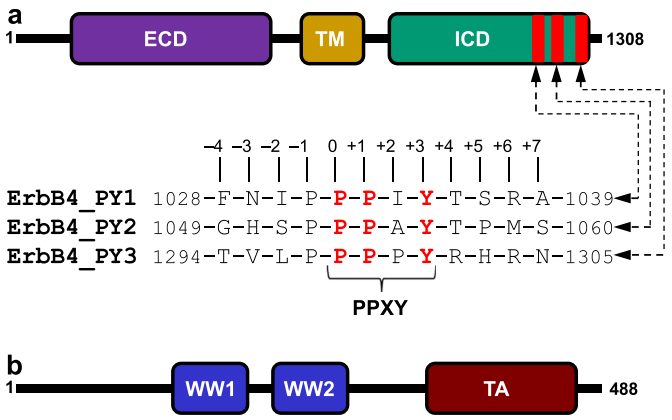


**Fig. 1.** Modular organization of human ErbB4 and YAP2 proteins. (a) ErbB4 contains the canonical ECD-TM-ICD receptor tyrosine kinase modular cassette, where the central single-helical transmembrane (TM) domain is flanked between an N-terminal extracellular domain (ECD) and a C-terminal intracellular domain (ICD). The three PPXY motifs (designated PY1, PY2 and PY3) within the ICD are located at the extreme C-terminus. Note that the amino acid sequence of 12-mer peptides containing the PPXY motifs and flanking residues are provided. The numerals indicate the nomenclature used in this study to distinguish residues within and flanking the PPXY motifs relative to the first consensus proline, which is arbitrarily assigned zero. (b) YAP2 is comprised of a tandem copy of WW domains, designated WW1 and WW2, located N-terminal to the transactivation (TA) domain.

mediating the transactivation function of ErbB4 receptor tyrosine kinase [3,4], YAP also serves as a transcriptional regulator of a multitude of cellular factors including p73, RUNX, TEAD, LATS1, ErbB4 and, in particular, plays a key role in mediating the Hippo signaling pathway [11–18]—involved in regulating the size of organs and in the suppression of tumors through inhibiting cellular proliferation and promoting apoptosis. Consistent with these observations, YAP-knockout in mice results in embryonic lethality [19]. Most importantly, the YAP–ICD interaction is mediated by the canonical binding of WW domains of YAP to PPXY motifs located within the ICD of ErbB4 (Fig. 1a and b). It should be noted here that ICD of ErbB4 is a much more potent co-activator of YAP2 than YAP1 [3]. This finding most likely argues in favor of a multivalent interaction between the tandem WW domains of YAP2 and PPXY motifs within ICD. In an effort to uncover the molecular basis of YAP–ErbB4 interaction, we report herein a detailed biophysical analysis of the binding of WW domains of YAP2 to PPXY motifs located within the ICD of ErbB4. Briefly, our data show that the WW1 domain of YAP2 binds to PPXY motifs within the ICD in a differential manner and that this behavior is by and large replicated by the WW2 domain. Remarkably, while both WW domains absolutely require the integrity of the PPXY consensus sequence, non-consensus residues within and flanking this motif do not appear to be critical for binding. In spite of this shared mode of binding, the WW domains of YAP2 display distinct conformational dynamics in complex with PPXY motifs derived from ErbB4.

## 2. Materials and methods

### 2.1. Protein preparation

WW1 domain (residues 171–205) and WW2 domain (residues 230–264) of human YAP2 were cloned into pET30 bacterial expression vectors with an N-terminal His-tag using Novagen ligation-independent cloning (LIC) as described earlier [20]. Recombinant proteins were subsequently expressed in *Escherichia coli* BL21*(DE3) bacterial strain (Invitrogen) and purified on a Ni-NTA affinity column using standard procedures [20]. Further treatment on a Hiload Superdex 200 size-exclusion chromatography (SEC) column coupled in-line with GE Akta FPLC system led to purification of WW domains to apparent homogeneity as judged by SDS-PAGE analysis. Final yield was typically between 50 and 100 mg protein of apparent homogeneity per liter of bacterial culture. Protein concentration was determined spectrophotometrically on the basis of extinction coefficients calculated for each protein construct using the online software ProtParam at ExPasy Server [21].

### 2.2. Peptide synthesis

12-mer wildtype and mutant peptides spanning various PPXY motifs within the ICD of human ErbB4 were commercially obtained from GenScript Corporation. The wildtype sequence of these peptides is shown in Fig. 1a. The peptide concentrations were measured gravimetrically.

### 2.3. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed on a Microcal VP-ITC instrument and data were acquired and processed using the integrated Microcal ORIGIN software. All measurements were repeated at least three times. Briefly, WW domains of YAP2 and ErbB4 peptides were dialyzed in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA and 5 mM β-mercaptoethanol at pH 7.0. The experiments were initiated by injecting 25 × 10 μl aliquots of 4 mM of each peptide from the syringe into the calorimetric cell containing 1.46 ml of 40–60 μM of each WW domain at 25 °C. The change in thermal power as a function of each injection was automatically recorded using the ORIGIN software and the raw data were further processed to yield binding isotherms of heat release per injection as a function of molar ratio of each peptide to WW domain construct. The heats of mixing and dilution were subtracted from the heat of binding per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was titrated against each peptide in an identical manner. To extract the binding constant ($K_d$) and binding enthalpy ($\Delta H$), the ITC isotherms were iteratively fit to a one-site model by non-linear least squares regression analysis using the integrated ORIGIN software as described earlier [22,20]. The free energy change ($\Delta G$) upon peptide binding was calculated from the relationship:

$$\Delta G = RT \ln K_d \quad (1)$$

where $R$ is the universal molar gas constant (1.99 cal/K/mol) and $T$ is the absolute temperature. The entropic contribution ($T\Delta S$) to the free energy of binding was calculated from the relationship:

$$T\Delta S = \Delta H - \Delta G \quad (2)$$

where $\Delta H$ and $\Delta G$ are as defined above.

### 2.4. Circular dichroism

Far-UV circular dichroism (CD) measurements were conducted on a Jasco J-815 spectropolarimeter thermostatically controlled at 25 °C. Briefly, ErbB4 peptides were dialyzed in 10 mM sodium phosphate at pH 7.0 and experiments were conducted on 100 μM sample of each peptide. Data were collected using a quartz cuvette with a 2-mm pathlength in the 185–255 nm wavelength range and with a slit bandwidth of 2 nm at a scan rate of 10 nm/min. All data were normalized against reference spectra to remove the contribution of buffer. Each data set represents an average of four scans acquired at 0.1 nm intervals. Data were converted to mean



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