

Engineering the serine/threonine protein kinase Raf-1 to utilise an orthogonal analogue of ATP substituted at the N^6 position

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Abstract One key area of protein kinase research is the identification of cognate substrates. The search for substrates is hampered by problems in unambiguously assigning substrates to a particular kinase in vitro and in vivo. One solution to this impasse is to engineer the kinase of interest to accept an ATP analogue which is orthogonal (unable to fit into the ATP binding site) for the wild-type enzyme and the majority of other kinases. The acceptance of structurally modified, γ - ^{32}P -labelled, nucleotide analogue by active site-modified kinase can provide a unique handle by which the direct substrates of any particular kinase can be displayed in crude mixtures or cell lysates. We have taken this approach with the serine/threonine kinase Raf-1, which plays an essential role in the transduction of stimuli through the Ras $\rightarrow$ Raf $\rightarrow$ MEK $\rightarrow$ ERK/MAP kinase cascade. This cascade plays essential roles in proliferation, differentiation and apoptosis. Here we detail the mutagenesis strategy for the ATP binding pocket of Raf-1, such that it can utilise an N^6 -substituted ATP analogue. We show that these mutations do not alter the substrate specificity and signal transduction through Raf-1. We screen a library of analogues to identify which are orthogonal for Raf-1, and show that mutant Raf-1 can utilise the orthogonal analogue N^6 (2-phenethyl) ATP in vitro to phosphorylate its currently only accepted substrate MEK. Importantly we show that our approach can be used to tag putative direct substrates of Raf-1 kinase with ^{32}P - N^6 (2-phenethyl) ATP in cell lysates.

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1. Introduction

It has been estimated that the mammalian genome encodes 518 putative protein kinase genes, which correspond to 1.7% of all human genes [1]. Kinases play essential roles in the communication of external stimuli into the cell by regulating the cell cycle and many other essential processes. The importance of kinases in cell regulation is underscored by the fact that 47% of all kinase genes map to disease loci or cancer amplicons [1].

The key challenge in the study of any one kinase is to identify how the kinase is activated and regulated, where the kinase is located within the cell, and what its effectors (substrates) are. The identification of kinase substrates has proved to be remarkably difficult due to several factors: the large number of kinases; the organisation of kinases into multiprotein complexes that often contain several kinases; the difficulty of rigorously demonstrating that a particular kinase has been biochemically purified from other contaminating kinases; the relaxed substrate specificity demonstrated by many kinases in vitro; the overlapping substrate specificities of many kinases; and the common catalytic requirement of ATP for all kinases.

Shokat and colleagues have developed a new method for identifying kinase substrates, using orthogonal ATP analogues. In their studies with the v-Src tyrosine kinase [2–5], the Shokat group used a site-directed mutagenesis approach to modify the ATP binding pocket of v-Src to accept an ATP analogue with a bulky adduct at the N^6 position of the purine ring. This analogue was orthogonal for wild-type v-Src and other kinases. To engineer v-Src to accept N^6 (benzyl) ATP the Src protein crystal structure was used to identify amino acids in close proximity to the N^6 position in the ATP–enzyme complex [3]. Proximal residues were then mutated to contain smaller side chains, thus creating an ATP binding pocket that allowed N^6 (benzyl) ATP to bind. The power of this approach was demonstrated by using the mutant v-Src and N^6 (benzyl) ATP to identify several new v-Src substrates, initially in cell lysate labelling experiments and then confirmed by in vitro kinase reactions [5].

We have chosen to apply this approach to the serine/threonine kinase Raf-1, which lacks a solved crystal structure, but

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is distantly related to Src. Raf kinases are the entry point to the archetypical extracellular signal-regulated kinase/mitogen-activated protein (ERK/MAP) kinase cascade, which regulates many fundamental cellular processes by integrating external growth factor signals into distinct biological responses. Typically, Raf-1 activation is initiated by binding to activated Ras which triggers a complex series of posttranslational modifications and changes in protein interactions that co-operate to activate Raf-1. Activated Raf-1 in turn activates MAP kinase/ERK kinase (MEK) or MAP kinase kinase by direct phosphorylation. MEK phosphorylates ERK or MAP kinase resulting in its activation. This signaling unit plays key roles in proliferation, differentiation and apoptosis (for a recent review see [6]). The Raf family consists of three kinases, A-Raf, B-Raf and Raf-1. Raf-1 is ubiquitously expressed and the most thoroughly studied member of the family. A-Raf and B-Raf show tissue-specific expression, although improved detection techniques have recently revealed that they are more widely expressed than originally suspected [7].

The only generally accepted substrate for Raf is MEK, which has two homologues, MEK1 and MEK2. However, there is increasing evidence that Raf-1 can also signal outside this established signaling unit in a MEK-independent fashion (for review see [8]). The most convincing evidence comes from Raf-1 knock-out mice [9,10]. In these studies, Raf-1 expression was abolished completely, and resulted in embryonic lethality. Interestingly, the phosphorylation of MEK appeared to be normal in Raf-1^{-/-} fibroblasts upon stimulation with growth factors and the Raf-1^{-/-} cells had normal proliferative capacity. Despite the apparent normal regulatory phosphorylation of MEK, Raf-1^{-/-} cells had an increased propensity towards apoptosis. This suggests that Raf-1 can function independent of MEK and that this signaling pathway impinges on the regulation of apoptosis.

In the present study, we have mutated Raf-1 in its ATP binding pocket, to create a mutant that can utilise an orthogonal ATP analogue, *N*⁶(2-phenethyl) ATP. We have based our choice of mutations on a three-dimensional homology model of Raf-1 and sequence alignment to kinases with known crystal structures. We demonstrate that the mutations in the ATP binding pocket do not affect Raf-1 signaling or the ability of Raf-1 to phosphorylate MEK. We show that mutant Raf-1 can utilise *N*⁶(2-phenethyl) ATP to phosphorylate MEK and tag putative direct Raf-1 substrates in cell free lysates.

2. Materials and methods

2.1. Cloning and mutagenesis

The mutations were constructed by polymerase chain reaction (PCR) with two sets of oligonucleotides as described previously [19]. PCR was performed with the Pwo polymerase (Boehringer Mannheim) using Raf-1 cDNA as the template. Amplified fragments were restricted with *Eco*RI and *Bam*HI and cloned into the FLAG-CMV-2 expression vector (Sigma). All amplified fragments were sequenced in their entirety. For expression in insect Sf9 cells mutated Raf-1 cDNAs were cloned into the baculovirus vector pAcGHLT-C (Pharmacia). Recombinant baculovirus was generated by cotransfection of Sf9 cells with linear baculovirus and recombinant transfer vector DNAs.

