Forum on Immunopathological Diseases and Therapeutics, 2(1), 59–70 (2011)

Phosphotyrosine Recognition by the Raf Kinase Inhibitor Protein

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ABSTRACT: Raf kinase inhibitor protein (RKIP) negatively regulates the mitogen-activated protein kinase (MAPK) signaling cascade by direct interaction with Raf-1. RKIP belongs to a family of proteins for which a wide range of structures have now been described; all are highly homologous and share a distinctive anion-binding pocket. Using X-ray crystallography, we show that phosphotyrosine can be neatly accommodated within this pocket, and suggest that RKIP proteins may therefore form a novel phosphotyrosine recognition motif. As studies have previously indicated that phosphorylation of the $S^{338}SYY^{341}$ region of Raf-1 enhances binding to RKIP leading to suppression of MEK activation, we also propose a model of the Raf-1 $DS^{338}SYpY^{341}W$ motif bound to RKIP based on the RKIP-pTyr crystal structure. Consistent with reported experimental data, this model suggests phosphorylation of Ser^{338} may additionally stabilize this complex. Together, these results imply a mechanism for RKIP/Raf-1 interaction in which RKIP regulates Raf-1 activity via direct steric inhibition of the Tyr^{341} region.

KEY WORDS: PEBP, phosphotyrosine, RKIP, X-ray crystallography, Raf-1, c-Raf.

ABBREVIATIONS

MAPK: mitogen-activated protein kinase; **MAPKKK:** MAP kinase kinase kinase; **PEBP:** phosphatidylethanolamine-binding protein; **RKIP:** Raf kinase inhibitor protein; **rmsd:** root-mean-square deviation; **Tris:** tris(hydroxymethyl)aminomethane

I. INTRODUCTION

The mitogen-activated protein kinase (MAPK) pathway forms one of the key signaling pathways for the regulation of cellular proliferation, differentiation, and apoptosis. Central to this pathway is Raf-1 (c-Raf), a ubiquitously expressed MAP kinase kinase kinase (MAPKKK) that is intricately regulated by a wide range of effectors and other proteins (reviewed by Kolch[1]). One such protein is the Raf kinase inhibitor protein (RKIP), which was first identified as a Raf-1 kinase inhibitor by Yeung et al.,[2] but has also been demonstrated to interact with several other kinases.[2–6] RKIP competitively forms an inhibitory complex with Raf-1, preventing the activation of MEK and hence switching off downstream ERK activity.[2] Reduced RKIP levels have been associated with a number of diseases including prostate cancer metastasis (reviewed by Keller et al.[7]). RKIP has also been shown to affect NF–κB signaling independently of the MAPK pathway,[4] and negatively regulates the G protein coupled receptor (GPCR) kinase 2 (GRK2).[5] A compound, locostatin,[8] has been reported to inhibit the RKIP–Raf-1 interaction, leading to reduced epithelial cell motility and is therefore of interest as an anticancer therapeutic. The central importance of Raf-1 activation to a wide range of cell growth and proliferation processes highlights the significance of understanding the molecular basis of its regulation.

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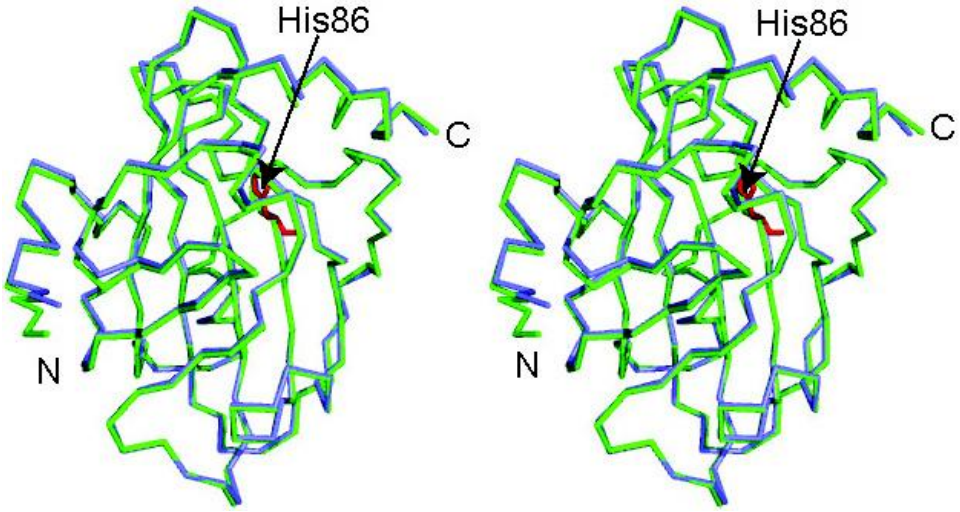

