Ligand Binding Study of Human PEBP1/RKIP: Interaction with Nucleotides and Raf-1 Peptides Evidenced by NMR and Mass Spectrometry

Lauretta Tavel1, Lucie Jaquillard2, Andreas I. Karsisiotis2, Fabienne Saab2,3, Laurence Jouvensal2, Alain Brans4, Agnès F. Delmas2, Françoise Schoentgen5, Martine Cadene2, Christian Damblon1*

1 Department of Chemistry, University of Liège, Liège, Belgium, 2 CBM, CNRS, Orléans, France, 3 Institut de Chimie Organique et Analytique (ICOA), University of Orléans, CNRS FR 2708, UMR 7311, Orléans, France, 4 IJP, University of Liège, Liège, Belgium, 5 IMPMC, University Pierre & Marie Curie (P6), Paris, France

Abstract

Background: Human Phosphatidylethanolamine binding protein 1 (hPEBP1) also known as Raf kinase inhibitory protein (RKIP), affects various cellular processes, and is implicated in metastasis formation and Alzheimer’s disease. Human PEBP1 has also been shown to inhibit the Raf/MEK/ERK pathway. Numerous reports concern various mammalian PEBP1 binding ligands. However, since PEBP1 proteins from many different species were investigated, drawing general conclusions regarding human PEBP1 binding properties is rather difficult. Moreover, the binding site of Raf-1 on hPEBP1 is still unknown.

Methods/Findings: In the present study, we investigated human PEBP1 by NMR to determine the binding site of four different ligands: GTP, FMN, and one Raf-1 peptide in tri-phosphorylated and non-phosphorylated forms. The study was carried out by NMR in near physiological conditions, allowing for the identification of the binding site and the determination of the affinity constants KD for different ligands. Native mass spectrometry was used as an alternative method for measuring KD values.

Conclusions/Significance: Our study demonstrates and/or confirms the binding of hPEBP1 to the four studied ligands. All of them bind to the same region centered on the conserved ligand-binding pocket of hPEBP1. Although the affinities for GTP and FMN decrease as pH, salt concentration and temperature increase from pH 6.5/NaCl 0 mM/20°C to pH 7.5/NaCl 100 mM/30°C, both ligands clearly do bind under conditions similar to what is found in cells regarding pH, salt concentration and temperature. In addition, our work confirms that residues in the vicinity of the pocket rather than those within the pocket seem to be required for interaction with Raf-1.

Introduction

Phosphatidylethanolamine binding protein 1 (PEBP1), also known as Raf kinase inhibitory protein (RKIP), is involved in several processes in living cells. Its physiological function, mechanism of action and binding properties have been studied by using various cells and tissues from human, bovine, rat and mouse. The main results have revealed that PEBP1/RKIP regulates three different processes in living cells. Its physiological function, mechanism of action and binding properties have been studied by using various cells and tissues from human, bovine, rat and mouse.
hPEBP1 is a member of the phosphatidylinositol binding protein (PEBP) family, which is a highly conserved group of more than 400 ubiquitous proteins found in a variety of tissues from a wide range of organisms (bacteria, yeasts, insects, mammals and plants). The crystal structures of PEBPs have revealed a remarkably conserved ligand-binding pocket. X-ray studies for bovine and human PEBP1s showed that ions such as acetate and α-phosphorylated phosphatidylserine (PE) [PDB 1A14; PDB 1B7A] [25], phosphate and α-phosphorythreonine (PDB 2QYQ) [26], or cadaverine (PDB 1BEH) [27] could bind to this conserved pocket. The conserved pocket is the only ligand-binding site of PEBP1s identified by X-ray.

