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Techniques to examine nucleotide binding by pseudokinases

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Abstract
Approximately 10% of the human kinome have been classified as pseudokinases due to the absence of one or more of three motifs known to play key roles in the catalytic activities of protein kinases. Structural and functional studies are now emerging, reclassifying this "dead" kinase family as essential signalling molecules that act as crucial modulators of signal transduction. This raises the prospect that pseudokinases may well represent an as-yet-unexplored class of drug targets. However, the extent to which nucleotide binding and catalytic activity contribute to the biological functions of pseudokinases still remain an area of great controversy. In this review, we discuss the advantages and disadvantages of the different methods employed to characterize the nucleotide binding properties and activity of pseudokinases.

Introduction
The breadth and functional diversity of the pseudokinase family have only started to emerge. While pseudokinases display a typical kinase fold, constructed of the ten canonical subdomains [1], subtle alterations in the constellation of amino acids that make up the key catalytic elements are observed, leading to their initial classification as "inactive" kinase. Notwithstanding the challenges, essential functions in signal transduction have now been attributed to some pseudokinases, mainly as modulators of the catalytic activities of *bona fide* protein kinases or as scaffolding proteins that promote the assembly of signaling complexes. Some have even been attributed kinase activity, albeit with a catalytic efficiency significantly lower than a "normal" protein kinase, which questions the relevance of this catalytic activity to the pseudokinase biological function in signal transduction.
While it is clear that the "true function" of pseudokinases cannot be predicted solely on bioinformatic studies and that each pseudokinase should be experimentally studied using a combination of techniques, the direct methods that have been employed to probe their nucleotide binding properties and potential catalytic activity have often resulted in conflicting results. Considering that relatively few pseudokinases have been characterized to date, it is important to review the methods currently used to evaluate nucleotide binding and provide best practices to allow the correct classification of pseudokinases as either active enzymes whose biological function requires their catalytic activity or else those that are catalytically inactive and therefore function via a different mechanism.

When is a pseudokinase a pseudokinase?
The first method that comes to mind in the characterization of the activity of a kinase is the use of standard kinase assay in presence of [γ-^{32}P]ATP and a suitable substrate. While this method is the method of choice for characterizing the catalytic activity of a kinase and determining its cognate substrates, when it comes to pseudokinases, it is more problematic. The catalytic efficiency of pseudokinases, if it exists, may be orders of magnitude lower than ordinary kinases either through a reduced affinity for ATP and/or substrate or else a reduced k_{cat}. For example pseudokinases for which kinase activity has been convincingly detected (e.g. JAK2, HER3/ErbB3 and KSR2) have all been reported to display a very low activity by comparison to their closest true kinase paralogue. Interestingly, HER3 [7, 8] and JAK2(JH2) [16] contain an asparagine residue (N815) in place of the catalytic aspartate that may explain their reduced catalytic output. Indeed, robust residual kinase activity is seen mainly via an autophosphorylation event, in which the apparent local concentration of "substrate" is extremely high, counteracting any weak $K_D^{substrate}$. This may be a common theme for other weakly active pseudokinases.

Because of this, the presence of even miniscule amounts of contaminating kinases from the original expression host (especially a eukaryotic expression host) can lead to one falsely ascribing catalytic activity to a "dead" pseudokinase. Therefore, as well as it being absolutely critical to obtain a highly pure sample though the inclusion of several purification steps, kinase assays should always be accompanied with the generation of kinase-dead mutants to ensure that the activity seen in the wild-type form is entirely due to the pseudokinase studied. A great example is the vertebrate mitotic checkpoint protein BubR1, long believed to have kinase activity due to the presence of the key residues within the VAIK, HRD and DFG motifs (reviewed in [21]) but elegantly shown recently,