FIGURE 1. Structure of human RKIP. Stereoview showing the C2 (in blue) and $P2_1$ (in green) crystal structures of human RKIP overlaid as Cα traces. The amino and carboxy termini are labeled N and C, respectively, and the conserved amino acid His^{86} is shown in red to highlight the site of the binding pocket.

RKIP is also known as the phosphatidylethanolamine-binding protein (PEBP), one of an extensive and highly conserved family of proteins that have been identified in a wide range of organisms.[9] In plants, a series of distinct flowering phenotypes are associated with amino acid changes in these proteins,[10,11] consistent with the concept that they act as regulators of a central growth and differentiation control pathway. The PEBP protein FT in *Arabidopsis* has recently been identified as the elusive "florigen" or signal for generating flowers in plants.[12,13]

A range of PEBP protein structures have been reported (e.g., in Refs. 14–19), all showing a remarkably highly conserved fold comprising a large central b-sheet sandwiched by a smaller sheet and several helices. This arrangement consistently generates a conserved cavity at one end of the molecule (Fig. 1), which in the various crystal structures has been found to bind a cacodylate ion (phosphate mimic),[14] O-phosphoethanolamine, in two alternate conformations,[15] an acetate ion[14,20] and, toward its surface, a Hepes molecule.[20] This family of proteins was originally noted[21,22] and named for its ability to bind phospholipids; however, the observed binding conformation of phosphoethanolamine within the pocket[15] and distribution of surface electrostatic charges are not consistent with this site providing a means to anchor RKIP to membranes. The anion pocket has also been postulated to be a phosphoamino acid recognition site.[14,16] Protein phosphorylation forms a key mechanism for protein-mediated signal transduction, and regulators of these pathways function by docking to phosphorylated motifs, e.g., phosphoserine (pSer) in the case of 14-3-3 proteins or phosphotyrosine (pTyr) in the case of Src homology 2 (SH2) domains, pTyr-binding (PTB), or protein kinase C δ (PKCδ) C2 domains (reviewed in Refs. 23, 24). RKIP proteins have no recognizable structural or sequence homology to these established phosphopeptide binding modules.

However, coimmunoprecipitation assays have indicated that RKIP binds to the first 100 amino acids of the Raf-1 kinase domain,[2,3] a region that contains two key residues, Ser^{338} and Tyr^{341}, whose phosphorylation is linked to Raf-1 activation. This is supported

by a recent study[25] in which the binding of a series of phosphopeptides to RKIP led to the identification of a 24–amino acid minimal binding region incorporating the $S^{338}SYY^{341}$ phosphorylation motif. In contrast to an earlier report suggesting that phosphorylation of Ser^{338} and $Tyr^{340/341}$ disrupted RKIP binding to Raf-1,[26] this more recent study reported an enhancement in RKIP binding when any of Ser^{338}, Ser^{339}, or particularly Tyr^{341} were phosphorylated. Direct visualization of the RKIP–Raf-1 complex is complicated by the difficulty in obtaining sufficient and stable quantities of Raf-1 suitable for crystallization and the low affinity of Raf-1 peptides for RKIP.[25]

Further to the existing data on the binding of RKIP to Raf-1, we here explore the hypothesis that the biological ligand for the RKIP anion pocket is phosphotyrosine. Using a detailed crystallographic analysis, we show that phosphotyrosine binds within and is highly complementary for the RKIP binding pocket. From our data, we hypothesize that RKIP forms a novel pTyr-binding motif that directly contacts one of the key activation sites of Raf-1. These results support the notion that RKIP acts to establish a threshold for MEK activation by sterically masking this crucial site.