Besides crystallographic data, binding studies have been reported using other techniques. A study by affinity chromatography at pH 7.5 revealed that nucleotides could bind to the bovine brain PEBP (hPEBP), in the decreasing affinity order FMN>GTP>GDP>GMP>FAD>ATP>NADP>GTP>UTP>ADP [28]. Interactions of human and bovine PEBP1s with morphine and morphine derivatives were characterized at pH 6.8 by noncovalent mass spectrometry [29]. Moreover, an NMR study of rat PEBP1 (rPEBP1) in near physiological conditions (pH, salt concentration, temperature) showed that the conserved pocket could accommodate various ligands such as 1,2-dihexanoyl-sn-glycero-3-phosphoethanolamine (DHPE), dihexanoylphosphatidylserine (DHPS), dihexanoylphosphatidylethanolamine (DHPE), and dihexanoylphosphatic acid (DHPA) [30]. The screening of a chemical library by NMR spectroscopy revealed three novel ligands for rPEBP1 that also bind to the protein pocket [31]. Shemon and co-workers [2009] were also interested in the interaction of rPEBP1 with locostatin (S)-(+)-4-benzyl-3-crotonyl-2-oxazolidinone), since it is known to be a cell migration inhibitor whose cellular target is PEBP1 in cell lines from different origins [6]. However, locostatin itself could not be analyzed by NMR because of its limited solubility and the fact that it induced protein precipitation [32]. Contrary to locostatin, its precursor (S)-4-benzyl-2-oxazolidinone was compatible with NMR studies, which indicated a binding to the conserved pocket of rPEBP1 [32]. Furthermore, interactions between rat, mouse or human PEBPs and an inhibitor of phosphodiesterase-5 (PDE5) were shown by combining affinity based enrichment and mass spectrometry [33]. The binding was confirmed by solution based assays using absorbance, fluorescence and NMR spectroscopy.

However, some of these studies have emphasized the importance of both experimental conditions and the species of the PEBP used in the binding studies. A comparative NMR study at pH 7.5 and 6.0 showed that some ligands of hPEBP1 and bPEBP1 previously identified did not interact with rPEBP1 at pH 7.4, particularly PE [30] and the nucleotides GDP and GTP [31]. Furthermore, the binding study involving PEBPs from rat, mouse and human (rPEBP2, mPEBP1, hPEBP1) and an inhibitor of PDE5 evidenced different behaviors depending on the species and the tissues of origin of the protein, in spite of high sequence homologies and high similarities in the protein tertiary structures [33].

As previously mentioned, PEBP1 from bovine, human or rat is able to bind small ligands as well as proteins such as the Raf-1, MEK and ERK kinases [1,11]. Although the mechanism of PEBP1 binding to Raf-1 remains unknown, several studies have provided information about the binding region of Raf-1 on the one hand, and the binding region of PEBP1 on the other hand. Yeung and co-workers (2000) showed that the binding domains of Raf-1 with rPEBP1 were subdomains I and II, a region of approximately 100 amino acids [10]. More recent studies revealed that the phosphorylated N-region of Raf-1, encompassing amino acids 331 to 349, was sufficient to bind to rPEBP1 [34,35]. These data are consistent with rat and human PEBP1s inhibiting Raf-1 by preventing its phosphorylation at S338 and Y341 [12]. Besides, it has been shown that binding to Raf-1 requires the integrity of the rPEBP1 pocket [30,35] and is influenced by rPEBP1 pocket occupancy by another ligand (DHPE) [30]. Furthermore, the P74L mutation of the rPEBP1 pocket affects Raf-1 binding, but not the binding of DHPE to rPEBP1 [30]. Thus, the rPEBP1 lipid binding site may be distinct from the kinase binding site, and at least some of the pocket residues may be involved directly or indirectly in the interaction between rPEBP1 and Raf-1 [31]. Another work did support the idea of an indirect binding of Raf-1 to the PEBP1 pocket. Indeed, in contrast to DHPE, the locostatin precursor binding to the rPEBP1 pocket was not sufficient to interfere with Raf-1 binding [32]. The authors suggested that other residues of rPEBP1 may be critical for Raf-1 binding.

