Techniques to examine nucleotide binding by pseudokinases

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Abstract
Approximately 10% of the human kinome has 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 remains an area of great controversy. In the present 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. Although 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’ kinases. 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 signalling 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.

Although it is clear that the ‘true function’ of pseudokinases cannot be predicted solely on bioinformatic studies and that each pseudokinase should be studied experimentally 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 the standard kinase assay in the presence of [γ-32P]ATP and a suitable substrate. Although 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 kcat. For example, pseudokinases for which kinase activity has been convincingly detected (e.g. JAK2 [Janus kinase 2] [JH2 (JAK homology 2)], HER3 (human epidermal growth factor receptor 3)/ErbB3 and KSR2 (kinase suppressor of Ras 2)) have all been reported to display a very low activity in comparison with their closest true kinase parologue. Interestingly, HER3 [2,3] and JAK2 (JH2) [4] contain an asparagine residue (Asn158) in the place of the catalytic aspartate residue 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 Kdsubstrate. This may be a common theme for other weakly active pseudokinases.

Because of this, the presence of even minuscule 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’
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 an NTP, 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 the present review, we discuss a number of methods used to determine ATP binding; however, an important caveat is that, although ATP binding is necessary for catalytic activity, it is not sufficient; this issue is 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 an atypical catalytic lysine residue in the protein kinase WNK (with no lysine) [11]. Several nucleotide-bound pseudokinases have been structurally characterized: ILK [8,12], JAK2 (JH2) [13], ROP (rhoptry protein) 5B1 [14], HER3 [2,3], STRADα (STE20-related adaptor α) [15,16] and CASK (Ca2+/calmodulin-dependent serine protein kinase) [17], 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 (vaccinia-related kinase 3) [18], ROP2 [19] and MviN [20] pseudokinase domains were crystallized as apoproteins 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 [19].

Fluorescence spectroscopy using fluorescent nucleotide analogues

Several methods are available today 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 [2′,3′-O-(2,4,6-trinitrophenyl)] and mant [2′(3′)-O-(N-methylanthraniloyl)] 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. The ATP analogues TNP-ATP and mant-ATP are both sensitive to environment polarity, with their fluorescence significantly enhanced upon binding to hydrophobic binding pockets, making them highly suitable for various assays of ATP binding to kinases [21]. 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 assays, the fluorescence excitation is set at 280 nm and the emission detection is set 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 NTPs 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 analogues, or may
bind in a mode non-conducive to fluorescence excitation. As a result, caution should also be taken in interpreting an absence of fluorescent excitation, since this may reflect as absence of ATP analogue binding rather than a lack of ATP-binding potential.

Using TNP-ATP, Zeqiraj et al. [15,16] demonstrated that STRADα bound nucleotide in the absence of its biological partner MO25α and cations. The $K_d^{TNP-ATP}$ was 1.1 μM (30–100-fold higher than for 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 tumour suppressor LKB1 seems to be mediated by the adoption of an overall ‘active-like conformation’ that is mediated via ATP and MO25α binding [15,16] (see below).

CASK was also shown to interact with TNP-ATP, but with a much weaker affinity ($K_a 0.563$ mM) [17]. In keeping with TNP-ATP binding 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, the HER3/ErbB3 pseudokinase domain was also shown to fully retain ATP-binding ability in the presence of Mg$^{2+}$ with a $K_a$ value of 1.1μM [3]. Similarly, JAK2 (JH2) has been reported to have a $K_a$ value for mant-ATP of 1 μM in the presence of Mg$^{2+}$ [4].

**Fluorescence polarization**

BODIPY (boron dipyrromethene) dye-labelled 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-hydrolysable nucleotides, a BODIPY fluorophore linked through the $\gamma$-thiol of ATPγS (adenosine 5′-[γ-thio]triphosphate) 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 assess the ATP-binding propensity of the mycobacterial pseudokinase MvIN, yielding no evidence of binding of MvIN to the fluorescent ATP analogue, a finding that can be rationalized by structural data showing that the ATP-binding site is filled with aliphatic and aromatic residues, including those from the degraded G-loop [20].

**Kinase-affinity chromatography using immobilized ATP and ATP-mimetics**

Affinity beads, to which ATP is linked via the $\gamma$-phosphate group via a long hydrophilic spacer, have been used extensively for identifying proteins with a nucleotide-binding site. Affinity pull-down experiments using ATP–agarose/Sepharose beads are relatively simple to set up using either cell lysates or purified protein. The KHD (kinase homology domain) of GC-C (guanylate cyclase C), also known as HSER (heat-stable enterotoxin receptor) [22], was first shown to interact directly with ATP from cell lysates using ATP–agarose beads and mutation of the conserved lysine residue in the VAIK motif was shown to abolish this interaction [23]. Similarly, the ability of the *Toxoplasma gondii* pseudokinase ROP5 to bind ATP was tested by incubation of purified recombinant ROP5 with $\gamma$-phospho-linked ATP–Sepharose and was shown to bind the resin in an Mg$^{2+}$-dependent manner, with excess ATP inhibiting this interaction [14]. More recently, the advances in proteomic technologies combined with the use of immobilized ATP–mimetics have opened new avenues in direct cellular kinase profiling. This affinity-based chemoproteomics strategy relies on an affinity matrix (best known as kinobeads) that captures nucleotide-binding proteins from cell extracts 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 MS. This method was used recently to assess directly the affinities of 200 kinases for the cellular nucleotide cofactors ATP, ADP and GTP in the presence of Mg$^{2+}$ and Mn$^{2+}$. Among these, EphB6, ILK and STRADα were reported to bind ATP in presence of Mg$^{2+}$ with affinities of 230, 34 and 30 μM respectively [24].