2.2. Protein expression and purification

FLAG-tagged Raf-1 proteins were expressed by transient transfection in COS-1 cells using Effectene (Promega). Thirty-six hours after transfection, the cells were washed with phosphate-buffered saline and serum-starved overnight. The following morning the cells were stimu-

lated with 12-*O*-tetradecanoylphorbol 13-acetate (1 µg/ml) for 20 min. Cells were lysed in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors. Extracts were cleared by centrifugation and the over-expressed proteins purified by immunoprecipitation overnight with the M2 FLAG monoclonal antibody (Sigma).

Glutathione *S*-transferase (GST)-tagged Raf-1 proteins were expressed in insect Sf9 cells and were activated by co-expression with Ras and Src baculovirus as described [20]. Infected Sf9 cells were collected and washed 44 h post infection. Cells were lysed using TBST and the lysates cleared by centrifugation. The over-expressed Raf proteins were purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech). GST-tagged kinase-dead MEK was expressed in Sf9 cells and purified as described above. Additionally, GST-MEK was eluted from the glutathione-Sepharose using competition with glutathione, and the protein eluent dialysed to remove residual glutathione.

2.3. Raf-1 kinase assays

In vitro kinase assays were performed in a 30 µl volume in Raf-1 kinase buffer (20 mM Tris-HCl pH 7.4, 20 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol) in the presence of 350 ng GST-MEK and 100 µM ATP or ATP analogue, as indicated in the text. The addition of similar quantities of Raf-1 and mutants was monitored by immunoblotting and/or Coomassie staining. Reactions were incubated at 32°C for 20 min and terminated with the addition of 5×Laemmli sample buffer. Reactions were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and blotted onto polyvinylidene difluoride membrane (Millipore). MEK phosphorylation was detected by probing with the MEK phosphospecific antibody 9121 (Cell Signaling), and MEK levels were checked using MEK antibody 9122 (Cell Signaling). To control for the amount of input Raf-1 proteins, all membranes were subsequently probed with the cRaf-1 monoclonal antibody (Transduction Laboratories).

In situ kinase assays were carried out in a volume of 100 µl using desalted serum-starved COS-1 lysates prepared as described in [17]. Desalted lysate was mixed with activated Raf-1 or mutants, 100 µM ATP or analogue, 5 mM magnesium chloride in Raf kinase assay buffer (50 mM Tris pH 7.5, 150 mM sodium chloride, 5 mM EGTA, 5 mM magnesium chloride). Reactions were incubated at 32°C for 1 h and terminated by adding 5×Laemmli sample buffer. The reactions were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted and probed as described in the figure legends. Radiolabelling of lysates was carried out in a volume of 100 µl by mixing desalted serum-starved COS-1 lysate, GST-Raf-1 or GST-Raf-1 mutants, 100 µM cold ATP, and 0.7 MBq of [³²P]ATP or [³²P]*N*⁶(2-phenethyl) ATP in Raf kinase assay buffer. Reactions were incubated at 32°C for 30 min and terminated by adding 5×Laemmli sample buffer. The reactions were separated by large-format SDS-PAGE, blotted and exposed to film at -70°C. The blots were probed as described in the figure legends.

2.4. Synthesis of ATP analogues

*N*⁶-modified adenosine analogues were synthesised by refluxing 6-chloropurine riboside (Aldrich) with the corresponding amines in ethanol overnight [21]. *N*⁶-substituted ATP analogues were synthesised by the method of Hecht and Kozarich [22] as described in [2]. The radiolabelled analogue was purified on DEAE (A-25) Sephadex (0.1–1 M triethylammonium bicarbonate buffer pH 7.5) and the triphosphate was identified by co-injection of the radiolabelled material with an authentic sample of *N*⁶ ATP analogue on a strong anion exchange high performance liquid chromatography column (5–750 mM ammonium phosphate pH 3.9 in 10 min at 0.5 ml/min).

2.5. Homology modelling

The tertiary structure for Raf-1 was modelled by means of homology modelling [23]. For the purpose, the homology modelling program MODELLER was employed [12]. In homology modelling, a homologous structure is used as a template for the unknown structure. MODELLER obtains the three-dimensional model of the unknown protein by satisfying spatial restraints in the form of probability density functions (pdfs) derived from the alignment of the unknown structure with one or more homologous structures. The pdfs restrain C_α–C_α distances, main-chain N–O distances, as well as main-chain and side-chain dihedral angles. The optimisation is carried

out by the variable target method that applies the conjugate gradient algorithm to positions of all non-hydrogen atoms. The initial model generated by MODELLER is further optimised by constrained energy minimisation with all the backbone atoms fixed using the program X-PLOR [24].

2.6. Reporter gene assays

Reporter gene assays were done as previously described [25]. Briefly, COS-1 cells were transfected using Effectene (Promega), with 1 µg DNA per well in a six-well plate. The DNA included an AP-1 luciferase reporter construct, an activated truncated Raf-1 (BXB) construct and a β-galactosidase construct. Each sample was done in triplicate. Cells were harvested 48 h post transfection and lysed using reporter assay lysis buffer (Promega). Lysates were assayed for luciferase activity (reagents from Promega) and for β-galactosidase activity. Each data point was adjusted for transfection efficiency (using β-galactosidase assay results) and is an average of three wells.

2.7. Radiolabelling of cell lysates for 2D gel electrophoresis

Rat-1 S100 cytosolic extracts (40 µg) were mixed with 10 mM MgCl₂, 100 µM ATP, 10 mM KCl, 0.5 mM EDTA, 20 µCi of [³²P]ATP or [³²P]N⁶(2-phenethyl) ATP and 100 µM ATP. FLAG-tagged Raf-1, Raf-1 TAFL, or Raf-1 TAFLGA was expressed in COS cells and immunopurified as described above. Raf-1 proteins were added and the reactions were incubated at room temperature for 30 min. Kinase reactions were clarified by PlusOne 2-D Clean-up Kit (Amersham Biosciences) and resuspended in rehydration buffer (8 M urea, 2% Triton X-100, IPG buffer (pH 3–10 NL), 0.002%

bromophenol blue) for the first dimensional isoelectric focusing (IEF). IEFs with Immobiline Drystrip (7 cm, pH 3–10 NL, Amersham Biosciences) were performed at 5000 V for 3 h using Ettan[®]IPG-phor[®] Isoelectric Focusing System (Amersham Biosciences). IEF strips were equilibrated in SDS equilibration buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) for 15 min before loading onto 12% SDS-PAGE. Gels were dried and were subject to autoradiography using Kodak X-Omat AR film.