Thus, in spite of the numerous papers concerning PEBP1 binding ligands, one another’s conclusions are not always in agreement. The works previously mentioned evidenced different binding behaviors as a function of (i) the species of PEBP1 (mouse, rat or human) [33], and (ii) the experimental conditions of binding, particularly the pH value [31]. Moreover, the binding of Raf-1 is complex and the binding site on PEBP1 is still unknown. In the present study, we investigated the human PEBP1 by NMR to determine the binding site of four different molecules: two nucleotides, GTP and FMN, because of their relatively high affinities for hPEBP1 [28], and a Raf-1 peptide of 19 amino acids in tri-phosphorylated and non-phosphorylated forms. The non-phosphorylated peptide RPRQQRDSYYWYEIEASEV is the minimal region 331–349 of Raf-1 required for rPEBP1 binding [34]. Three phospho-amino acids were incorporated at the positions Ser338/Tyr339 and Tyr341, since the phosphorylation enhanced the binding to rPEBP1 as studied by surface plasmon resonance [34]. In order to examine the effects of experimental conditions such as pH, salt concentration, and temperature on binding, we investigated hPEBP1 in two sets of conditions: MES 10 mM pH 6.5 at 20°C, and HEPES 10 mM, NaCl 100 mM, pH 7.5 at 30°C (near physiological conditions). NMR titrations were also used to derive the affinity constants Ki of the ligands with hPEBP1. Native mass spectrometry (MS) was used as an alternative method for measuring Ki at pH 7.4/37°C for GTP and FMN and at pH 7.4/25°C or pH 6.6/20°C for the tri-phosphorylated Raf-1 peptide.

Results

15N-1H heteronuclear single quantum coherence (HSQC) NMR experiment was used to study the interaction between hPEBP1 and four different ligands under two sets of experimental conditions. The HSQC spectrum of a protein monitors peptides NH groups, giving one signal per amino acid at the level of the protein backbone. Since the chemical shift is very sensitive to the environment of the observed nuclei, the binding of the ligand affects the chemical shifts of both peptide nitrogen and proton within the binding area. Hence, the residues involved in a binding can be determined using HSQC spectra of hPEBP1 in the presence or absence of a ligand.

Mammalian PEBP1s crystal structures (PDB 2QYQ [26]) have revealed a remarkably conserved ligand-binding pocket. The hPEBP1 pocket can be defined by 16 residues at the surface of the protein: D70, A73, P74, Y81, W84, H86, V107, G108, G110, P111, P112, H118, Y120, L180, Y181, and L184 (Figure 1).

GTP and FMN do bind to the ligand-binding pocket of hPEBP1

NMR titration of GTP in MES 10 mM pH 6.5 at 20°C revealed 34 residues in fast exchange on the NMR time scale.
GTP and FMN do bind to hPEBP1 in near physiological conditions

Since the experimental conditions can affect the binding behavior [31], the binding of GTP and FMN was also investigated under near physiological conditions: HEPES 10 mM pH 7.5, NaCl 100 mM, at 30°C.

The binding of GTP in near physiological conditions exhibited the same features as in MES 10 mM pH 6.5, 20°C, that is, the same binding site and a fast exchange on the NMR time scale. Among the 34 residues affected at pH 6.5/20°C, 26 were also perturbed at pH 7.5/NaCl 100 mM/30°C. However, the chemical shift perturbations were smaller in near physiological conditions (\( \Delta \text{CSP} > 2 \sigma = 0.058 \) ppm) than at pH 6.5/20°C (\( \Delta \text{CSP} > 2 \sigma = 0.087 \) ppm) (Figure 4). Moreover, the binding constant measured for GTP at pH 7.5/NaCl 100 mM/30°C was 3425 ± 1967 μM, which was higher than 669 ± 140 μM at pH 6.5/20°C (Table 1).

Similarly to the study performed at pH 6.5/20°C, the titration of FMN in near physiological conditions revealed residues in slow exchange on the NMR time scale, as well as residues in intermediate and fast exchange. The data evidenced the conserved hPEBP1 pocket as the binding surface in both conditions. Nevertheless, regarding the residues in slow exchange, the loss in intensity occurred at a higher FMN concentration at pH 7.5/NaCl 100 mM/30°C than at pH 6.5/20°C (data not shown). The estimation of \( K_D \) from CSP data of residues in fast exchange confirmed a lower affinity in near physiological conditions: \( K_D = 252 \pm 84 \) μM at pH 7.5/NaCl 100 mM/30°C versus \( K_D = 14.9 \) μM at pH 6.5/20°C (Table 1).