II. EXPERIMENTAL PROCEDURES

Human RKIP (PEBP; PIR accession number P04049) was recombinantly expressed in *Escherichia coli* and purified as described in Banfield et al.[14]

II.A. Crystallographic Studies of RKIP–Phosphoamino Acid Interactions

An alternative crystal (C2) form of human RKIP, differing from that previously reported,[14] was obtained by vapor diffusion from 4.3 mg/ml RKIP, 0.1 M sodium acetate, pH 4.0, and 32–34% PEG 4000, diffracted to atomic (1.2 Å) resolution (Table 1). Due to the incorporation of 20 mM tris pH 7.5 buffer in the protein solution, the final pH in the crystallization drop was found to be approximately 6. Solution and refinement of the structure of these crystals was as described for mPEBP-2[17] and showed improved access to the binding site; however, an acetate ion occupied the site (as previously observed for related structures[14,15]). This was removed by back-soaking against a solution in which the acetate was replaced by 0.2 M NaCl and 0.1 M Hepes, pH 7.0, and to which 0.1 M concentrations of phosphorylated amino acids were added. Multiple attempts to either cocrystallize or introduce by soaking pSer and pThr into the RKIP crystals were nonproductive. In contrast, soaking the crystals for 3–7 days in 100 mM pTyr produced a stable complex from which diffraction data were collected to 1.95 Å (Table 1). The structure was solved and refined as for the native C2 crystals (Table 1), and difference maps were used to locate the bound ligand. The integrity of the final model was validated with PROCHECK[27]; 88.4% of residues are in the most favored regions of the Ramachandran plot, 11.6% in the additionally allowed regions, and there are no outliers. Coordinates and structure factors for the RKIP–pTyr complex have been deposited in the Protein Data Bank (PDB) under the accession code 2QYQ.

II.B. Molecular Modeling

Molecular modeling was performed using SYBYL7.0 software (Tripos Inc., St Louis, Missouri) and was based on coordinates of the RKIP–pTyr complex. The pTyr backbone was extended to include Raf-1 residues 337–341 (DSSYYW) using the peptide composer features of the software, maintaining the experimental placement of the pTyr ligand. Manual adjustment was performed to rectify steric clashes prior to energy minimization using the Amber99 forcefield.[28] Stereochemistry was assessed with PROCHECK.[27] Images were

TABLE 1. X-ray Data Collection, Processing, and Refinement Statistics for the Native C2 hRKIP Structure and the pTyr-hRKIP Complex

Data collection and processing	Human RKIP	Human RKIP-pTyr complex
Unit cell parameters (Å; deg)	*a* = 82.39, *b* = 33.72, *c* = 59.46; β = 97.14 deg	*a* = 83.67, *b* = 33.89, *c* = 59.71; β = 96.09 deg
Space group	C2	C2
X-ray source; wavelength	SRS synchrotron, PX14.2; 0.975 Å	Bruker Proteum R; 1.542 Å
Resolution range (Å)	60–1.2 (1.24–1.20)	60–1.95 (2.02–1.95)
Number of unique reflections	50,137	11,833
Redundancy	3.7 (3.0)	5.7 (3.4)
Completeness (%)	98.5 (85.5)	95.4 (78.7)
R_{merge} (%)	5.2 (23.6)	8.1 (23.4)
Final model		
R-factors: R_{work} (%)	12.6	15.4 (18.0)
R_{free} (%)	16.9	20.8 (30.2)
Rmsd, bond lengths (Å)	0.021	0.016
Rmsd, bond angles (deg)	1.985	1.49
Number of non–hydrogen protein atoms	1714	1659
Number of water molecules	240	169

Values in parentheses correspond to the highest-resolution shell.

created with PyMOL (DeLano, W.L., http://www.pymol.org).

III. RESULTS

III.A. Human RKIP Structure in C2 Crystal Form

An earlier determination of the human RKIP structure[14] was based on crystals grown at pH 6.5 and with $P2_1$ symmetry. The structure has been described in two essentially identical forms, i.e., with and without cacodylate bound within the anion-binding pocket. The C2 crystal form in the current study, grown from a mixture of acetate and tris buffers resulting in an overall pH in the crystallization liquor of about 6, was found to have a very similar structure. A superimposition of the a-carbon backbones of both structures (Fig. 1) resulted in a root-mean-square displacement (rmsd) of 0.31 Å. Discernible differences were limited to minor shifts in the placement of the C-terminal helix and N-terminal region, and variations in the positions of surface amino acid side chains; however, in view of the differing resolutions of the two structures (1.2 and 1.8 Å) and altered crystal packing, these changes are unlikely to be of significance. Similarly, the C2 structure showed no discernible differences (rmsd 0.23 Å for one chain, 0.27 Å for the other) from a second $P2_1$ form obtained at pH 7.5 under conditions described previously for murine PEBP2[17] (data not shown). The acetate ion observed in the binding pocket of the C2 form could be successfully removed by back-soaking against acetate-free buffer. This did not induce any significant changes in the structure (rmsd 0.25 Å) apart from a small expansion of the unit cell associated with the