3. Results

3.1. The Raf-1 kinase domain: mutagenesis strategy based on structure prediction

No crystal structure of the Raf-1 kinase domain has been determined. Raf-1 is a serine/threonine-specific kinase and its kinase domain shows highest similarity to Src family tyrosine kinases [11]. As shown in Fig. 1a, the primary sequence of the kinase domain of Raf-1 and that of c-Src share 33% identity and 53% homology. Therefore, we based our mutagenesis strategy on the strategy utilised by Shokat et al. [2] to obtain v-Src mutants that accept orthogonal ATP analogues. These authors replaced the isoleucine at position 338 in v-Src with a glycine to create an ATP binding pocket that could accommodate N⁶(benzyl) ATP. A subsequent mutational analysis of v-Src showed that Ile338 was the key determinant of specific-

(a)

Identities = 96/283 (33%), Positives = 154/283 (53%)

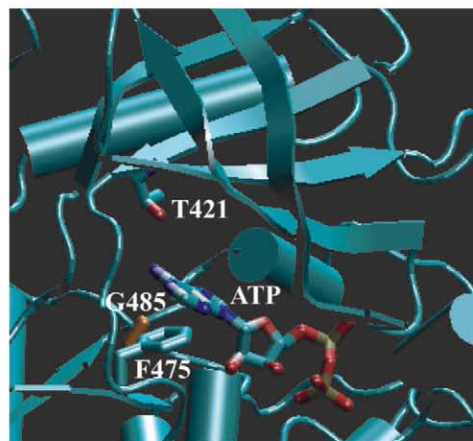
Raf	331	RPRQRDSSYYWEIEASEVMLSTRIGSGSFGTVYKGRHGD--VAVKILKVVDPTEQFQ	388
Alignment		++T·GL·KDA···PRES·R·EV++·Q·C·E·+M·T·+·T···+T·PGTM···	
Src		KPQTQGLAKDAWEIPRESLRLEVLKQGCFCGEVWMTWNGTTRVAIKTLKPGTMSPE---	
Raf	389	APRNEVAVLRKTRHVNILLPMGYMTKDNLAIVTQWCEGSSLYKHLHVQETKP-QMPOLID	447
Alignment		·LQ·AQ·++·L·EK++QLYAV++P+Y···+MSKG·LDF·KG·TG·+·++P··+	
Src		AFLEQAQVMKLRHKLRLQYAVVSEPIYIVTEYMSKSLDPLFGSETGKYLRPLQLVD	
Raf	448	IARQTAQGMVYLHAKNIHREDMKSNINILHEGLTVKIGDFGLATV--KSRWSGSQVEQP	505
Alignment		+A·I·S·A·A·ERN·Y····++A·L·G·N·VC·+A····R·D+E·+AR·GA·F·	
Src		MAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADFGRLARLIEDNEYTARQGAKEP	
Raf	506	TGSVLWMAPEVIRMQDNNFFSFQSDVYSYGVLYELMT-GELPYSHINNRDQIIFMVGRG	564
Alignment		+K·T···AALY GR·+I····++·+·T·T·R·+·PG·V··++DQ·E··	
Src		---IKWTAPAEALY---GRPTKSDVSWFGILLTRLTTRGRVYPVPMVNR-EVLDOVERG	
Raf	565	YASFDLSKLYKNCPKAMKRLVADCVKVKKEERPLFPQILSSIE 607	
Alignment		·RM·CPPE·++HD·+CQ·W·EP···T·EY·Q·P·+	
Src		YRMP---CPPECPESLHDLQCKWRKEPEERPTFEYLQAFLE	

(c)

C-Raf-1	KIL KVVDPTEQFQAFRNEVAVLRKTRHVNILLPM GYMTKDNLAIVTQWCEG	(426)
v-Src	KTLKPGTM···SPEAFLEQAQVMKLRHKLRLQYAVVSE EPIYIVIEYMSK	(343)
Cdk2	KKIRLDTEG·VPSTAIRESLLKELNHPNIVKLLDVIHTENKLYLVTEFLHQ	(85)
PKA	KILDKQVKVKKQIEHTLNKRIQAVNFFLVKLEFSFKDQNSNLYMVEYVPG	(125)

C-Raf-1	IARQTAQGMVYLHAKNIHREDMKSNINILHEGLTVKIGDFGLATVKSRSWSGSQ-	(500)
v-Src	MAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADFGRLARLIEDNEYT--	(417)
Cdk2	YLFOLLQGLAFCHSHRVLHRLDKPQLNINTEGAIKLADFGARAFGVVPR---	(153)
PKA	YAAQIVLTFFEYLHSLDLIYRDLKPNELIDQGGYIQVDFGFAKRVKG-----	(193)

(b)



(d)

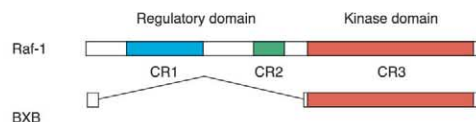


Fig. 1. Mutagenesis strategy and modelling of the Raf-1 ATP binding pocket. a: Alignment of the primary sequence of the kinase domains of Raf-1 and c-Src (PDB accession number 2SRC). Threonine 338 in c-Src and 421 in Raf-1 are indicated. b: Homology model of Raf-1 with bound ATP. The peptide is shown in a cartoon representation. The three mutated residues T421, F475 and G485 and their side chains are shown as is ATP which has been modelled into the binding site. c: Alignment of Raf-1, v-Src, Cdk2 and PKA, showing the sites targeted for mutagenesis in Raf-1 in this study. d: Schematic representation of the constructs used in this study.

ity for N^6 -substituted ATP analogues [4]. These results have been confirmed in mutational studies of Thr339 of Fyn, which occupies the position corresponding to Ile338 of v-Src [4]. Ile338 is a threonine in c-Src that corresponds to Thr421 in Raf-1. Therefore, we changed Thr421 to alanine or glycine. Although mutation at position Thr421 did confer orthogonal ATP analogue specificity on Raf-1 mutant kinase, it resulted in a low catalytic efficiency (data not shown).

