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Dawn of the Dead: protein pseudokinases signal new adventures in cell biology

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Abstract

Recent studies of proteins containing kinase-like domains that lack catalytic residue(s) classically required for phosphotransfer, termed pseudokinases, have uncovered important roles in cell signaling across the kingdoms of life. Additionally, mutations within pseudokinase domains are known to underlie human diseases, suggesting that these proteins may represent new and unexplored therapeutic targets. To date, few pseudokinases have been studied in intricate detail, but as described below and in this issue of BST, several new studies have provided an advanced template and an improved framework for interrogating the roles of pseudokinases in signal transduction. Here, we review landmarks in the establishment of this field of study, highlight some experimental challenges and propose a simple scheme for definition of these domains based on their primary sequences, rather than experimentally-defined nucleotide-binding or catalytic activities.

Background

Protein kinase complements, or "kinomes", are found within, and regulate cell biology across, all annotated phyla, including archaea, bacteria, plants and animals. The human kinome comprises >500 proteins that are responsible for the transient phosphorylation of approximately two-thirds of the human proteome to convey biological information and direct cellular fates, including proliferation, differentiation and death. The kinase domain represents the conserved functional unit of the kinase superfamily and contains a series of nearly invariant amino acids embedded in motifs that help position substrates and ATP prior to enzymatic transfer of the γ-phosphate to Serine, Threonine or Tyrosine acceptor amino acids. ATP binding, allosteric control and protein phosphorylation thus represent basic signalling 'currencies' within the kinome, and are absolutely fundamental for the cellular responses to complex environmental and intracellular cues.

The pioneers of kinome exploration set sail.

Several landmark papers have set the scene for our current understanding of kinomes and the more recent unveiling of "pseudokinomes", a ubiquitous and integral subclass. The sequence conservation of 11 subdomains within protein kinase sequences was first described by Hanks, Quinn and Hunter in 1988 [1], from which several invariant, catalytic residues could be experimentally linked to aspects of nucleotide binding and catalysis [2]. Subsequently, a widespread (and sometimes conflicting) literature has emerged in which specific roles have been assigned to each motif with respect to nucleotide positioning, the phosphotransfer reaction and ADP release (Table 1). Another groundbreaking study in this field was the definition of the human kinase complement in 2002 in the wake of the human genome project [3]. In this landmark paper, Manning and colleagues coined the term "kinome", and categorized proteins with kinase homology into evolutionary-related groups, families and 'sub-families', distinct from their biochemical division into Ser/Thr and Tyr kinases that had previously been the norm. Moreover, this glut of bioninformatic data also permitted a 'split' in the kinome between the conventional protein kinases and the pseudokinases, which are defined as lacking one or more of the key residues known to mediate phosphoryl transfer in active protein kinases (Table 1 and Figure 1). Bioinformatic comparisons between kinomes has taught us that, far from being degenerate kinases that have merely lost potential catalytic function, pseudokinases frequently harbour highly-conserved (or invariant) motifs in place of conventional protein kinase catalytic motifs, suggesting that these signature motifs are now essential to the biological functions of these domains. Subsequently, a 2006 review by Alessi and colleagues [4] was instrumental in defining the diverse forms and functions of pseudokinases, and highlighting their emergence as essential players in cellular signalling. The flourishing field of pseudokinase biology, which ultimately led to a recent international gathering including many kinome pioneers (Exploring kinomes: pseudokinases and beyond, Cambridge, UK, March 2013), forms the basis of the series of reviews presented herein. Each discusses recent experimental advances that can be utilized in analyses of pseudokinases, atypical kinases and protein Histidine kinases [5-22], the latter field likely to mushroom with the development of new tools to study this most experimentally challenging of amino acid modifications [23, 24]. The review articles that follow in this volume of Biochemical Society Transactions are contributions from key participants at the meeting, and contain a wealth of new information relevant to devotees of cell signaling.

To be or not to be active, that is the question.