The formation of hPEBP1-nucleotide complexes was also monitored by native MS (data not shown). In ammonium bicarbonate (ABC) 20 mM at pH 7.4 and 37°C, hPEBP1 was found to bind with GTP and FMN with \( K_D \) values of 89 ± 48 μM and 5 ± 2 μM, respectively (Table 1). As in NMR, hPEBP1 showed a higher affinity for FMN than for GTP. In native MS, \( K_D \) values were measured in the absence of NaCl. In contrast, NMR measurements were performed in the presence of NaCl 100 mM, leading to a partial screening of electrostatic charges, and consequently to higher \( K_D \) values.

The Raf-1 peptide does bind in tri-phosphorylated and non-phosphorylated forms to the ligand-binding pocket of hPEBP1

HSQC spectra displayed a total of 73 perturbed residues upon titration of the tri-phosphorylated Raf-1 peptide in MES 10 mM pH 6.5 at 20°C: 54 residues in slow exchange, 11 residues in intermediate exchange, and 8 residues in fast exchange (Figure 3C). Among these 73 perturbed residues, 11 residues were buried (V27, V65, L68, T69, S109, V121, V122, V124, V151, and C146) and three were isolated at the surface of hPEBP1 (Q13, G57, and L58). Thus, after discrimination, we determined a single binding surface composed of 59 residues including and surrounding the conserved pocket. Neither the intensity data of the residues in slow exchange, nor the CSP data of the residues in fast exchange did allow us to estimate the affinity of the tri-phosphorylated Raf-1 peptide. Indeed, on the one hand, the peak intensities dropped sharply when the Raf-1 peptide concentration increased. And, on the other hand, the plot of CSP versus Raf-1 peptide concentration revealed no saturation upon titration.

However, the affinity of hPEBP1 for the tri-phosphorylated Raf-1 peptide was measured by native MS. A \( K_D \) value of 45 ± 12 μM was obtained in conditions of incubation similar to the conditions used for NMR, in ammonium acetate at pH 6.6 and 20°C.
In ABC at pH 7.4 and 25°C, a $K_D$ of $11.63 \text{ mM}$ was found, a value close to the $K_D$ of $20 \text{ mM}$ determined in solution with rPEBP1 [34]. All these values are within the same order of magnitude.

For the NMR titration with the non-phosphorylated Raf-1 peptide in MES 10 mM pH 6.5 at 20°C, only 33 residues were perturbed: 16 residues in slow exchange, 6 residues in intermediate exchange, and 11 residues in fast exchange (Figure 3D). After discrimination of the buried residues (V27, V46, D72, and S109) and those isolated at the surface (Y29), three surface patches were identified. Two small surfaces were formed by L25-H26-V34-G166 and W55/D56/G57/L58/V164 on the opposite side of the conserved pocket of hPEBP1, but were not large enough to be considered as potential binding surfaces. Besides, 19 perturbed residues defined a surface centered on the conserved pocket similarly to the other ligands. It is worth noticing that the corresponding binding surface was larger for the tri-phosphorylated peptide. In addition, comparison of the peak intensities for
both Raf-1 peptides titrations at similar protein/ligand ratios revealed that the intensity loss was less severe for the non-phosphorylated peptide (data not shown). Altogether, these data suggest a lower affinity of hPEBP1 for the non phosphorylated Raf-1 peptide compared to the tri-phosphorylated Raf-1 peptide, in agreement with literature reports [34]. However, we could not confirm this with a binding constant value. The severe drop of peak intensities for the residues in slow exchange and the non-saturation of CSP for the residues in fast exchange upon titration did not allow us to estimate the $K_D$ as mentioned for the tri-phosphorylated Raf-1 peptide.