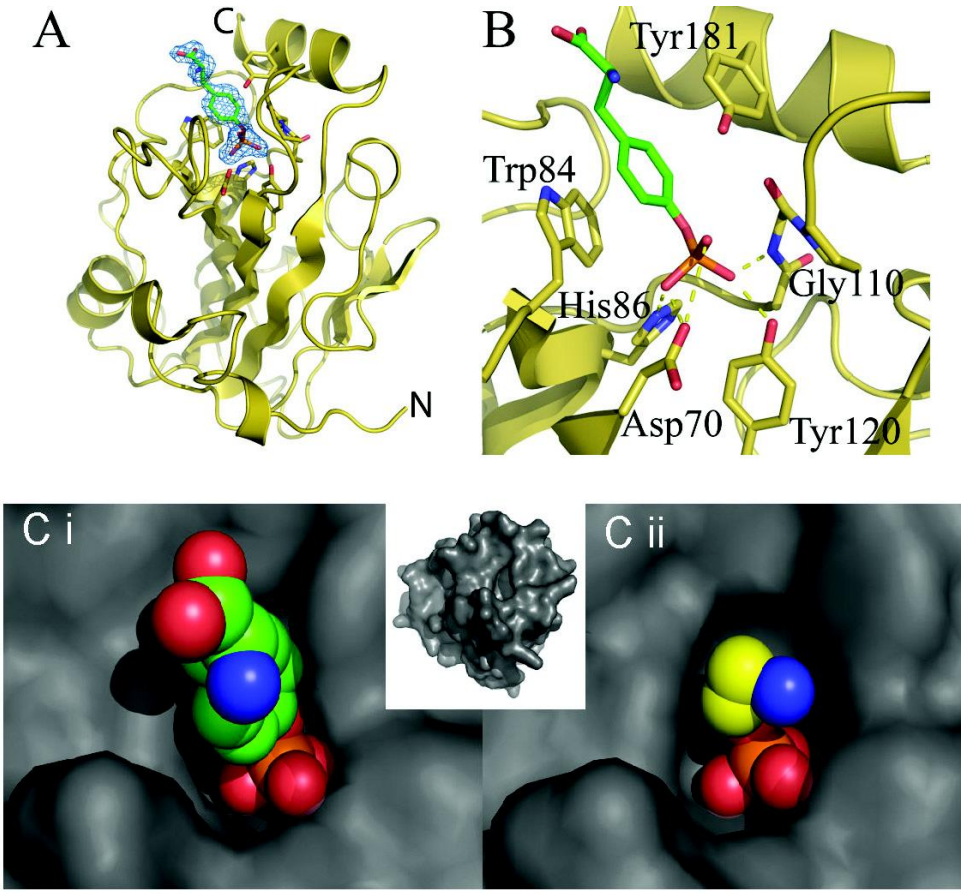

FIGURE 2. Structure of the pTyr-hRKIP complex. (**A**) The pTyr ligand (green sticks) is modeled into refined electron density from a 2m*F*o-D*F*c map contoured at 1.0σ (blue). N and C represent the chain termini. (**B**) Close-up view of the pTyr molecule (green) in the RKIP-binding site (gold). Possible H-bonds (yellow dashes) are shown between the phosphate moiety and the conserved residues: Asp[70], His[86], Gly[110] and Tyr[120] (main chain). pTyr stacks against Trp[84] and is within contact distance of Tyr[181]. (**C**) Surface representations of the binding pocket region showing the prominence of the binding cavity in the surface of RKIP (inset): (i) the distinctive shape complementarity for the pTyr molecule shown in sphere representation bound within the cavity, with RKIP shown as a semitransparent surface; and (ii) in contrast, the lipid head group, O-phosphoethanolamine (spheres), in the higher occupancy form of its two alternate binding conformations in the equivalent binding pocket in the bovine PEBP structure.[15] Clearly, as part of a lipid molecule, this head group would likely be incapable of binding in this manner.

soaking procedure. Together with the more open crystal-packing arrangement in the C2 form, removal of the bound acetate allowed access to the binding pocket, enabling pTyr but not pSer or pThr to be readily bound within the pocket at neutral pH. This differed from earlier experience with the $P2_1$ crystal form in which the packing did not appear to allow sufficient access to this site. Furthermore, no additional pTyr-binding sites were revealed in the electron density difference maps.