**Fluorescence-based thermal shift assay**

Thermal denaturation-based methods are also independent of protein function, which is especially useful for characterizing the 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 (i.e. pH or 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 and the liganded protein. This assay confers several advantages over other techniques: it requires minimal amounts of recombinant protein (2–5 μg per condition), it is rapid, it provides highly reproducible $\Delta T_m$ which has been shown to correlate well with binding strength and IC$^{50}$ values [25], and it 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 approximately 100 μM to observe a reproducible $\Delta T_m$ (above 2–3° C). Hence fluorescence-based thermal shift assays have been widely used for hit identification in kinase drug discovery programmes and we anticipate that it will be the method of choice for the search of pseudokinase inhibitors when inhibitor binding

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Table 1 | List of the techniques used to characterize pseudokinases and data obtained

For the structural data rows, PDB codes are listed.

<table>
<thead>
<tr>
<th>Pseudokinase</th>
<th>Method</th>
<th>Data</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRADα</td>
<td>Fluorescent ATP analogue</td>
<td>$K_d$ (TNP-ATP) 1.1 μM</td>
<td>[15,16]</td>
</tr>
<tr>
<td></td>
<td>Kinobeads</td>
<td>$K_d$ (ATP-Mg$^{2+}$) 30 μM</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>Structural data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASK</td>
<td>Fluorescent ATP analogue</td>
<td>$K_d$ (TNP-ATP) 0.563 mM</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Structural data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Her3/ErbB3</td>
<td>Fluorescent ATP analogue</td>
<td>$K_d$ (mant-ATP-Mg$^{2+}$) 1.1 μM</td>
<td>[2,3]</td>
</tr>
<tr>
<td></td>
<td>[γ-32P]ATP/kinase-deficient mutants</td>
<td>Catalytic activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Structural data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK2 (H2)</td>
<td>Fluorescent ATP analogue</td>
<td>$K_d$ (mant-ATP-Mg$^{2+}$) 1.1 μM</td>
<td>[4,13]</td>
</tr>
<tr>
<td></td>
<td>[γ-32P]ATP/kinase-deficient mutants</td>
<td>Catalytic activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Structural data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRAK2</td>
<td>[γ-32P]ATP (from immunoprecipitated lysate)</td>
<td>Catalytic activity</td>
<td>[26]</td>
</tr>
<tr>
<td>MviN</td>
<td>Fluorescence polarization</td>
<td>No nucleotide binding</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>Structural data</td>
<td>3UQC</td>
<td></td>
</tr>
<tr>
<td>HSER</td>
<td>Immobilized ATP</td>
<td>Binding to ATP-agarose</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>Conformation-specific antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROP5</td>
<td>Immobilized ATP</td>
<td>Binding to ATP-Sepharose</td>
<td>[14]</td>
</tr>
<tr>
<td>EphB6</td>
<td>Kinobeads</td>
<td>$K_d$ (ATP-Mg$^{2+}$) 230 μM</td>
<td>[24]</td>
</tr>
<tr>
<td>ILK</td>
<td>Kinobeads</td>
<td>$K_d$ (ATP-Mg$^{2+}$) 34 μM</td>
<td>[24]</td>
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<tr>
<td></td>
<td>Structural data</td>
<td>3KMW, 3REP</td>
<td></td>
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<td></td>
<td>ITC</td>
<td>$K_d$ (ATP) 3.6 μM</td>
<td>[8,12]</td>
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<tr>
<td></td>
<td>[γ-32P]ATP</td>
<td>No catalytic activity</td>
<td></td>
</tr>
<tr>
<td>VRK3</td>
<td>Thermal shift assay</td>
<td>No nucleotide binding</td>
<td>[18]</td>
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<td></td>
<td>Structural data</td>
<td>2JI</td>
<td></td>
</tr>
<tr>
<td>MLKL</td>
<td>Thermal shift assay</td>
<td>Nucleotide binding</td>
<td>[28]</td>
</tr>
</tbody>
</table>

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 [8,12].

**Nucleotide binding compared with 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 pseudokine 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 KHD of GC-C/HSER [23] and the pseudokinase STRADα [16].

We believe that a strong ‘hint’ as to whether a pseudokine 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 cations, this suggests there has...
been selective pressure for the protein to retain this ability and may indicate that it is active.

So far, only IRAK2 (interleukin-1 receptor-associated kinase 2) kinase activity has been shown to be crucial to its function in vivo [26]. However, studies of BubR1 [6] and ILK [8] 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 signalling. JAK2 (JH2) was shown to autophosphorylate two negative regulatory sites: Ser523 and Tyr970 [4,13]. A combination of structural, biochemical and chemical biology approaches were recently employed to dissect the function of KSR2, a pseudokinase effector in the MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase]/Raf/ERK signalling pathway [27]. That work, in which KSR2’s nucleotide-binding pocket was mutated to accommodate a ‘bumped’ ATP analogue, illustrates how emerging chemical biology approaches may provide an elegant and direct means to unequivocally attribute 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 across 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 in the present article. A subset of pseudokinases have been reported to bind ATP and exhibit a weak catalytic activity (e.g. IRAK2, JAK2, HER3/ErbB3 and 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 a true molecular switch (e.g. STRADα and HSER), which is likely to underlie their functions as signalling scaffolds. Additionally, ATP-binding studies have unveiled a class of pseudokinase domains that do not bind nucleotides (e.g. VRK3 and 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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