One additional difference could be observed between the two peptides regarding the perturbation of the pocket itself. The conserved pocket of hPEBP1 is formed by 16 residues at the

Figure 3. Binding site of ligands at hPEBP1 surface at pH 6.5/20°C. Mapping of amino acid residues whose HSQC peak is significantly affected by (A) GTP, (B) FMN, (C) the tri-phosphorylated Raf-1 peptide, and (D) the non-phosphorylated Raf-1 peptide at the surface of hPEBP1 (X-Ray; PDB 2QYQ). Red = residues in slow exchange; orange = residues in intermediate exchange; yellow = residues in fast exchange. Prolines 74, 111 and 112, which belong to the hPEBP1 pocket but are not detected by HSQC spectrum, are indicated in green. Serine 153 is indicated in cyan as a reference point. (E) hPEBP1 sequence alignment (accession number P30086) indicating the residues defining the binding surface of GTP, FMN, the tri-phosphorylated Raf-1 peptide (3P. Raf-1 peptide), and the non-phosphorylated Raf-1 peptide (Raf-1 peptide). The color code is similar to (D). doi:10.1371/journal.pone.0036187.g003

Table 1. $K_D$ values of nucleotides derived from NMR and MS spectrometry.

| Compound | NMR $K_D$ (μM) pH 6.5/20°C | NMR $K_D$ (μM) pH 7.5/NaCl 100 mM/30°C | MS $K_D$ (μM) pH 7.4/37°C |
|----------|-----------------|-----------------|-----------------|
| GTP      | 669±140         | 3425±1967       | 89±48           |
| FMN      | 14±9            | 252±84          | 5±4             |

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surface of the structure PDB 2QYQ [26]. Three prolines are among these 16 residues, hence only 13 residues of the pocket can be detected by HSQC. Whereas all these 13 residues were perturbed upon binding of the tri-phosphorylated Raf-1 peptide, only four of them were affected by the binding of the non-phosphorylated peptide: three residues (A73, Y81, and G110) were located on the edge of the pocket, and only G110 was in the bottom of the pocket.

Discussion

The present study demonstrates and/or confirms the binding of hPEBP1 to four ligands, two nucleotides and one Raf-1 peptide in tri-phosphorylated and non-phosphorylated forms. Although the affinities for GTP and FMN decrease as pH, salt concentration, and temperature increased from pH 6.5/NaCl 0 mM/20°C to pH 7.5/NaCl 100 mM/30°C according to our NMR data, both ligands clearly do bind under near physiological conditions. Moreover, all four ligands bind to the same region centered on the conserved pocket previously identified by X-ray crystallography.

The binding of the two nucleotides

The binding of GTP and FMN was evidenced in two sets of conditions (MES 10 mM pH 6.5 at 20°C, and HEPES 10 mM, NaCl 100 mM, pH 7.5 at 30°C) and involved hPEBP1 pocket as well. However, hPEBP1 shows a higher affinity for FMN than for GTP, in agreement with literature reports concerning bPEBP1 [28]. Moreover, a higher affinity was observed at pH 6.5/20°C than at pH 7.5/NaCl 100 mM/30°C (Table 1). We carried out complementary experiments to differentiate the effect of the pH alone. Therefore, FMN was studied in HEPES 10 mM pH 7.5 at 20°C to compare with the binding study in MES 10 mM pH 6.5 at 20°C. Similar to the data at pH 6.5, the titration of FMN at pH 7.5 showed a majority of residues in slow exchange, but also residues in intermediate and fast exchange. Altogether, the perturbed residues defined the hPEBP1 pocket as the binding site of FMN at pH 7.5/20°C (data not shown). The measured affinity indicated no significant effect of the pH: $K_D = 14 \pm 11 \mu M$ at pH 7.5/20°C (estimation from CSP data of 9 residues in fast exchange) versus $K_D = 14 \pm 9 \mu M$ at pH 6.5/20°C (estimation from CSP data of 14 residues in fast exchange). Concerning the effect of salt alone, it is important to note that the presence of NaCl 100 mM induced no change on the
The binding of the Raf-1 peptide in tri-phosphorylated and non-phosphorylated forms

hPEBP1 pocket did bind the tri-phosphorylated Raf-1 peptide (Figure 3C), as previously shown by surface plasmon resonance [34], or for rPEBP1 [30,35]. In particular, our data showed that residues A73 and S75 surrounding P74 as well as residue H86 were involved in the binding, supporting the study of Granovsky and co-workers (2009) that showed the effect of the mutations P74L and H86A in the pocket on the binding of rPEBP1 with Raf-1 kinase. Besides, although S153 was not perturbed itself, residues K150, V151, A152 immediately preceding S153 in an-helix H1 of hPEBP1 were affected by the tri-phosphorylated Raf-1 peptide binding. This could agree with the fact that rPEBP1 dissociates from Raf-1 upon phosphorylation by PKC on S153 (Figure 3C) [13,14].