In order to further improve the catalytic efficiency of the Thr421 Raf-1 mutants, additional targets for mutation were sought. To aid this search we modelled the Raf-1 kinase domain by means of homology modelling using the program MODELLER [12] based on the 1.5 Å resolution crystal structure of human tyrosine protein kinase c-Src (PDB accession number 2SRC [13]). The modelled three-dimensional structure for the Raf-1 peptide includes residues 331–607. The nucleotide ATP was placed into the protein analogous to its position in the crystal structure of c-Src [13]. The complex structure of Raf-1 with ATP was then optimised with molecular mechanics energy minimisation. The final model of Raf-1, as analysed by PROCHECK [14], has all the residues in the allowed regions of Ramachandran plot and an acceptable overall geometry. The full details of the Raf-1 kinase domain model will be presented in a separate communication.

The ATP binding pocket in the modelled three-dimensional structure (Fig. 1b) and sequence alignment of Raf-1 to other kinases (Fig. 1c) guided our selection of residues for site-directed mutagenesis. The goal was to enlarge the binding pocket of the adenine moiety of ATP so that a large substituent at the N^6 position can be accommodated spatially. Thr421 stands out in terms of geometric proximity to the N^6 atom of adenine. The closest atom to atom distance between the side chain of Thr421 and the N^6 amino group of ATP is 4.8 Å. This geometric proximity of Thr421 in the modelled Raf-1 structure is consistent with known crystal structures of two serine/threonine kinases, cAMP-dependent protein kinase (PKA) and cyclin-dependent protein kinase 2 (Cdk2), and three tyrosine kinases in the Src family (Src, Hck and Lck). Analysis of these structures revealed that in all cases the side chains of residues homologous to Thr338 in c-Src or Met120 in PKA are within a 5 Å sphere of the N^6 amino group of ATP [2]. In Fig. 1c, the primary sequence of Raf-1 is aligned with those of PKA, Cdk2 and v-Src. It shows that the residue Thr421 of Raf-1 is aligned to the corresponding residues in v-Src (Ile338) or PKA (Met120).

A careful inspection of the Raf-1 modelled structure revealed other residues with side chains that are in close proximity to the adenine base of ATP. For instance, Leu406 is located only 5 Å away from the N^6 amino group of ATP. Therefore, we mutated Leu406 to alanine. However, the resulting mutant kinase showed very low catalytic efficiency (data not shown). Phe475 is in close geometric proximity to the adenine base with a closest atom to atom distance of 3.5 Å. Interestingly, in our modelled structure (Fig. 1b) Phe475 is positioned at the entrance of the ATP binding pocket. We reasoned that the low catalytic efficiency of the Thr421 Raf-1 mutants could be due to the bulky Phe475 residue restricting the access of the bulky substituted ATP analogues to the ATP binding pocket. A sequence alignment between Raf-1, v-Src, Cdk2 and PKA (Fig. 1c) showed that Phe475 of Raf-1 corresponds to a leucine in the other three kinases. Therefore, we mutated Phe475 to leucine, creating a double mutant (T421A

and F475L). Hereafter, this double mutation is denoted Raf-TAFL. We also engineered a triple mutant by mutating an additional residue, Gly485, to alanine. The rationale behind this mutation was to better emulate the structure of the v-Src kinase. As seen in the sequence alignment between Raf-1 and v-Src (Fig. 1c), Gly485 of Raf-1 corresponds to Ala403 in v-Src. The G485A mutation enhances the similarity between Raf-1 and v-Src. This triple mutant (T421A, F475L and G485A) is denoted Raf-TAFLGA throughout the text.

The following studies characterise these mutants in more detail. In addition to full-length Raf-1 these mutations were also introduced into the isolated Raf-1 kinase domain, BXB (Fig. 1d). BXB contains a deletion of the N-terminal regulatory domain and is functionally equivalent to v-Raf [15].

3.2. *In vitro* and *in vivo* activities of mutated Raf-1 proteins

The main purpose of generating these Raf-1 mutants was to develop them as tools for the identification of physiological substrates. Therefore, it was important to show that the mutations did not alter the biochemical or biological properties of the Raf-1 kinase. First, we examined the ability of the mutant kinases to phosphorylate the MEK substrate (Fig. 2a). Wild-type Raf-1 and the different mutants were tagged at the amino-terminus with the FLAG epitope, over-expressed by transient transfection in COS-1 cells, and purified by immunoaffinity chromatography. The kinase activities of the purified proteins were determined using recombinant MEK as the substrate. MEK phosphorylation was detected with an antibody directed against the phosphorylated form of MEK. This antibody detects MEK only when it is phosphorylated on the activating serine residues Ser217 and Ser221. FLAG-tagged wild-type Raf-1 isolated from serum-deprived cells had very low activity (data not shown). Raf-1 kinase activity was substantially stimulated upon treatment with epidermal growth factor (Fig. 2a, lane 1). The specificity of the kinase activity was demonstrated by the observation that almost no measurable activity was detected in preparations of kinase-dead Raf-1 K375W (lane 2). Under these conditions the activity of the Raf-1 TAFL and TAFLGA kinases was only slightly reduced relative to wild-type Raf-1 (compare lane 1 with lanes 4 and 5). In contrast, the activity of the Raf-1 TA kinase was still detectable, but at a significantly reduced level (lane 3). For that reason only the double and triple mutants, Raf-1 TAFL and TAFLGA kinases, were selected for further experiments.

The above results could be explained by a reduced interaction of the mutants with MEK or ATP. However, kinase-dead Raf-1 is very efficient in interacting with MEK and actually acts as a substrate trap, presumably because it can bind substrate but not phosphorylate it (data not shown). Therefore, we examined the ability of the mutant kinases to utilise ATP (Fig. 2c). The wild-type, TAFL and TAFLGA Raf-1 proteins displayed a comparable K_M for ATP (3.3 μ M, 6.0 μ M, and 1.2 μ M, respectively). The K_M of Raf-1 for ATP was previously reported as 11.6 μ M [16]. This is in agreement with our measurements, given that Force et al. [16] used a different kinase assay and a differently tagged version of the Raf-1 protein. The widening of the ATP binding pocket to accommodate N^6 -substituted ATP analogues usually does not preclude the binding of ATP [2]. While this is often viewed as an unwanted effect, it has the advantage that such mutant kinases can function normally when expressed in cells. This is

