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Something from Nothing: The First Phosphorylation

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Abstract
Protein and lipid kinases are known to play key roles in maintaining cellular health. Disruption in kinase signaling cascades can lead to a host of diseases and conditions, including diabetes, cancer, Alzheimer's disease, and bipolar disorder. A subset of kinases requires an activating phosphorylation within a region of their catalytic domains known as the activation loop to achieve maximum enzymatic activity. While this can be mediated via an upstream kinase, often it is the result of an autophosphorylation event, which sets up the paradoxical question of how the first kinase becomes phosphorylated. Recently, mechanisms for this first autophosphorylation have been elucidated for a handful of kinases and they broadly fall into two categories: cis-autophosphorylation, where a translational intermediate has distinct kinase activity or trans-autophosphorylation, where two kinase domains come together to facilitate phosphoryl transfer. At a time when kinase inhibition holds tremendous promise for drug therapies but high specificity is paramount, these mechanisms that underpin the very way kinases are activated may hold the key to the development of new specific inhibitors for both laboratory work focusing on discrete kinases and therapeutics for disease treatment.

Introduction

Signaling pathways involving cell growth, differentiation, proliferation, apoptosis, and metabolism are governed in large part by the reversible phosphorylation of proteins (Matthews and Gerritsen, 2010). A protein's phosphorylation state, and thus its activity, is dependent upon both the kinases and phosphatases that act upon it (Alberts, 2002). Phosphate groups are rapidly turned over allowing for quick changes between a protein's active and inactive states and such swift modifications are necessary for cells to respond efficiently to stimuli (Alberts, 2002). Mutations in kinases are known to disrupt cell signaling events and have been implicated in diabetes and cancer as well as disorders in cardiovascular health, neurodegeneration, development, and behavior (Lahiry et al., 2010), thus kinases play key roles in maintaining cellular health (Duong-Ly and Peterson, 2012; Zhang et al., 2009). Because of this, these enzymes have become important chemotherapeutic targets with notable success stories involving the tyrosine kinase inhibitors imatinib mesylate (Gleevec, Novartis), gefitinib (Iressa), and erlotinib (Tarceva), which all demonstrate antitumor activity (Arora and Scholar, 2005). Unfortunately, most inhibitors work by targeting the highly conserved ATP-binding pocket, which can result in reduced specificity and off-target effects. In addition, many kinases in the human kinome, including several that are clinically relevant, lack validated inhibitors that would be beneficial to both the medical world needing therapeutics and laboratory work seeking to understand kinase function (Anastassiadis et al., 2011; Fedorov et al., 2007). Given the high profile nature of kinases in disease, new and insightful inhibitor strategies are necessary (Duong-Ly and Peterson, 2012).

Phosphorylation is the enzyme-catalyzed transfer of the gamma phosphate from either ATP or GTP to the side chain hydroxyl group of a substrate protein's serine, threonine or tyrosine or to a lipid head group (Alberts, 2002). The kinase domain is responsible for binding nucleotide, phosphate acceptor, and the required cofactor Mg2+ as well as facilitating phosphate transfer and eventual release of products. This process is unidirectional due to the large amount of free energy that is released upon ATP/GTP hydrolysis (Alberts, 2002).

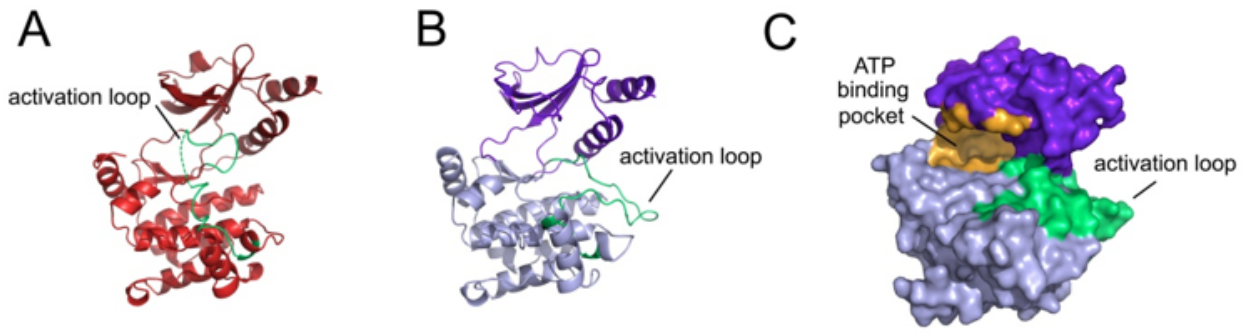

Figure 1. The structure of the ePK domain. (A) Inactive Pak1 kinase domain (PDB = 1F3M) shown in cartoon representation. The N-lobe (upper) is darker red while the C-lobe (lower) is lighter red. The activation loop is colored green, but missing amino acids due to disorder in the crystal structure. (B) Active Pak1 kinase domain (PDB = 1YHV) shown in cartoon representation. The N-lobe (upper) is dark blue while the C-lobe (lower) is lighter blue. The activation loop is colored green and in a conformation competent for catalysis. (C) The same active Pak1 kinase domain and coloring as (B) but now shown in surface representation. The conserved ATP binding pocket between the domains is colored orange.