Raf-1 peptide binds more tightly when it is phosphorylated, as previously demonstrated by Park and co-workers [34]. As expected, the binding site of the non-phosphorylated Raf-1 peptide was centered on the conserved pocket (Figure 3D), involving residue G110 at the bottom of the pocket. However, most residues of the pocket were not involved in the binding. Indeed, residues in the vicinity of the pocket, rather than those within the pocket, were perturbed and hence, seemed to be required for interaction with Raf-1, as previously suggested by Shemon and co-workers (2009, 2010) [31,32].

Since our data demonstrated differences between rat and human PEBPs for GTP binding, we investigated the interaction with the locostatin precursor [S]-4-benzyl-2-oxazolidinone (Sigma #294640) for which the binding to rPEBP1 has been evidenced by NMR under near physiological conditions (Tris-HCl 50 mM pH 7.4, NaCl 100 mM, 30°C). The titration of the locostatin precursor with hPEBP1 (in HEPES 10 mM pH 7.3, NaCl 100 mM, 30°C) revealed 19 residues in fast exchange on the NMR time scale (data not shown). Analysis of the CSP values of these 19 residues provided a binding constant equal to 6 ± 1 μM. The mapping of the perturbed residues on the X-ray structure of hPEBP1 shows that the locostatin precursor binds to the hPEBP1 pocket as previously shown for rPEBP1 under similar conditions (no KD value was measured for rPEBP1) [32].

Figure 6. Multiple sequence alignment of human PEBP1 (hPEBP1, accession number P30086), rat PEBP1 (rPEBP1, accession number P31044) and mouse PEBP2 (mPEBP2, accession number Q8VIN1). The hPEBP1 residues defining the binding surface of GTP at pH 6.5 and 20°C are colored yellow.
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In the present study, we confirm the binding of the human PEBP1 to two nucleotides (GTP and FMN) and a Raf-1 peptide (in tri-phosphorylated and non-phosphorylated forms) in different conditions using NMR and mass spectrometry. All ligands bind to the same region centered on the conserved ligand-binding pocket of hPEBP1 previously identified by X-ray crystallography. Our work confirms that residues in the vicinity of the pocket rather than those within the pocket seem to be required for interaction with Raf-1 [31,32]. The affinity constants $K_d$ were estimated by NMR titration and/or native mass spectrometry. Although the affinities for GTP and FMN were lower at pH 7.5/NaCl 100 mM/30°C than at pH 6.5/20°C, both nucleotides clearly did bind under near physiological conditions. Since no interaction was shown between the rat PEBP and GTP by NMR in near physiological conditions [31], our study demonstrates the specific binding behavior of the human PEBP1 and highlights the importance of the studied species. In a therapeutic perspective, the choice to study human PEBP1 is a critical factor in drawing conclusions on human pathologies.

Materials and Methods

Interaction of hPEBP1 was studied with four different ligands: two nucleotides, GTP and FMN, and a Raf-1 peptide of 19 amino acids in tri-phosphorylated and non-phosphorylated forms.

Materials

Guanosine triphosphate (GTP), flavin mononucleotide (FMN), β-mercaptoethanol (BME), and ammonium bicarbonate (ABC) were purchased from Sigma (St. Louis, MO). Ammonium chloride $^{15}$N 98% ($^{15}$NH$_4$Cl) was purchased from Cortecnet (Voisins-Le-Bretonneux, France). Ammonium acetate (NH$_4$OAc) was purchased from Merck (Darmstadt, Germany) and formic acid 90% (FA) from Fisher (Loughborough, UK). The Raf-1 peptide in tri-phosphorylated and non-phosphorylated forms was prepared by conventional solid-phase peptide synthesis using the Fmoc strategy. Fmoc-Ser(PO(OBzl)OH)-OH and Fmoc-Tyr(PO(OBzl)OH)-OH were used as phosphoderivatives. They were obtained by combining both a manual chain assembly method and an automated one with a ABI 433A synthesizer (Applied Biosystems). Details of the synthesis strategy will be described elsewhere. All solvents and buffers were prepared using 18 M purified water.