using four-kinase deficient mutants and deletion mutants, that the activity reported was more likely due to co-purified contaminated kinases [22]. Instead, residues lining the nucleotide-binding pocket were shown to be essential for BubR1 conformational stability, classifying BubR1 as an unusual pseudokinase. Similarly, ILK was previously shown to have weak kinase activity in presence of manganese [23] but has been recently reported to be devoid of catalytic activity, instead playing a major role as a scaffolding protein through its ankyrin repeat domain and pseudokinase domain [3]. The reported catalytic activities of some pseudokinase domains require further examination with appropriate kinase-deficient mutants included as controls, since the greatest technical challenge to studying domains, which may possess weak catalytic activities, is to eliminate the possibility that this activity is entirely attributable to trace amounts of contaminating *bona fide* protein kinases from the expression host. As a result, it remains to be established beyond doubt whether proteins such as PEAK1/SgK269 [24] and Scyl2/CVAK104 [25] are capable of catalyzing phosphoryl transfer.

Methods used to characterize the structural integrity of the ATP binding site

The issues regarding contamination and kinase assays have led to the search for other methods to determine whether a pseudokinase is catalytically active or not. In order to be catalytically active, a pseudokinase must bind a nucleotide triphosphate, thereby allowing ATP binding to be assayed to determine potential activity. Most binding assays are far less sensitive to contamination than enzymatic assays as the conditions can usually be manipulated to observe only the dominant protein species in the assay mixture. In this review we discuss a number of methods used to determine ATP binding however an important caveat is that whilst ATP binding is *necessary* for catalytic activity it is not *sufficient*, this issue will be discussed below.

X-ray crystallography and NMR spectroscopy

Although arguably the most technically challenging approach, X-ray crystallography enables the most definitive examination of a pseudokinase's nucleotide binding pocket and propensity to engage ATP. In particular, the crystal structures of pseudokinases in complex with nucleotide ligands provide both definitive evidence for nucleotide-binding propensity as well as atomic level insights into the nucleotide binding mode. In addition, such structures allow the examination of the presence or absence of crucial residues typical of conventional protein kinases within a pseudokinase domain's nucleotide binding pocket and may permit the identification of non-canonical catalytic residues, such as atypical catalytic lysine in the protein kinase, WNK [2]. Several nucleotide-bound pseudokinases have recently been structurally characterized: ILK [3];[4], JAK2(JH2) [5], ROP5B$_I$ [6], HER3[7, 8]; STRADα [9, 10]; and CASK[11], and these reports were accompanied with complementary biophysical and biochemical techniques (described below) in order to fully unravel their true function. In contrast, the VRK3[12], ROP2 [13] and MviN [14] pseudokinase domains, were crystallised as apo proteins and additional biophysical data were presented to support an absence of ATP binding propensity. In the case of ROP2, NMR spectroscopy – arguably the most sensitive biophysical technique for examining weak intermolecular interactions – was employed to confirm an absence of ATP binding [13].

Fluorescence spectroscopy using fluorescent nucleotide analogs

Several methods are today available to assess the propensity of pseudokinases to bind nucleotides using a variety of nucleotide analogues that act as ATP-competitive inhibitors.

The fluorescent probes TNP and mant are suitable tools to label various nucleotides for enzymatic studies, as they are rather small and attached to the nucleotide's ribose moiety, rendering steric inhibition of enzymatic reactions more unlikely than modifications at the base or the phosphoryl moiety. Both the ATP analog 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) and mant[2′-(3′)-O-(N-methylanthraniloyl)]-ATP are sensitive to environment polarity, with their fluorescence significantly enhanced upon binding to hydrophobic binding pocket, making them highly suitable for various assays of ATP binding to kinases [15]. For TNP-ATP binding assays, fluorescence emission spectra are usually recorded with the excitation wavelength set at 410 nm and

the emission wavelength scanned from 480 to 650 nm. For mant-ATP binding assay, the fluorescence excitation is set at 280 nm and the emission detection at 450 nm. Competition between ATP and the fluorescent nucleotide analogue can be used to characterize the binding of ATP to the test protein. However, in some cases TNP and mant-substituted nucleoside 5′-triphosphates have been reported to bind much more tightly to kinases and pseudokinases than ATP itself, therefore the K_d values obtained for these analogues may not be indicative of the K_d values of ATP. Conversely, the active sites or pseudoactive sites of kinase-like proteins may not accommodate binding of these modified analogs, or may bind in a mode non-conducive to fluorescent excitation. As a result, caution should also be taken in interpreting an absence of fluorescent excitation, since this may reflect as absence of ATP-analog binding rather than a lack of ATP binding potential.