III.B. The RKIP–pTyr Complex

The crystal structure of the human RKIP–pTyr complex provides a clear demonstration of the complementarity between the RKIP pocket and pTyr. Figure 2A depicts the RKIP

protein complex with the phospholigand enveloped by refined electron density (2mFo-DFc map), bound within the conserved cavity. The phosphate group is deeply buried and primarily interacts through H-bonding to the side chains of the highly conserved residues Asp^{70}, His^{86}, and Tyr^{120}, and the main chain amine group of Gly^{110} (Fig. 2B). These contacts are characterized by distances of ~2.5 Å, implying tight interactions. The hydrophobic tyrosyl group of the ligand stacks against the Trp^{84} side chain (Fig. 1B), with the aromatic ring systems separated by typical van der Waals distances of 3.7–4.1 Å. The other aromatic residue within the site, Tyr^{181}, is also within a similar distance of one edge of the tyrosyl ring (3.7 Å), but does not stack against it. However, the position of Tyr^{181} may influence the entry of hydrophobic groups into the cavity. All of these residues are highly conserved throughout the RKIP/PEBP family, supporting the notion that these interactions are central to the biological function of RKIP proteins.

Examination of the surface of the binding pocket (Fig. 2C) indicated a close shape and chemical complementarity of the pocket with its bound pTyr ligand. This differed from the binding previously described for O-phosphoethanolamine (the head group from the lipid phosphatidylethanolamine) to bovine RKIP,[15] in which the ligand only partially occupies the volume of the pocket, enabling it to adopt two alternate conformations in the crystal structure (Fig. 2Cii). This disorder appears to arise from the nonspecificity of the interactions of the aminoethyl chain with the hydrophobic groups that form the walls of the cavity. We briefly mention that the mode of binding observed for the lipid head group would be highly improbable as part of an intact lipid molecule given steric constraints in this deep cavity, since the phosphate group buried here normally serves to bridge the ethanolamine head with the fatty acid tail, i.e., it is in fact positioned in an implausible orientation (see Fig. 2Cii). The phosphate groups of both these ligands are bound at the base of the pocket in the same position previously observed for the cacodylate ion. It is evident from both the depth of the phosphate-binding region from the surrounding protein surface and the shape of the cavity that pThr or pSer would be unlikely to bind at this site. Nonetheless, unphosphorylated Tyr could still be accommodated, though we presume that this would likely incur a significant loss of binding affinity.

IV. DISCUSSION

IV.A. pTyr Binds Within the RKIP Conserved Pocket

Structures from about 10 RKIP homologues are present within the PDB and have been crystallized from a variety of conditions in the pH range 4.3–8.5. In all cases, the binding pocket arrangements are highly similar; there is no obvious change in the structure in relation to variations in pH or crystallization conditions. Crystal packing, however, appeared to be a limiting factor in obtaining ligand complexes using a range of peptides with demonstrated low micromolar binding to RKIP.[25] Our discovery of a new crystal form with altered packing allowed us to successfully introduce monomeric amino acids into the crystals, but not longer peptides. The direct equivalence of the human RKIP structures from two separate crystal forms, especially within the binding pocket, indicates a robust structure unlikely to be affected by changes in pH, although the amphiphilic carboxy terminal helix exhibits some mobility, as discussed previously.[14]

The crystal structure of the RKIP-pTyr complex provides clear evidence that pTyr forms a viable ligand for the RKIP anion-binding pocket. Although its optimal fit and chemical compatibility with this cavity might be considered fortuitous, it is difficult