Production and purification of $^{15}$N hPEBP1

$^{15}$N hPEBP1 was produced according to the method described by Marley [41]. The CDNA coding the human PEBP1 has been inserted in pET31b plasmid [29]; E. coli BL21 DE3 cells were used to overexpress hPEBP1. The general protocol is as follows: 2 L of an E. coli BL21 (DE3, pET31b) overnight preculture were inoculated into 60 L of LB. Upon reaching OD$_{600}$ ~0.7, the cells were pelleted by centrifugation. The cell pellet was resuspended in 15 L of M9 medium with 15NH$_4$Cl 1 g/L, ampicillin 50 μg mL$^{-1}$, and then incubated to allow the recovery of growth and the clearance of unlabeled metabolites. After 1 h, protein expression was induced by addition of isopropyl-1-thio-β-galactoside (IPTG) to a final concentration of 1 mM. After a 2–3 h incubation period, the cells were harvested and frozen at −20°C.

The purification of hPEBP1 was performed according to a two-step procedure involving two different ion exchange chromatography columns. The frozen cell pellet was resuspended in water and loaded into a French Press cell disruptor. The cell lysate was centrifugated at 14,000 g for 20 min at 4°C. The clear supernatant was dialysed overnight against Tris 20 mM, EDTA 1 mM, BME 1 mM, pH 8.0. The dialysed cell lysate was loaded onto an anion exchange chromatography column (Q Sepharose Fast Flow, Amersham) and eluted with Tris 20 mM, BME 1 mM, pH 8.0. The fractions containing hPEBP1, identified with 18% SDS-PAGE, were gathered and dialysed overnight against NaAc 10 mM, BME 1 mM, pH 5.5. The dialysed sample was loaded onto a cation exchange chromatography column (Sp Sepharose High Performance, Amersham). hPEBP1 was eluted with a linear gradient 0–1 M NaCl. The fractions containing the protein were gathered and dialysed against MES 10 mM, BME 1 mM, pH 6.5. The protein solution was aliquoted and stored at 4°C. The final protein purity was assessed according to 18% SDS-PAGE gel and mass spectrometry.

hPEBP1 and nucleotides purification for mass spectrometry analysis

Non-labeled recombinant hPEBP1 purified as previously described [29] was used for mass spectrometry analysis. To prevent Na$^+$ adduct formation, the commercial GTP and FMN nucleotides used in native MS were desalted. For this purpose, a protocol derived from the RNA-desalting procedure of Limbach et al. (1995) [42] was set up [43].

NMR measurements

The interactions between hPEBP1 100–270 μM and the four selected ligands were investigated by $^{15}$N-$^1$H heteronuclear single quantum coherence (HSQC) NMR experiments with a sensitivity enhancement and gradient selected coherence. $^1$H, $^{15}$N HSQC spectra were recorded at 20 or 30°C on a Bruker 500 MHz or a Varian Inova 600 MHz spectrometer. Two experimental sets of conditions were tested: MES 10 mM pH 6.5 at 20°C, and HEPES 10 mM, NaCl 100 mM pH 7.5 at 30°C.

Although the backbone assignment is available for the human protein at pH 4/25°C at the BMRB (BMRB 16992) [44], we performed our own backbone amide assignment of free hPEBP1 at pH 6.5/25°C (BMRB 18204) using 3D TROSY-based HNCA, HN/C/CA, HNCACB, HN/C/CA/CB, HNCO and HN/C/CO/ experiments [45]. $^1$H and $^{15}$N chemical shifts were assigned for 96.5% of non-prolines residues: all residues except Met1, Val3, Asp35, Glu43, Lys47 and Lys187 (total residues: 187; non-prolines residues: 172; assigned residues: 166/172). Measurements were performed on a Bruker Avance spectrometer 800 MHz equipped with a cryogenic $^1$H/$^{15}$C/$^{15}$N triple-resonance probe.