While the catalytic activity of conventional protein kinases can usually be readily established by measuring ATP hydrolysis directly, deducing whether a pseudokinase domain possesses any residual kinase activity or is catalytically inert is far from trivial. The loss of one or more of the three crucial protein kinase catalytic motifs was predicted by Manning *et al.* [3] to obviate protein kinase activity, yet several pseudokinases have since been reported to exhibit (weak) catalytic activity *in vitro*. This remains a potential source of controversy in the field, since the possibility of contamination of recombinant protein preparations by host kinases (especially, but not limited to,

those purified from eukaryotic cells) increases the likelihood of misleading results, and the biological relevance of an artificially measured catalytic rate of turnover is neither universally accepted, nor trivial to control for experimentally. For example, kinome members are reported to exhibit a variety of ATP affinities and *in vitro* activities that range from low micromoles of ATP hydrolysed per minute per mg (e.g. PKA) to picomole-femtomole amounts per minute per mg (e.g. CASK). Whether rate of turnover is directly proportional to signaling potential in cells is difficult to ascertain unequivocally, although supporting evidence that activity is intrinsic *in vitro* and of biological relevance *in vivo* can usually be provided by mutational analysis [25]. Nonetheless, caveats exist for an intact cellular analysis, unless closely supported by additional approaches such as chemical biology, since different mutational strategies (e.g. mutation of Lys *vs*. catalytic or Mg^{2+}-binding Asp residues) can lead to differential preservation of intrinsic catalytic activity [26]. Furthermore, the 'absence' of catalytic activity in pseudokinase (or other) preparations does not constitute an unequivocal proof for an inactive state, since post-translational modifications, allosteric interactions or substrate requirements that more closely mimic a cellular environment might be required for catalysis under defined assay conditions [27]. Although not definitive, techniques that allow examination of nucleotide-binding propensity *in vitro* (as reviewed [9]), offer a useful experimental starting point to help understand the catalytic potential of any pseudokinase.

In many cases, it appears that the inability of many pseudokinases to catalyse a phosphotransfer reaction is likely to be a moot point with respect to their cellular functions. The detailed characterization of pseudokinases, including STRAD α [28, 29] and ROP5B$_I$ [30], illustrates that ATP binding likely serves an important role in governing protein conformation and that this, rather than any vestigial catalytic activity, underlies *in vivo* function. In other cases, as vividly illustrated by studies of ILK [19, 31, 32], ATP binding does not modulate protein conformation, suggesting that an ATP binding conformation, rather than ATP binding itself, is crucial to the scaffolding function of some pseudokinases. While a handful of well-characterized examples have greatly advanced our understanding of pseudokinase biology, much remains unknown, even amongst the human kinome. For example, a dozen or so human gene products that lack non-chordate homologues have received almost no scientific attention since their annotation in vertebrate genomes, and these include very interesting signaling molecules such as the cancer-associated kinases NOK/SuRTK106/STYK1 and PSKH2, SgK071, SgK495/SHIK/STK40 and SgK223/Pragmin. Notably, all of these pseudokinases are highly phosphorylated when isolated from vertebrate cells based on proteomic evidence (data collated from www.phosphosite.org in June 2013), although whether this pertains to autophosphorylation driven by intrinsic nucleotide binding or catalytic activity, or is due to exogenous phosphorylation by 'upstream' kinases, remains to be discovered. The advent of gene knockdown and knock-in technologies, coupled with genetically-tractable chemical biology approaches [5], means that we are now in a strong position to bring more up-to-date experimental approaches to the expanding pseudokinase arena. The obvious prize of potential new drug targets and disease markers is one reason for such an effort, but the scientific knowledge to be gained through an understanding of the molecular basis of pseudokinase mechanism and biology, be it catalytic, allosteric, scaffolding or a combination, is also attractive.