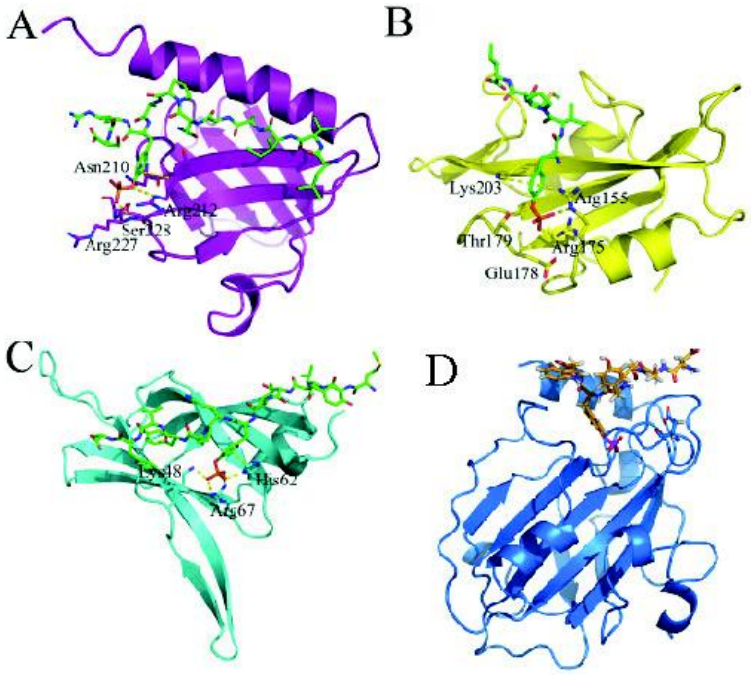

FIGURE 3. pTyr-binding motifs. pTyr-containing peptides (green sticks) bound to: (**A**) The IRS PTB domain (purple ribbon[29]; PDB code: 1IRS); (**B**) The v-Src SH2 domain (yellow ribbon[30]; PDB code: 1SHA); (**C**) The PKCδ C2 domain (cyan ribbon[24]; PDB code: 1YRK). Some possible noncovalent interactions are shown as yellow, dashed lines; (**D**) For comparison, the distinctive structure of RKIP with the modeled peptide in orange is shown.

to envisage other common biological entities that would be more compatible. Because the majority of contacts with the protein are made through the aromatic ring, electrostatic effects do not appear to be the primary determinant of complex formation. Importantly, our soaking experiments revealed no other binding site for pTyr on RKIP other than this pocket. This provides a convincing argument that the pTyr-binding site reported herein is specific. When combined with previous data confirming that the interface with Raf-1 incorporates a tyrosine residue known to be phosphorylated when Raf-1 is activated,[25] and the consistency of the modeled Raf-1 complex with existing data, this study strongly supports the notion that the RKIP pocket, as a pTyr-recognition motif, forms a central part of the RKIP interface with Raf-1 and possibly other protein partners.

IV.B. RKIP Contains a Novel Phosphotyrosine Recognition Motif

The arrangement of the pTyr-binding site in RKIP differs substantially from previously reported pTyr-binding motifs, the best studied of which are SH2 and PTB domains (reviewed by Kuriyan and Cowburn[23]). Unlike RKIP/PEBP proteins, these are usually found as signaling modules within multidomain protein chains. Each is smaller (typically containing 100 and 60 residues, respectively) and structurally dissimilar to RKIP (Fig. 3). For example, the IRS PTB domain[29] and the v-Src SH2 domain[30] bind the pTyr moiety in shallow surface grooves (Figs. 3A and 3B), rather than in a deep cavity, as for RKIP. The predominant contacts to the pTyr phosphate oxygen atoms, in contrast to RKIP, are formed with basic Arg residues in the SH2 and PTB

domains. These domains interact with the pTyr benzyl ring by means of the aliphatic chains of basic residues, unlike the π-stacking arrangement with Trp[84] in RKIP. These same principles are also seen in the interaction of the C2 domain of PKCd with a pTyr peptide from the Src-binding glycoprotein, CDCP1[24] (Fig. 3C), although this domain does utilize a histidine side chain to stack against the tyrosine ring, reminiscent of the role of Trp[84] in RKIP. In all of these cases, however, the tyrosine is accommodated within a shallow surface groove (Fig. 3). In contrast, RKIP with its deep binding pocket and distinctive overall architecture seems to define a novel family of pTyr-recognition motifs.