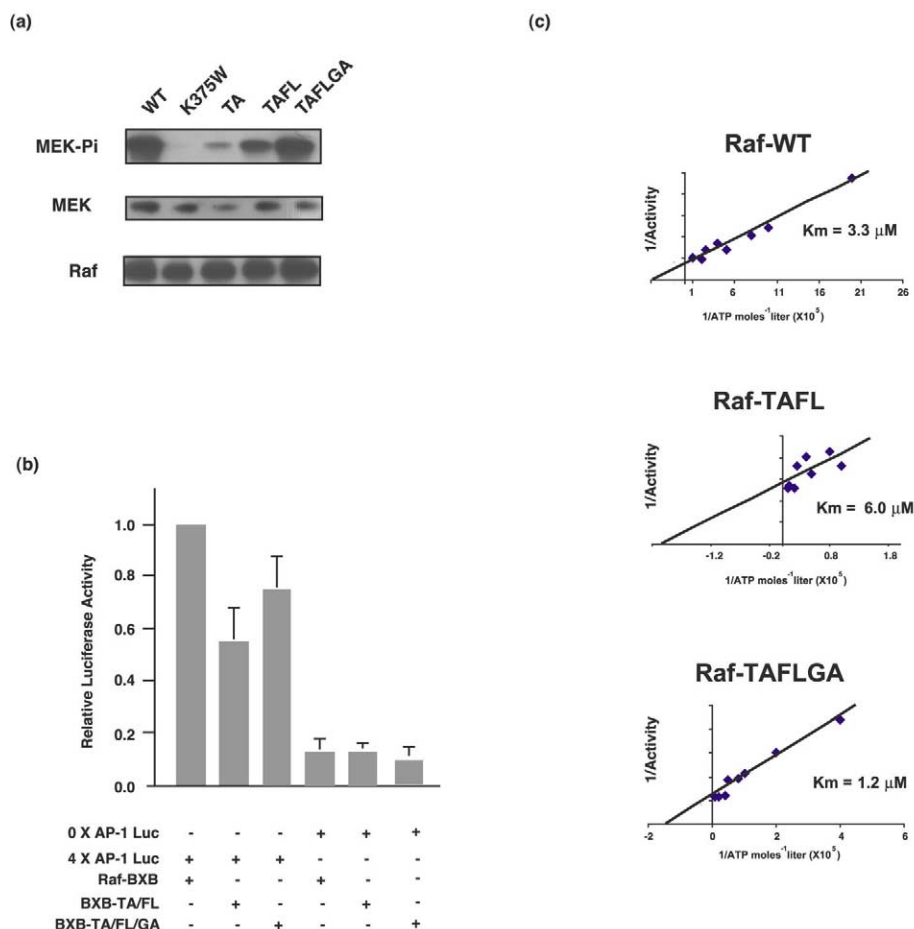


Fig. 2. Mutations in the ATP binding pocket do not affect the biochemical or biological properties of Raf-1. a: In vitro kinase assays. Raf-1, Raf-K375W (kinase-negative mutant) and ATP binding site mutants were expressed in COS-1 cells as described in Section 2. Raf-1 proteins were immunoprecipitated and incubated with purified recombinant MEK protein. Reactions were separated by SDS-PAGE and blotted. The blots were probed with anti-phospho-MEK (upper panel), anti-MEK (middle panel) and anti-Raf-1 (lower panel). b: AP-1 luciferase reporter assay data. COS-1 cells were transfected with the indicated plasmids and β -galactosidase expression vector. 4 $\times$ AP-1 Luc contains a quadruplicate AP-1 binding site in its promoter, 0 $\times$ AP-1 Luc is the corresponding control plasmid. Luciferase expression was adjusted for transfection efficiency using β -galactosidase activity. Data are shown as fraction of wild-type Raf-BXB activity. Each data point is representative of four separate experiments performed in triplicate. c: K_M (ATP) determination for Raf-1 and mutants. Assays were done as described in Section 2 and data are presented as Lineweaver–Burk plots.

important as kinase-dead mutants often behave as dominant negative mutants that sequester activators and substrates.

To measure the effects of the mutations on the biological activity of Raf-1, we made use of the well documented observation that the AP-1 transcription factor is one of its main intracellular effectors. In order to ensure that AP-1 activation was elicited by the transfected Raf-1 protein and not endogenous Raf-1, we incorporated the Raf-1 TAFL double mutations and Raf-1 TAFLGA triple mutations into a constitutively active form of Raf-1, BXB (Fig. 1b). BXB or its mutated derivatives were transiently expressed in COS-1 cells in the presence of a luciferase reporter gene whose expression was dependent on AP-1 activity. The control was an equivalent reporter plasmid without AP-1 binding sites. The data (Fig. 2b) are presented as proportions of wild-type Raf-BXB activity. AP-1 reporter activity of the double and triple mutants was similar to BXB, although a little reduced. In fact, the reduction is proportionally the same as the reduction in MEK phosphorylation seen in Fig. 2a. Activation of the reporter was AP-1-specific, since a reporter without AP-1 bind-

ing sites (AP-1 control) was not activated by any of the BXB constructs. Similarly, Raf-BXB constructs with TAFL or TAFLGA mutations were capable of conferring anchorage-independent growth in soft agar colony assays (data not shown).

Taken together, these results confirm that the mutations introduced into the ATP binding site do not significantly affect the biochemical, biological properties, and the substrate specificity of Raf-1. Our next step was to identify an optimal orthogonal ATP analogue for these mutants.

3.3. Specificity of wild-type and mutated Raf-1 proteins for ATP analogues

To find the optimal ATP analogue for the double and triple Raf-1 mutants, we screened a panel of N^6 -substituted ATP analogues which included aliphatic and aromatic substituents (Fig. 3a). This assay employed immunoprecipitated FLAG-tagged Raf-1 and mutant proteins, and recombinant kinase-dead MEK. The efficacy of each ATP analogue was assessed by blotting for phosphorylated MEK (Fig. 3b). Wild-type