The semantics of pseudokinase definition

The rather weak catalytic activities reported for several pseudokinases, including HER3/ErbB3 [7, 27], JAK2 [17, 33], the Kinase Suppressor of Ras family [5, 21, 34], and kinases that employ variant sequence motifs, such as WNK1 and Haspin, can be rationalized from their respective structural characterizations [27, 35-38]. However, not all data supporting weak catalytic activities among kinase-like proteins lacking one or more key catalytic residue are as convincing. Indeed, we firmly believe that all 49 human pseudokinases (Figure 1) should remain defined as pseudokinases, whether they can or cannot bind nucleotides, or can or cannot hydrolyse the γ-phosphate under a defined *in vitro* condition. This definition is absolute rather than biased, since it relies on the

absence of catalytic residues from the primary sequence, whereas biophysical definitions, such as whether these proteins can hydrolyse (or even engage) ATP will depend on overcoming a 'Catch-22' situation, in which the appropriate experimental conditions must first be established in order to reveal an activity, yet such an activity might not exist. Not surprisingly, this has led to much mirth and debate in the literature. For example, ILK was proposed to be catalytically-active [39], even though structural, biochemical [31, 32] and mouse knock-in [19, 40] data suggest that the catalytic core of the protein is highly-degraded and that a scaffolding, rather than enzymatic, function underlies its biological function.

In addition, rightly or wrongly, the term pseudokinase is historically-associated with kinase-like proteins that lack catalytic activity. However, several domains that contain apparently conventional protein kinase catalytic residues, such as Ror1 [41, 42], RYK [7, 43] and BubR1 [44], appear to be catalytically inactive *in vitro*. Although clearly a functional, rather than bioinformatic, definition, we believe that these domains should logically be grouped with the human pseudokinases (Figure 1). Indeed, recent studies of Ror2 [7, 45] have provided a structural explanation for why (at least some) of these domains are catalytically inactive. In the Ror2 crystal structure, the adenine binding pocket is occupied by a bulky sidechain that would likely preclude ATP binding, as previously demonstrated for VRK3 [46]. These studies provide a window into the origins of pseudokinase domains and suggest that catalytic activity is perhaps only one function of a conventional protein kinase domain and, as for pseudokinase domains, conventional kinase domains are likely to have evolved additional scaffolding and allosteric regulation functions that are important to their cellular activities.

Pseudokinases as drug targets

The recent identification of catalytic activity among pseudokinase domains that are mutated in human disease, including the JAK family [16, 17], HER3/ErbB3 [7, 11, 27], and the KSR family [5, 21, 34], raise the prospect of targeting these and other pseudokinases with specific small molecules in a manner akin to the clinically successful targeting of protein kinases. Intriguingly, as discussed in reference [7], it remains unclear how important any 'weak' catalytic activity of pseudokinases is with respect to *in vivo* functions, and naturally leads to the question of whether ATP-mimetic small molecules could modulate the cellular functions of these domains by modulating conformation. Put more broadly, can pseudokinases that bind (but not hydrolyse) ATP, such as STRADα [29] or MLKL [47] be targeted in a similar fashion as catalytically active kinases, in order to select for signaling-competent or incompetent conformations? Taken a step further, might distinct small molecule interactors also modulate the scaffolding functions of non-ATP binding pseudokinases? The emergence of new techniques to enable rapid screening for small molecules that bind pseudokinases, such as thermal stability shift assays and NMR-based methologies [9, 46, 47], suggests that answers to these questions will be available in the near future. In particular, these and other chemical biology approaches [5, 34] will be especially important to the advancement our understanding of the cellular signaling functions of pseudokinases in their physiological environments.

Outlook

The first human kinome analysis produced the conclusion that 'surprisingly, nearly 10% of all kinase domains appear to lack catalytic activity. However, these domains are otherwise well conserved and are likely to maintain the typical kinase domain fold' [3]. This assertion might have lessened the perceived importance of some of these proteins as signalling molecules (and potential drug targets) originally. However, the situation has now changed for the better, and based upon the reviews presented in this edition of BST, we appear to be on the edge of a new and explosive era in the study of protein pseudokinases. We eagerly await the next decade of kinome 'exploration', and feel certain that many more pseudokinase surprises await us, as the 'dead' begin to walk into our collective signaling consciousness.