NMR titrations

In the simple case of protein-ligand interactions, the free and the bound states are observed during the titration. The interpretation of an NMR spectrum, such as an HSQC, depends on the rate of exchange between the bound and the free forms. Three different cases can be observed. If the complex rate of dissociation is very slow, two separate resonances are observed at the positions corresponding to the chemical shifts characteristic of the two states (free and bound). During the titration, the intensity of the free resonance decreases while the bound resonance one appears and goes up. This regime corresponds to slow chemical exchange on the NMR time scale. If the complex rate of dissociation is very fast, only a single resonance is observed, whose position is the average of the chemical shifts of the two states, weighted by their relative populations. In this case, Chemical Shift Perturbations (CSP) are observed, i.e. the chemical shift evolves as the ligand concentration increases. This regime corresponds to fast exchange on the NMR time scale, and is typical for weaker affinity complexes. In the intermediate chemical exchange case, in addition to CSP, complex changes will affect the line shape that results in the observation of very broad signals with low intensity.
In the fast exchange regime, CSP can be measured from $^{15}$N-HSQC spectra using the equation:

$$CSP = \sqrt{(\delta_{1}\delta_{2})^2 + (0.17 \times \delta_{15N})^2},$$

(1)

with $\delta$ being the chemical shift in ppm [37].

A threshold value was estimated in order to determine significant CSP. In a first step, all the CSP are considered and the average <CSP> plus two times the standard deviation (σ) is calculated. Then, the highest CSP (CSP > <CSP> + 2σ) are removed from the data and new average and new standard deviation calculation. The operation is repeated until the convergence is reached. The final value <CSP> + 2σ for the residues not significantly perturbed corresponds to the threshold.

Once the residues involved in the binding were selected, the experimental data were fitted with the quadratic equation 2 using SigmaPlot 9.0 in order to obtain the dissociation constant value ($K_D$):

$$CSP = \frac{CSP_{max}}{2 \times [P]_0} \left[ ([L] + [P]_0 + K_D) - \sqrt{([L] + [P]_0 + K_D)^2 - 4 \times [P]_0 \times [L]} \right],$$

(2)

where $[P]_0$ and [L] are the total protein and ligand concentrations, respectively [46]. A $K_D$ value was estimated for each residue involved in the binding, and then an average was calculated.

In the slow exchange regime, intensity ratios $I/I_0$ can be calculated upon titration with the peak intensity at a fixed concentration of ligand and $I_0$ the initial peak intensity. A method similar to the one explained for CSP was used to discriminate the significant loss of intensity. The threshold corresponds to the average of the intensity ratios values ($<I/I_0>$) minus two times the standard deviation (σ) for the residues not significantly perturbed.

Once the significant perturbations were discriminated, the perturbed residues were taken into account for the determination of the binding surface when (i) the perturbation reaches saturation upon titration, (ii) the residues are located at the surface, and (iii) define a contiguous surface patch [56].

In the case of the slow exchange regime, the binding constant can rarely be calculated because peak intensities are not measured with enough accuracy.

**Native mass spectrometry**

All MS measurements were performed in an ESI-ion trap model Esquire HCT or Ultra HCT PTM Discovery (Bruker, Bremen, Germany), or in a maXis ESI-UHR-QqQ-TOF (Bruker). Complexes were formed by incubating hPEBP1 with a range of ligand concentrations in ammonium bicarbonate 20 mM/formic acid buffer, pH 7.4 at 37°C or in ammonium acetate 20 mM, pH 6.6 at 20°C. After incubation, samples were treated with a Zeba micro gel filtration device with a 7 kDa cut-off (Thermo Scientific, Waltham, MA) prior to MS measurement, or analyzed directly in MS. The $K_D$ was determined by measuring the bound protein fraction by native MS. Details of the development of the native MS method for $K_D$ determination are described in the work of Jaquillard et al. (in press) [43].

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**Author Contributions**

Conceived and designed the experiments: LT L. Jaquillard L. Jouvensal F. Schoentgen MC CD. Performed the experiments: LT L. Jaquillard L. Jouvensal CD. Analyzed the data: LT L. Jaquillard MC CD. Contributed reagents/materials/analysis tools: Aik F. Saab AD AB. Wrote the paper: LT F. Schoentgen CD.

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