IV.C. The RKIP–Raf-1 Complex

It has previously been shown[25] that residues in the vicinity of S[338]SYY[341] from Raf-1 bind directly to RKIP. This binding is enhanced by phosphorylation of Tyr[341] but not Tyr[340], and appears to be improved by phosphorylation of one or both serines.[25] Since phosphorylation of Tyr[341] and Ser[338] is linked to Raf-1 activation, whereas binding of RKIP to Raf-1 inhibits its activity on MEK, we explored the possibility that in the RKIP–Raf-1 complex Tyr[341] (the only Tyr from Raf-1 definitively proven to be phosphorylated in vivo[31]) corresponds to the pTyr observed in the crystal complex. On this basis, a model of the Raf-1[337–342] peptide bound to RKIP was constructed by extension of the high-resolution crystal structure of the pTyr–RKIP complex, noting the protrusion of the pTyr main chain into a distinctive groove on the RKIP surface. This tentative model is consistent with a number of experimentally observed features of the RKIP–Raf-1 interaction. First, Tyr[340], which lies adjacent to Tyr[341] (Fig. 4), would be expected to point toward the core of the Raf-1 domain, consistent with reports that it is unlikely to be phosphorylated in vivo.[25,31] Second, the side chain of Ser[338] is located sufficiently close to

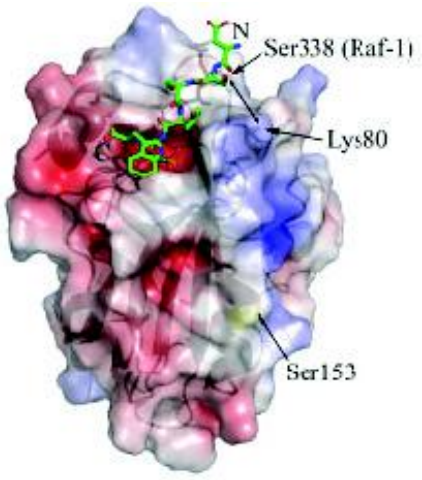

FIGURE 4. Binding of the Raf-1 peptide to RKIP. Surface view of RKIP showing the proposed Raf-1-binding site, colored by electrostatic potential (red, negative; blue, positive). The Raf-1 peptide (D[337]SSYpYW[342], green sticks) is modeled into the pTyr pocket and surface groove, based on the conformation of pTyr[341] as observed in the crystal structure. Note the position of Ser[153], and of Lys[80] adjacent to Ser[338] on the Raf-1 peptide.

the conserved RKIP residue Lys[80] such that, if phosphorylated, the serine could salt bridge to it directly. Ser[338] is known to be phosphorylated in activated forms of Raf-1 in vivo,[31] and Raf-1 peptides incorporating pSer[338] show enhanced binding to RKIP.[25] There is no obvious equivalent pairing for Raf-1 pSer[339], which is also reported to enhance RKIP binding,[25] although the physiological prevalence of this modification is less clear. Finally, PKC isoforms can phosphorylate RKIP Ser[153] and diminish Raf-1 association.[32] Although Ser[153] is not adjacent to our Raf-1 peptide model (Fig. 4), moderate extension of the association surface on Raf-1 could potentially bring Ser[153] into contact. Indeed, experimental evidence from Park et al.[25] suggests that sequences extended by up to 20 amino acids either side of the Raf-1–SSYY motif may contribute to an extended RKIP–Raf-1 interface.

A distinguishing feature of the pTyr binding pocket in RKIP is that the electrostatic potential map (Fig. 4) indicates that the

phosphate-binding pocket is itself highly electronegative, unlike in all previously described pTyr-binding domains. Rather than being unfavorable for pTyr binding, it seems likely that this provides a mechanism to achieve high selectivity for the biological anion, as has previously been observed in phosphate- and sulfate-binding proteins.[33] This electrostatic potential representation is too imprecise to portray the specific hydrogen-bonding networks defined by the local polarization states of the interacting atoms which, together with the distinctive shape complementarity of the pocket, provide selectivity for the pTyr ligand. This mode of recognition with reduced reliance on ion pairing of a strongly negatively charged phosphate group may additionally promote binding of unphosphorylated tyrosine, albeit with expected lower affinity.

IV.D. Biological Implications

Several studies have established the role of RKIP as an inhibitor of Raf-1 activation of MEK.[2,3,32] However, it is not certain whether formation of the RKIP–Raf-1 complex prevents activation (phosphorylation) of Raf-1, or is formed postactivation and acts simply by sterically blocking the association with MEK.