Using TNP-ATP, Zeqiraj and colleagues demonstrated that STRADα bound nucleotide in the absence of its biological partner MO25α and cations. The $K_D^{TNP\text{-}ATP}$ was 1.1μM (30 to 100 fold higher than ATP). The structure of STRADα later revealed an "active" conformation with ATP bound but no cation. Consistent with these data, no catalytic activity was detected for STRADα, rather its ability to activate the tumor suppressor LKB1 seems to be mediated by the adoption of an overall "active-like conformation" that is mediated via ATP and MO25α binding [9, 10] (see below).

CASK CaM-kinase domain was also shown to interact with TNP-ATP but with a much weaker affinity (K_D = 0.563 mM) [11]. In keeping with TNP-ATP exhibiting markedly higher affinities than ATP for some kinase-like domains, a 500-fold excess of ATP was insufficient to displace all bound TNP-ATP. In addition to a millimolar affinity for ATP, CASK's affinity for ATP was abrogated by the presence of divalent cations, posing an interesting conundrum of how tightly a kinase needs to bind to ATP for catalytic activity, how can phosphotransfer be mediated in the absence of cations, and can such an activity serve an important physiological function?

Using mant-ATP, HER3/ErbB3 TKD was also shown to fully retain ATP-binding ability in presence of Mg^{2+} with a K_D value of 1.1μM [7]. Similarly, JAK2(JH2) has been reported to have a K_D value for mant-ATP of 1μM in presence of Mg^{2+} [16].

Fluorescence polarization

Boron Dipyrromethene (BODIPY) dye–labeled nucleotides have also been used as long-wavelength probes of nucleotide binding sites, enzyme substrates and for screening applications. For protein-binding studies that require non-hydrolyzable nucleotides, a BODIPY fluorophore linked through the γ-thiol of ATP-γ-*S* is commonly used. Fluorescence polarization measurements are measured using an excitation energy of 485 nm and a 535 nm emission filter with a multilabel counter. This approach was used to assay the ATP binding propensity of the mycobacterial pseudokinase MviN, yielding no evidence of binding of MviN to the fluorescent ATP analog, a finding that can be rationalised by structural data showing the ATP binding site is filled with aliphatic and aromatic residues, including those from the degraded G-loop [14].

Kinase-affinity chromatography using immobilised ATP and ATP-mimetics

Affinity beads, in which ATP is linked via the γ-phosphate group via a long hydrophilic spacer, have been extensively used for identifying proteins with a nucleotide-binding site. Affinity pull-down experiments using ATP agarose/sepaharose beads are relatively simple to set up using either cell lysates or purified protein. The kinase homology domain (KHD) of the Guanylyl Cyclase C (GC-C), also known as HSER [17], was first shown to directly interact with ATP from cell lysates using ATP-agarose beads and mutation of the conserved lysine in the VAIK motif was shown to abolish this interaction [18]. Similarly, the ability of the *Toxoplasma gondii* pseudokinase ROP5 to bind ATP was tested by incubation of purified recombinant ROP5 with γ-phospho linked ATP-Sepharose and was shown to bind the resin in an Mg^{2+}dependent manner with excess ATP inhibiting this interaction [6]. More recently, the advances in proteomic technologies combined with the use of immobilised ATP-mimetics has opened new avenues in direct cellular kinome profiling. This affinity-based chemoproteomics strategy relies on an affinity matrix (best known as kinobeads) that captures nucleotide-binding proteins from cell extract via their nucleotide-binding site. Typically, ligand

affinities are determined by adding free ligand (ATP or ATP-analogue) to the cell extract and by quantifying the remaining captured kinases by mass spectrometry. This method was recently used to directly assess the affinities of 200 kinases for the cellular nucleotide cofactors, ATP, ADP and GTP in presence of Mg^{2+} and Mn^{2+}. Among these, EphB6, ILK, STRADα were reported to bind ATP in presence of Mg^{2+} with affinities of 230, 34 and 30 μM, respectively [19].