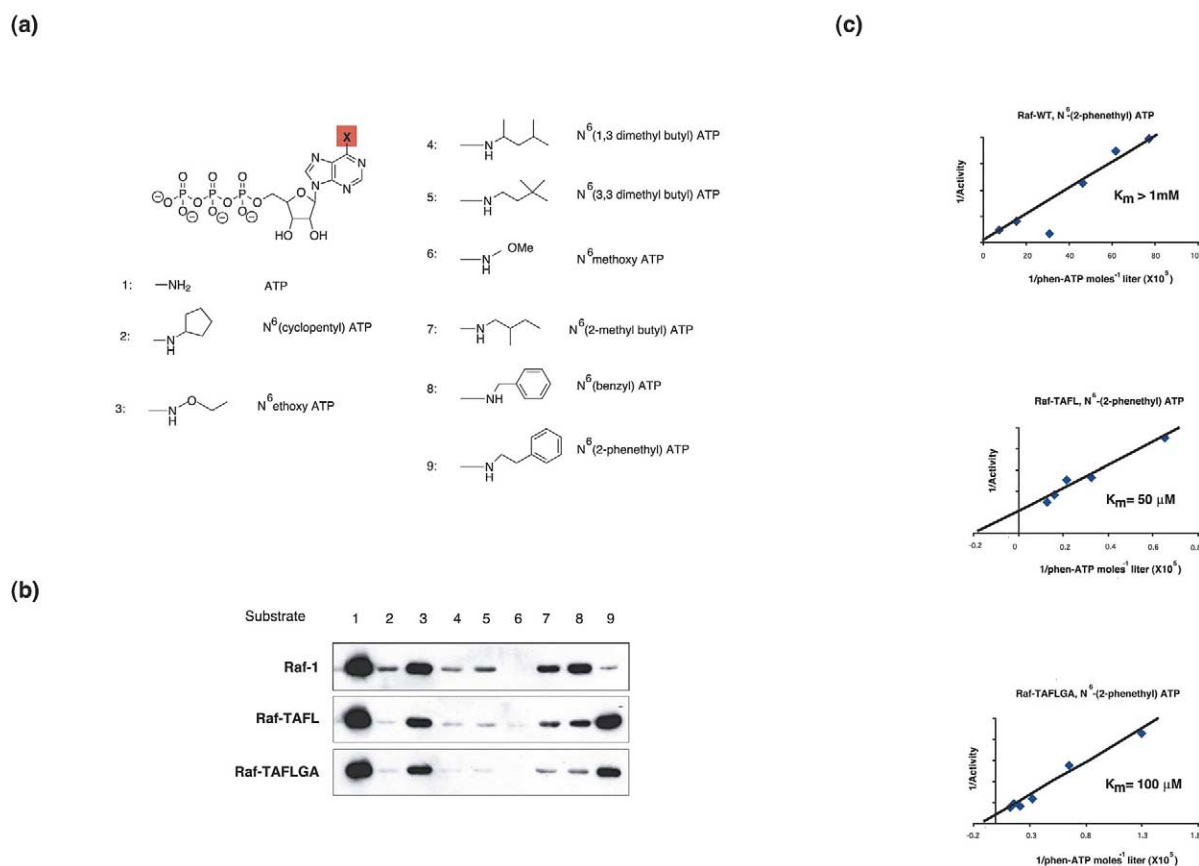


Fig. 3. Identification of ATP analogue utilised by Raf-1 ATP binding site mutants. a: The panel of ATP analogues used in screen with Raf-1 and mutants. b: In vitro kinase assays with immunoprecipitated Raf-1 and mutants and purified MEK as substrate, with the ATP analogues shown in panel a. Kinase assays were done as described in Section 2 and phosphorylated MEK was visualised by immunoblotting with anti-phospho-MEK antibody. c: K_M (N^6 (2-phenethyl) ATP) determination for Raf-1 and mutants. Data are presented as Lineweaver–Burk plots.

Raf-1 and the double and triple mutants showed a similar usage of ATP. Surprisingly, we found that wild-type Raf-1 could utilise a number of N^6 -substituted ATP analogues including N^6 (ethoxy) ATP, N^6 (2-methylbutyl) ATP as well as N^6 (benzyl) ATP. The double and triple mutants showed similar activity with regard to the analogues as the wild-type enzyme, with the exception of N^6 (2-phenethyl) ATP. In this assay N^6 (2-phenethyl) ATP emerged as the most reasonable orthogonal ATP analogue. Both Raf-TAFL and Raf-TAFLGA could utilise this analogue, but wild-type Raf-1 was clearly compromised. The double mutant, Raf-1 TAFL, had an approximately twofold better kinase activity towards MEK with N^6 (2-phenethyl) ATP than the triple mutant. To investigate whether these differences in MEK substrate phosphorylation can be explained by a differential utilisation of N^6 (2-phenethyl) ATP the K_M values of the Raf-1 TAFL and TAFLGA mutants for N^6 (2-phenethyl) ATP were determined (Fig. 3c). The K_M was 50 μM for Raf-1 TAFL and 100 μM for Raf-1 TAFLGA. This twofold difference closely correlates with the ability to phosphorylate MEK. Thus, these mutations make substrate phosphorylation by Raf dependent on the ability to use the corresponding ATP analogue without disturbing the interaction with protein substrates. Of similar importance was the finding that wild-type Raf-1 had an at least 10-fold or greater K_M for N^6 (2-phenethyl) ATP than the mutants.

3.4. In vitro labelling of cell extracts with [^{32}P] N^6 (2-phenethyl) ATP

As judged by the above results the Raf TAFL and Raf TAFLGA mutants appeared to be suitable tools for the identification of new Raf-1 substrates. Unfortunately, N^6 (2-phenethyl) ATP was not cell-permeable precluding in vivo labelling. Therefore, we used a cell-free system, termed KESTREL, that was devised to identify new substrates for protein kinases [17]. This system uses crude cell lysates that are desalted using a PD-10 ion exchange column to remove small molecules including ATP. This guarantees that the only source of ATP is exogenously added ATP. The kinase of interest is added as a purified protein along with [$\gamma\text{-}^{32}\text{P}$]ATP in order to allow the phosphorylation of substrates in the lysate [17]. In contrast to most other protein kinases Raf-1 only phosphorylates native MEK – Raf-1 does not phosphorylate denatured MEK or peptide substrates [16]. Therefore, to validate the KESTREL system we determined if the Raf TAFL mutant could phosphorylate MEK in desalted lysate prepared from COS-1 cells. The desalted lysates were incubated with ATP or N^6 (2-phenethyl) ATP plus purified recombinant GST-tagged Raf-1 proteins produced in Sf9 insect cells. Phosphorylated MEK was detected by a phosphospecific antibody (Fig. 4a). These results show that Raf and the Raf TAFL mutant could utilise ATP with similar efficiency to phosphorylate MEK in crude cell lysates. The Raf TAFL