Although phosphorylated Raf-1 peptides display limited affinity in their interactions with RKIP (typically 10–50 mM[25]) this association is expected to be significantly enhanced in the extended binding surface available when intact Raf-1 is the ligand. An interesting observation is that phosphorylation of Tyr^{341}—when part of a peptide—leads to tighter binding by only one order of magnitude.[25] This relatively modest discriminatory ability may derive from the nature of the amino acid side chains in the pocket used to accommodate the pTyr. Unlike in SH2 and PTB domains, ionic bonds between the ligand phosphate and conserved arginine or lysine residues at the base of the pocket are not a feature of the RKIP pocket. The distinctive shape complementarity of the RKIP binding groove for Tyr, in which the tyrosyl ring is sandwiched between two aromatic residues, suggests a mode of binding not solely reliant on incorporation of the phosphate. We therefore suggest that the RKIP structure is compatible with a gradated mode of binding to Raf-1, namely, $pY^{341} > Y^{341} > D^{341}$, that can be modulated by the addition and removal of phosphate groups. This may explain reports that RKIP also binds unphosphorylated Raf-1,[2] although the methods used generally do not allow an accurate comparison of the relative strengths of binding. The latter (D^{341}) is found in B-Raf, for which some binding to RKIP has been reported,[6] although disputed by others.[26] It is unclear whether the latter two modes of binding are physiologically relevant, although both have been demonstrated experimentally.[2] The relative concentrations of RKIP and each Raf-1 form may influence the formation of each potential complex.

Unlike SH2, PTB, or C2 domains, RKIP proteins exist in isolation. They are ubiquitously abundant throughout a wide range of cell types, and highly conserved. The level of RKIP within a cell has been proposed to serve as a molecular "sink" setting a threshold for Raf-1 activation with subsequent MEK activation only occurring when levels of activated Raf-1 are sufficient to exceed complex formation with endogenous RKIP.[1,3] The features of RKIP described in this study are consistent with this proposed role for RKIP. The suggestion that RKIP activity itself may be changed after PKC phosphorylation of Ser^{153} also provides a means by which this activation threshold can be dynamically altered.[5] Importantly, since RKIP is capable of inhibiting a partially activated form of Raf-1, this implies it may provide a vital "checkpoint" before full activation and downstream signaling are permitted to proceed. This appears consistent with its reported capacity to act as a tumor suppressor.[34]

The molecular features that define the pTyr-binding pocket of RKIP are shared across the family of RKIP/PEBP proteins. Of particular interest is the identification of the *Arabidopsis* RKIP homologue FT as the long-sought "florigen" or molecular switch by which flowering is induced in plants.[12,13] Association of FT with a transcription factor, FD, leads to cellular differentiation.[13] Competition between several RKIP homologues appears to take place, the function of each being determined by small changes in the surface regions adjacent to the pTyr-binding pocket.[18] In fact, Hanzawa et al.[35] have shown that substitution of His^{88} in the binding pocket of the *Arabidopsis* RKIP orthologue, TFL1, for its equivalent in FT (Tyr^{85}, corresponding to Trp^{84} in RKIP), and vice versa, is sufficient to swap their phenotypes. The involvement of other Raf-like kinases in this process is poorly understood, although these events are consistent with the RKIP proteins acting as competitive inhibitory-based regulators of these processes.

Finally, mammalian RKIP displays a degree of promiscuity, given its ability to associate with multiple partners. The physiological relevance of some of these interactions is unclear. That all reported binding partners are kinases suggests a commonality in the general mechanism of recognition, e.g., recognition of common kinase features including phosphomotifs. Nonetheless, we note that some of the reported interacting proteins, such as MEK1 and TAK1, are believed to lack phosphorylated Tyr residues. Further studies are required to confirm the mode, composition, and physiological relevance of these potential complexes, and indeed to confirm our proposal that the RKIP/PEBP proteins are novel pTyr-recognition motifs.

ACKNOWLEDGMENTS

We thank the staff at the Daresbury SRS Synchrotron and Diamond Light Source (DLS) for access to data collection facilities. This work was supported by a grant from the UK BBSRC (Ref: C502414) and through studentship awards to PCS (from the BBSRC) and NMB (from the UK MRC).

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