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Figure legend
Figure 1: Alignment of conventional protein kinase G-loop, VAIK, HRD and DFG motifs with their counterparts in the human pseudokinome.
Pseudokinases as defined by Manning *et al.* [3] and later extended by Scheeff *et al*. [46], but with the omission of SgK424, which possesses a highly truncated kinase-like domain. Although generally categorized as an atypical kinase rather than a pseudokinase, WNK1 is included in this table for comparison with pseudokinase sequences. Grey shaded regions indicate that no obvious candidate for a G-loop sequence could be deduced from the primary sequence. References to related articles in this issue of BST are listed to the right of the kinase or pseudokinase in question.

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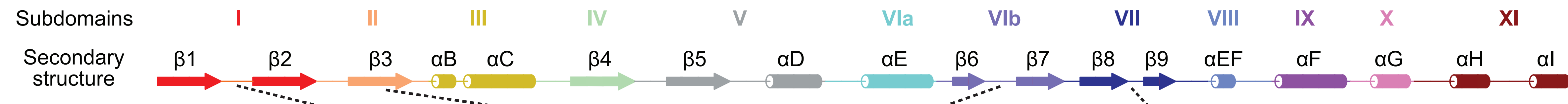
Table 1. Amino acid motifs that help to define the protein kinase superfamily

Name of motif	Biochemical Function	Sequence	Variability in kinome seen?[a]	Examples of important variants[b]	Notes
Gly-rich loop/P-loop	Flexible ATP-binding motif, usually unstructured in absence of nucleotide	GXGXXG	Yes, especially at Gly position 3	~4% of ATP-binding kinases, including WNK1-4 and STRADα	Often partially or completely degraded, compensation documented (*e.g.* HSER)
Lys-containing β3 strand	Charged Lys stabilises α and β phosphates, positioning γ phosphate for substrate transfer	X[AIL]XK	Yes, absent in ~5 % of active protein kinases	WNK1-4/PRPK active, despite lacking motif. β2 Lys compensates in WNK1	Present in most protein kinases, whether catalytically active or not. Nature of [AIL] residue occasionally important
Glu-containing □C helix	Glu ion pairs with β3 Lys to drive kinase 'active' conformation	E	Yes, absent in ~10% of protein kinases	Many lack motif, but are catalytically active	Not present in all protein kinases, dispensable for activity
Catalytic loop	Arg often conserved in kinases subject to phosphoregulation. Catalytic Asp abstracts a proton from the substrate, Asn helps position the Mg^{2+} co-factor	HRDXXXXN	Yes	HER3 lacks a "catalytic" Asp residue yet reported active. CASK lacks Asn residue but is reported to be active.	Catalytic Asp not always present. Asn not always needed to chelate metal co-factor
DFG	Asp chelates Mg^{2+} co-factor, Phe can modify the ATP binding site (DFG-in and DFG-out conformers)	DFG	Yes	CASK lacks Asp, replaced by Gly. Haspin lacks classical DFG, but also binds ATP and is active.	Due to its Mg^{2+}-independence, CASK reported to be active despite lacking the Asp in this motif.
APE	End of activation segment, Ion pairing	APE	Yes	'Ala' position widely substituted	Malleable: Pro and, especially Glu, highly conserved

[a]Most variability is seen at the 'X' positions. Underlined residues are especially important, but do not absolutely define ability to either bind nucleotide or transfer phosphate, as demonstrated by 'unusual' kinases such as WNK1 and Haspin

[b] Many examples of catalytically active kinases lacking some, or all, of these residues are now known. Hydrophobic "spine" motifs, which assemble productively in active kinases are difficult to predict from primary sequence alone, so are not considered in this analysis