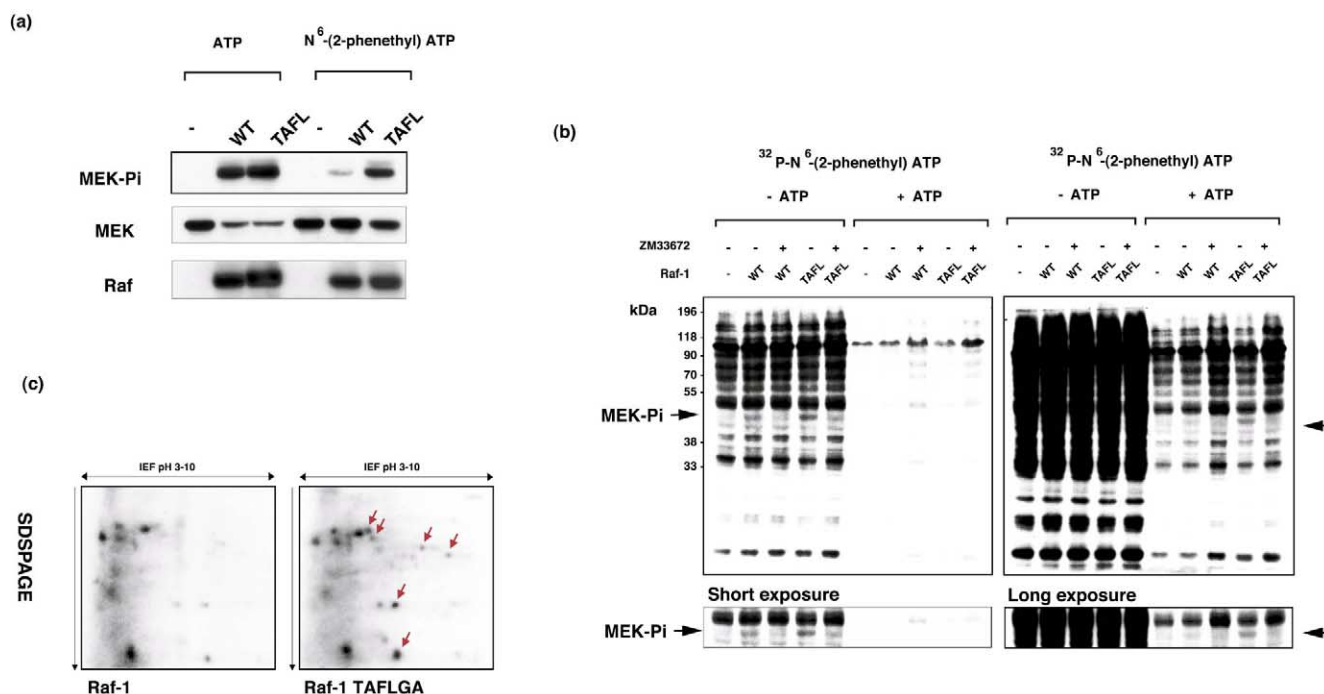


Fig. 4. Labelling of MEK and putative new substrates by Raf-1 mutants and N⁶(2-phenethyl) ATP in cell lysates. a: Phosphorylation of MEK in cell lysates by purified GST-tagged Raf-1 and Raf-1 TAFL in the presence of ATP or N⁶(2-phenethyl) ATP. Kinase assays were done as described in Section 2. Phosphorylated MEK was detected by immunoblotting with an anti-phospho-MEK antibody (upper panel). Total MEK and added GST-Raf proteins were detected using anti-MEK (middle panel) and anti-Raf-1 (lower panel) antibodies. b: Whole-cell lysate labelling with [³²P]ATP and [³²P]N⁶(2-phenethyl) ATP. Desalted COS-1 lysates were mixed with purified GST-tagged Raf-1 or Raf-1 TAFL in the presence or absence of Raf-1 inhibitor ZM33672 (10 μ M) as indicated. 20 μ Ci of [³²P]ATP or [³²P]N⁶(2-phenethyl) ATP and 100 μ M ATP were added. After incubation at room temperature for 30 min samples were separated by SDS-PAGE, blotted and autoradiographed. A short and a long exposure of the autoradiogram is shown. Arrows indicates the radiolabelled MEK in the lysates. The lower panel is an enlargement of the region containing phosphorylated MEK. c: 2D gel electrophoresis of kinase reactions using [³²P]N⁶(2-phenethyl) ATP. An in vitro kinase assay was performed using desalted Rat-1 extract, 20 μ Ci [³²P]N⁶(2-phenethyl) ATP, 100 μ M ATP, and FLAG-tagged Raf-1 or Raf TAFLGA. Samples were subject to IEF in the first dimension and SDS-PAGE in the second dimension as indicated. Shown are autoradiograms with differentially phosphorylated proteins indicated by arrows.

mutant could also readily utilise N⁶(2-phenethyl) ATP to phosphorylate MEK. The wild-type Raf-1 protein was much less efficient although a low level of MEK phosphorylation was observed. The results confirm that the KESTREL cell-free system preserves protein/substrate folding and hence is suitable for the identification of new candidates for Raf-1 substrates.

We next used radiolabelled [³²P]N⁶(2-phenethyl) ATP to label desalted COS-1 lysates in the presence of wild-type Raf-1 or Raf TAFL (Fig. 4b). Surprisingly, we observed a rather high background of kinase activity in lysates incubated with [³²P]N⁶(2-phenethyl) ATP alone in the absence of added Raf proteins. This was unexpected and we tried various conditions to reduce this background. The lowest background was obtained when radiolabeling with [³²P]N⁶(2-phenethyl) ATP was carried out in the presence of 100 μ M cold ATP (compare lanes 1–5 with 6–10). The excess cold ATP presumably quenches kinases in the lysate which are promiscuous for ATP substrate. The addition of recombinant Raf-1 proteins did result in the phosphorylation of MEK as indicated by the use of a Raf-1 inhibitor, ZM33672, to assure that phosphorylations were indeed Raf-1-dependent. A radiolabelled band at the position of MEK (as indicated by the arrow in Fig. 4b and the lower panel) was observed when the lysate was incubated with Raf TAFL and N⁶(2-phenethyl) [³²P]ATP, but abolished in the presence of ZM33672. The identity of the radiolabelled band was subsequently confirmed by immuno-

blotting (data not shown). Similar results were obtained with TAFLGA mutants (data not shown). Thus, both TAFL and TAFLGA mutants can facilitate the identification of Raf-1 substrates.