Fluorescence-based thermal shift assay

Thermal denaturation-based methods are also independent of protein function, which is especially useful for characterizing ligand-binding capacity of pseudokinases. In addition, thermal denaturation-based methods provide critical information on protein stability and hence provide a direct method to assay optimal buffer conditions (ie pH, ions) that may be used for subsequent assays and structural studies.

The assay takes advantage of a change in fluorescence arising from binding of the fluorescent dye, SYPRO Orange, to hydrophobic patches that become exposed when the protein undergoes thermal unfolding. Ligand binding to a protein is known to enhance a protein's thermal stability, and is assessed by a shift observed in the unfolding temperature (T_m) between the unliganded protein versus the liganded protein. This assay confers several advantages over other techniques; it requires minimal amounts of recombinant protein (2-5 μg per condition), is rapid, provides highly reproducible ΔTm which has been shown to correlate well with binding strength and IC_{50} values [20], and can be run in a 96/384 well plate format allowing high-throughput screening. The upper limit of the assay is mainly dictated by the solubility of the ligand and its K_D, which appears to be around 100μM to observe a reproducible ΔTm (above 2-3°C). Hence, fluorescence-based thermal shift assays have been widely used for hit identification in kinase drug discovery programs and we anticipate that it will be the method of choice for the search of pseudokinase inhibitors when inhibitor binding to a pseudokinase has been shown to modify downstream signalling.

Using this methodology, VRK3, a pseudokinase member of the vaccinia related kinase (VRK) family, was screened against ATP and a range of pan-kinase inhibitors and was shown to be devoid of any nucleotide/nucleotide analogue binding in keeping with the structural data showing that although VRK3 has kept its structural integrity, adopting an active-like conformation, required for binding to biological partners, it displays a highly degraded, non-functional ATP binding site [12].

Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is a robust method for quantitative analysis of molecular interaction by providing a complete picture of the thermodynamics of a binding reaction by determining dissociation constant (K_D), stoichiometry (*i.e.*, number of binding sites) and enthalpy. The major drawbacks are the need for high concentrations of purified protein, preparation time and buffer limitations.

Insight into the ATP-ILK interaction was obtained by ITC and a K_D of 3.6 μM was reported but unlike the STRADα/ATP complex, ATP binding did not induce any gross conformational change of ILK [3, 4].

Nucleotide binding versus catalytic activity

The important finding that a number of pseudokinases have retained the ability to bind ATP, but are devoid of any catalytic activity, shows that ATP binding can serve a non-catalytic role and therefore does not necessarily signify an active kinase. ATP binding to regular kinases induces a major rearrangement of the N- and C-lobes of the kinases, resulting in a more compact and stable structure. Likewise, ATP binding to a pseudokinase can induce a similar conformational change. Such a change may be envisaged to act as a "conformational switch", modulating the binding of interaction partners and thereby effecting downstream signalling. This has been explicitly demonstrated for the kinase homology domain (KHD) of the receptor guanylyl cyclases GC-C/ HSER, [18] and the pseudokinase STRADα [10].

We believe that a strong "hint" as to whether a pseudokinase is active or not is whether it binds ATP *in the presence of* Mg^{2+} (or Mn^{2+}). It is generally assumed that cation binding by active kinases is a required event in order for phosphate-transfer to occur. Therefore those pseudokinases that bind ATP but only in the absence of cations may be expected to be catalytically dead. In a similar vein, if a pseudokinase only binds ATP in the presence of cation this suggests there has been selective pressure for the protein to retain this ability and may indicate it is active.