						Reference
	Consensus	φ G δ G X Φ G X V	V A I K X	H R D φ K X δ N φ φ	D F G	
Bona fide protein kinases	*PKA*	49 L G T G S F G R V 57	69 Y A M K I 73	164 Y R D L K P E N L L 173	180 I Q V T D F G 186	[8]
	JAK2(JH1)	855 L G K G N F G S V 863	879 V A V K K 883	974 H R D L A T R N I L 983	990 V K I G D F G 996	[10]
	Ror1	479 L G E C A F G K I 487	503 V A I K T 507	613 H K D L A A R N I L 635	629 V K I S D L G 635	[7]
	BubR1	761 L G N E D Y C I K 769	792 T V I K V 796	880 H G D L S P R C L I 889	907 L K I V D F S 913	
	RYK	258 L Q E G T F G R I 266	283 A F V K T 287	385 H K D L A A R N C V 394	401 V K I T D N A 407	[7]
DFG motif Asp absent	*CASK*	18 I G K G P F S V V 26	38 F A V K I 42	139 H R D V K P H C V L 148	158 V K L G G F G 164	[12]
	PTK7/CCK4	802 L G K S E F G E V 810	827 V L V K S 831	928 H K D L A A R N C L 937	944 V K V S A L G 950	[7]
	SgK223/Pragmin	998 C C D S G D A I Y 1006	1019 Y A V K I 1023	1141 H R D L C L E N L L 1150	1208 L I I S N F L 1214	
	SgK269/PEAK1	1335 C C E A G D A V Y 1343	1356 Y A V K I 1360	1514 H C D L R L E N L L 1523	1551 L I V S N F S 1557	
	SgK495/STK40		63 Y Q L K I 67	195 H R D L K L G N M V 204	212 I T I T N F C 218	
	NOK/SuRTK106	120 I C S G S C G P I 128	144 V I L K A 148	249 H G D V A A R N I L 258	265 A K L C G L G 271	[7]
	RSKL1/RPS6KC1	338 K A F R V L G V I 346	361 F I L K G 365	927 C R D L N P N N I L 936	943 I Q L T Y F S 949	
	RSKL2/RPS6KL1	151 R G C R V V G V I 159	174 F V V K S 178	410 C R D L H P G N L L 419	426 I R L T Y F G 432	
	Titin	32184 L G R G E F G I V 32192	32204 Y M A K F 32208	32296 H F D I R P E N I I 32305	32314 I K I I E F G 32320	[20]
	Trb1	100 A E R E H V S R A 108	117 L R C K V 121	203 L G D L K L R K F V 212	221 L R L E S L E 227	[15]
	Trb2	70 L E G D H V F R A 78	87 L V C K V 91	173 L R D L K L R K F I 182	191 V K L E S L E 197	[15]
	Trb3	77 E E G G R A Y Q A 85	94 Y T C K V 98	180 L R D L K L C R F V 189	198 L V L E N L E 204	[15]
HRD motif Asp absent	*ANPα*	534 S G R G S N Y G S 542	564 V A V K R 568	658 H G N L K S S N C V 667	674 L K I T D Y G 680	
	ANPβ	518 S L R G S S Y G S 526	548 V A I K H 552	642 H G S L K S S N C V 651	658 L K I T D Y G 664	
	HSER/GUCY2C	495 K R R D T I Q R L 503	513 V I L K D 517	612 H G R L K S T N C V 621	628 V K I T D F G 634	
	CYGD/GUCY2D	539 I R S G P S Q H L 547	560 V W L K K 564	664 H G R L K S R N C I 673	680 L K I R D H G 686	
	CYGF/GUCY2F	538 V Q S G R S P R L 546	571 V W L K K 575	668 H G R L K S R N C V 677	684 L K V T D Y G 690	
	HER3/ErbB3	715 L G S G V F G T V 723	739 V C I K V 743	832 H R N L A A R N V L 841	848 V Q V A D F G 854	[7, 11]
	ILK	199 L N E N H S G E L 207	217 I V V K V 221	317 R H A L N S R S V M 326	335 I S M A D V K 341	[12]
	IRAK3	171 I G E G E I F E V 179	189 Y A V K L 193	291 C Q S I S S A N I L 300	307 P K L T D F A 313	
	JAK1(JH2)	589 L G R G T R T H I 597	619 V I L K V 623	712 H G N V C T K N L L 721	735 I K L S D P G 741	[16]