Despite numerous attempts and different regimens we could not further reduce the background phosphorylation. Therefore, we investigated whether a higher resolution gel technique such as 2D gel electrophoresis could reveal differences in substrate phosphorylation between Raf-1 and the Raf-1 mutants in the presence of [³²P]N⁶(2-phenethyl) ATP. For this purpose we prepared cytosolic extracts from serum-starved Rat-1 cells. Serum starvation will render many cellular kinases inactive and thus reduces the background. Purified recombinant Raf-1 or Raf-1 TAFLGA kinases were added to the Rat-1 extract and incubated with [³²P]N⁶(2-phenethyl) ATP and 100 μ M cold ATP. The proteins were analysed by 2D gel electrophoresis. The higher resolution of this method revealed additional phosphorylated proteins (marked by arrows), which are present exclusively or at much higher abundance in extracts labelled with [³²P]N⁶(2-phenethyl) ATP and TAFLGA mutant kinase (Fig. 4c). Identical results were obtained with Raf-1 TAFL (data not shown). These results strongly suggest that Raf-1 has more substrates in addition to MEK. The phosphorylated proteins could be detected by autoradiography, but their detection by various protein stains was unreliable. This indicates low abundance and has so far hindered their identification.

4. Discussion

We have carried out a mutagenesis study of the serine/threonine kinase Raf-1 with the aim of generating a mutant Raf-1 that can utilise orthogonal ATP analogues while preserving its ability to faithfully phosphorylate its substrate MEK and to mediate Raf-1-dependent signaling in cells. Our choice of mutations was based on a successful studies with v-Src [2,4,5], which was mutated to utilise N^6 (benzyl) ATP. The choice of mutations in the Shokat group studies was guided by homology to kinases with known structures, followed by a refinement round of mutagenesis and screening of a library of ATP analogues. A similar strategy was adopted here. As the three-dimensional structure of Raf-1 is still unknown, we developed a homology model of Raf-1 based on the 1.5 Å resolution crystal structure of v-Src. The homology model along with sequence alignment to other kinases with known crystal structures was used to identify residues in geometric proximity of the adenine base in the ATP binding pocket. Those proximal amino acids were Thr421 (analogous to v-Src Ile338), Phe475 (analogous to v-Src Leu398) and Gly485 (analogous to v-Src Ala405). They were mutated to alanine, leucine and alanine respectively, to mirror the studies with v-Src relative to position 421 in Raf-1, and to mirror the v-Src ATP binding site for positions 475 and 485. The initial stage of our studies involved a Raf-1 T421A single mutant. However, this single mutation had a very low catalytic activity (data not shown). Thus, additional double and triple mutations were made. The data presented in this study compare the catalytic properties of wild-type Raf-1 with the double mutant Raf-1 T421A F475L (TAFL) and triple mutant Raf-1 T421A F475L G485A (TAFLGA). We show that both the double and triple mutant of Raf-1 proteins can utilise N^6 (2-phenethyl) ATP, which is orthogonal for wild-type Raf-1.

While it is not currently possible to predict with certainty an optimal mutant–analogue combination, we show here that this can be achieved by an iterative process of optimisation. In our experience there are two key requirements for performing the optimisation process in a timely fashion: a non-radioactive kinase assay for the phosphotransfer reaction and a large library of ATP analogues. We have found probing with a phospho-specific antibody against substrate(s) to be a rapid and sensitive assay of phosphotransfer. For the tyrosine kinase family of enzymes probing with phospho-tyrosine antibodies can be adapted for this purpose [4]. This avoids the otherwise necessary use of radiolabelled ATP analogues in the early engineering stage of a study such as this. To aid the search for an optimal analogue, we synthesised a library of over 30 analogues, which include a variety of aliphatic, cyclic, and aromatic substituents.

The Raf-1 TAFL and TAFLGA mutants utilised several ATP analogues with varying efficiency. These results suggest that the mutations do not affect catalysis, but only the affinity of the enzyme for the ATP analogue. That is, the catalytic efficiency of any given enzyme–analogue pair is determined primarily by the affinity of the enzyme for the ATP analogue. When considered in combination with the data on v-Src [2,4,5], it is becoming increasingly apparent that the ATP binding domain is a robust structure whose ATP binding pocket can be subjected to extensive engineering without affecting the binding of protein substrates. A recent crystal structure of c-Src complexed with N^6 (benzyl) ADP showed

that the mutation of the ATP binding pocket does not alter the phospho-acceptor binding site of the kinase [18]. It is interesting to note that, although the Raf-1 mutations have been made to mirror the v-Src mutant, the behaviour of the mutant Raf-1 and mutant v-Src proteins towards the panel of analogues was different. While N^6 (benzyl) ATP was not accepted by wild-type v-Src, it was accepted by wild-type Raf-1 (Fig. 3). The N^6 substituent had to be enlarged to 2-phenethyl, which is bulkier than benzyl, in order to obtain an ATP analogue not accepted by wild-type Raf-1 and yet capable of being accepted by a mutant form. Thus, Raf-1 may have an intrinsically larger ATP binding pocket than v-Src.

Our results also suggest that the guiding principle of ‘bulkiness’ in redesigning ATP binding pockets of protein kinases is likely to be an oversimplification. Specific contacts between amino acid side chains and the ATP substituent play an increasingly important role as the interaction between the protein and the analogue is optimised. For example, while v-Src accepted both N^6 (cyclopentyl) ATP and N^6 (benzyl) ATP, the Raf-1 double and triple mutants accepted only N^6 (benzyl) ATP and did not accept N^6 (cyclopentyl) ATP, both of which are less bulky than N^6 (2-phenethyl) ATP. Thus, Raf-1 seems to prefer aromatic N^6 substituents and discriminate against the cyclic aliphatic substituents. A recent study has explored the shape of the ATP binding pocket of v-Src by engineering different *N*-substituted ATP analogues. This study revealed that the mutation of Ile338 in v-Src to the smaller Ala or Gly residues did not create an entirely new ATP binding pocket, but rather the mutations opened a gate for the N^6 substituent on ATP to gain access to an existing pocket in the ATP binding site [4]. Therefore, a large library of ATP analogues is crucial for the optimisation and fine tuning of enzyme–analogue interactions. Our observations show that it may require more than one mutation and an iterative optimisation process in order to generate suitable mutants that lend themselves as tools for the discovery and characterisation of substrates.

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