So far, only IRAK2 kinase activity has been shown to be crucial to its function *in vivo* [26]. However, studies of BubR1 [22] and ILK [3] indicate that VAIK motif mutations may render a pseudokinase unstable, rather than catalytically-compromised, demonstrating that caution should be exerted in interpreting the biological effects of IRAK2 mutation, especially in the absence of supporting biochemical data derived from highly-purified recombinant proteins. HER3/ErbB3 was also shown to exhibit robust residual kinase activity that may be crucial for ErbB signaling. JAK2(JH2) was shown to autophosphorylate two negative regulatory sites on itself: Ser523 and Tyr570 [5, 16]. A combination of structural, biochemical and chemical biology approaches were recently employed to dissect the function of Kinase Suppressor of Ras 2 (KSR2), a pseudokinase effector in the MEK-Raf-ERK signaling pathway [27]. This work, in which KSR2's nucleotide binding pocket was mutated to accommodate a "bumped" ATP-analog, illustrates how emerging chemical biology approaches may provide an elegant and direct means to unequivocally attribute a catalytic activity to a pseudokinase domain in future studies.

Concluding remarks

Following the recent boom in genomic information, examples of pseudokinase domains have been identified in all kingdoms, suggesting important, conserved functions among this protein family throughout nature. An important challenge ahead is to deduce what functions these domains serve *in vivo*, and to what extent this function relies on nucleotide binding. Establishing whether these domains are catalytically active or kinase-dead represents an important challenge, which must begin with defining whether a pseudokinase is capable of binding ATP using one of the techniques reviewed herein. A subset of pseudokinases have been reported to bind ATP and exhibit a weak catalytic activity (e.g. IRAK2, JAK2, HER3/ErbB3, KSR2), which may be important to their functions as modulators of *bona fide* kinases, although much is yet to be done to fully unravel the true biological relevance of such activities. The most exciting prospect is that despite lacking catalytic activity, some pseudokinases have retained the ability to adopt a distinct conformation upon ATP binding that would classify them as true molecular switch (e.g. STRADα, HSER), which is likely to underlie their functions as signaling scaffolds. Additionally, ATP binding studies have unveiled a class of pseudokinase domains that do not bind nucleotides (e.g. VRK3, MviN), illustrating that the kinase-like fold can serve crucial scaffolding roles that are divorced from nucleotide-binding. We propose that the most robust technique for assessing nucleotide binding propensity is the fluorescence-based thermal shift assay, an assay that simultaneously provides crucial information about the stability of the protein in various buffers in the presence of different ligands that will help further studies, including crystallization for X-ray crystallography.

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Pseudokinases	Methods	Data	References
STRADα	Fluorescent ATP analogue Kinobeads SPR Structural data	TNP-ATP: Kd = 1.1 μM Kd (ATP-Mg^{2+})= 30 μM 3GNI, 2WTK	[9, 10] [19]
CASK	Fluorescent ATP analogue Structural data	TNP-ATP; Kd= 0.563 mM 3C0H, 3C0I	[11]
Her3/ErbB3	Fluorescent ATP analogue [32P] ATP/Kinase deficient mutants Structural data	Mant-ATP-Mg; Kd= 1.1 μM Catalytic activity 3KEX, 3LMG	[7, 8]
JAK2(JH2)	Fluorescent ATP analogue [32P] ATP/ Kinase deficient mutants Structural data	Mant-ATP-Mg; Kd=1.1 μM Catalytic activity 4FVQ	[5, 16]
IRAK2	[32P] ATP (from immunoprecipitated lysate)	Catalytic activity	[26]
MviN	Fluorescence polarization Structural data	No nucleotide binding 3UQC	[14]
HSER	Immobilized ATP Conformation-specific antibody	Binding to ATP-agarose	[18]
ROP5	Immobilized ATP	Binding to ATP sepharose	[6]
EphB6	Kinobeads	Kd (ATP-Mg^{2+})=230 μM	[19]
ILK	Kinobeads Structural data ITC [32P] ATP	Kd (ATP-Mg^{2+})=34 μM 3KMW, 3REP Kd (ATP) = 3.6 μM No catalytic activity	[19] [3, 4]
VRK3	Thermal shift assay Structural data	No nucleotide binding 2JII	[12]
BubR1	[32P] ATP/ Kinase deficient mutants	No catalytic activity	[22]

Table I: List of the techniques used to characterize pseudokinases and data obtained.

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