	JAK2(JH2)	551 L G Q G T F T K I 559	578 V L L K V 582	671 H G N V C A K N I L 680	695 I K L S D P G 701	[16, 17]
	JAK3(JH2)	527 L G H G S F T K I 535	553 V L L K V 557	645 H G N V S A R K V L 654	667 I K L S D P G 773	[16]
	TYK2(JH2)	595 L G Q G T R T N V 603	639 V L L K V 643	732 H G N V C G R N I L 741	755 I K L S D P G 761	[16]
	PSKH2	69 I G T G S F S R V 77	89 F A I K V 93	181 H R N L K P E N L L 190	200 I L I T D F G 206	
	SgK071	34 L N P G A L G V N 42	54 H V I K Q 58	155 H R N L K P S N I I 164	171 C K L Q D L S 177	
	SgK396/STK31		755 I L L K G 759	852 H G S L H Q N N V F 861	868 G I V G D F D 874	
	VACAMKL	30 I K T E E F C E I 38	50 H T C K K 54	143 H R N L K L E N L V 152	162 I V I S D F H 168	
VAIK Lys absent	*WNK1*	227 I G R G S F K T V 235	247 V A W C E 251	347 H R D L K C D N I F 356	364 V K I G D L G 370	
	KSR1	482 I G Q G R W G R V 490	499 V A I R L 503	594 H K D L K S K N V F 603	609 V V I T D F G 615	[21]
	KSR2	549 I G K G R F G Q V 557	566 V A I R L 570	661 H K D L K S K N V F 670	676 V V I T D F G 682	[5]
VAIK Lys/HRD Asp absent	*GCN2*	292 L G K L V Y N A L 300	308 V L L Y E 312	423 H K V L S A S N V L 432	439 V K I T D Y S 445	
	PXK/Slob	155 I G W R I R K K Y 163	176 L V L S W 180	286 Y G H L H A S N V M 295	301 C R L L D L E 307	
VAIK Lys/DFG Asp absent	*ULK4*	10 I G R G S K T V V 18	30 V A I L C 34	119 F C D I S P R K I L 128	135 L K F S N F C 141	
HRD Asp/DFG Asp absent	*IRAK2*	216 I S Q G T F A D V 224	234 F V F K K 238	333 H S N V K S S N V L 342	349 P K L A H P M 355	
	MLKL	209 L R E N E V S T L 217	227 V A I K V 231	329 H G K I R S S N F L 338	345 V K L A G F E 351	
	SgK307/TEX14		270 V T V K E 274	374 H R S L S S Y A V H 383	390 A R L T N L E 396	
	STRADβ	64 I G R G F D N L T 72	86 V T I K I 90	182 H R S I K A S H I L 191	197 V T L S G L S 204	
	VRK3	172 Q T R D N Q G I L 180	200 F S L K L 204	304 H G N V T A E N I F 313	322 V T L A G Y G 328	
VAIK Lys/HRD Asp/DFG Asp absent	*EphA10*	651 L G G G R F G E L 659	674 V A V H M 678	768 H R G L A A R H V L 777	784 C K I S G F G 790	[7]
	EphB6	676 I G T G S F G E V 684	699 V A I Q A 703	793 H R S L S A H S V L 802	809 C K V A R L G 815	[7]
	NRBP1	71 V N Q R N V P G I 79	94 V V W N E 98	200 H G N L T C D T I F 209	216 I K I G S V A 222	[6]
	NRBP2	44 V N Q G N M P G L 52	67 V V W N E 71	173 H G N L T S D T I F 182	189 I K I G S V W 195	
	SCYL1		43 V S I F V 47	139 H N N V C M A A V F 148	155 W K L G G L D 161	
	SCYL2	38 I A S G G N G L A 46	61 V A V F V 65	176 H G N I T P E N I I 185	192 W K I M G F D 198	
	SCYL3		37 A S V F V 41	122 H N N V C L S S V F 131	138 W K L G G M E 144	
	SgK196	87 V G E G A V K R V 95	105 V A L S Q 109	199 T R V M C D S N D L 208	216 L L T S N F S 222	
	STRADα	75 I G K G F E D L M 83	97 V T V R R 101	193 H R S V K A S H I L 202	209 V Y L S G L R 215	
	TBCK	7 A E M G A F T F F 15	31 L T P N S 35	114 H R A L S P H N I L 123	130 I K L A K F G 136	
	TRRAP		3499 Y L V M N 3503	3659 L N R L N P E M L Q 3668	3676 L N V A Y F R 3682	