518 protein kinases (Manning et al., 2002) and twenty lipid kinases (Fabbro et al., 2012; Heath et al., 2003) comprise the human kinome, which is 1.7% of all human genes. Of the protein kinases, 478 share the eukaryotic protein kinase (ePK) domain and are further broken down into eight groups based on sequence similarity (Duong-Ly and Peterson, 2012). While the remaining 40 are classified as atypical protein kinases (aPKs), they all have been shown to have phosphoryl-transfer activity (Duong-Ly and Peterson, 2012). The twenty lipid kinases are responsible for the phosphorylation of sphingolipids and phosphoinositides found within cellular membranes (Duong-Ly and Peterson, 2012).

The ePK domain is comprised of 250 amino acids that form the N terminal and C terminal lobes (N-lobe and C-lobe respectively), which are connected by a hinge region (Alberts, 2002; Duong-Ly and Peterson, 2012). The N-lobe is smaller and has a mixture of alpha helices, including the notable C helix, and several beta strands while the larger C-lobe is composed primarily of alpha helices (Duong-Ly and Peterson, 2012) (Figures 1A and B). The highly conserved ATP binding pocket sits in the cleft between the lobes while the C lobe is primarily responsible for interacting with the substrate (Figure 1C). All protein kinases have a strictly conserved aspartate residue that is critical for catalysis (D389 in Pak1, (Johnson et al., 1996)). Kinases that have an asparagine residue (R) immediately preceding the catalytic aspartate (D) are common amongst most serine/threonine kinases and all tyrosine kinases and are known as RD kinases (Johnson et al., 1996).

Because correct spatial and temporal control of phosphorylation is necessary, kinases have adopted several different regulatory mechanisms to control their catalytic activity. Some kinases need to complex with other subunits that are under strict transcriptional control before reaching full catalytic activity. This type of regulation is most common among cyclin-dependent kinases (CDKs), which form heterodimeric complexes with cyclin proteins, whose concentrations change during the cell cycle (Johnson et al., 1996; Lodish et al., 2004). Requiring the binding of secondary messengers such as cyclic AMP (cAMP), diacylglycerol, or Ca2+/calmodulin to cAMP-dependent kinase, protein kinase C or calmodulin-dependent kinase, respectively, is another type of regulation (Johnson et al., 1996). Other kinases, such as Pak1, require correct localization via multivalent basic motifs before becoming active, but others will use pleckstrin homology (PH) domains or SH3 domains (Johnson

et al., 1996; Strochlic et al., 2010). The final and best understood mechanism for activation is through the phosphorylation of the kinase itself. It became apparent through research in the late 20th century that phosphorylation of key residues within an area alternatively called the activation segment, the activation loop or the T-loop were essential to obtain full catalytic activity for a particular subset of kinases (Johnson et al., 1996; Nolen et al., 2004). In fact, Johnson et al. declared that "…control by phosphorylation in the activation segment is a property of most, but not all, protein kinases." (Johnson et al., 1996).

In the early 1980s, autophosphorylation of Y416 in pp60v-src was observed and it was then demonstrated that this phosphorylation event was significant for controlling the activity of the cellular Src kinase (Hunter, 1987). Currently, a substantial number of kinases, particularly RD kinases, are known to require a phosphorylation within the activation loop to achieve full activity (Oliver et al., 2007). A comparison of catalytic domains from several different kinases in the active state shows that their conformations are highly conserved. When phosphorylated, the activation loop, defined as the approximately thirty amino acids that span between the conserved DFG (aspartate-phenylalanine-glycine) and APE (alanine-proline-glutamine) motifs, pulls away from the active site and is held in place by an extensive charge network (Lei et al., 2005; Lowe et al., 19997; Zhou et al., 2004). The strictly conserved aspartate residue (D389 in Pak1), which is part of the DFG motif, coordinates with an Mg2+ ion to position the phosphate groups of ATP for phosphoryl transfer (Duong-Ly and Peterson, 2012). Additionally, the DFG group is typically found pointing towards the ATP binding site in active kinase states; this conformation is called DFG-in. In contrast to their active states, inactive kinase conformations vary widely. For example, the activation loop of Pak1 is disordered in the crystal structure indicating its lack of fixed position (Figure 1A). Pak1 is an autoinhibited kinase due to its N-terminal regulatory domain, which occupies the active site and prohibits substrate binding (Lei et al., 2000). In most inactive kinase states, the DFG motif's aspartate residue is pointing away from the ATP binding site and referred to as DFG-out.

The necessity of activation loop phosphorylation for catalytic activity indeed depicts a perplexing paradox. If phosphorylation of this loop is absolutely required for catalysis, how then does the first phosphorylation occur? One simple explanation is the requirement of another kinase to treat an inactive kinase as a substrate. For some mitogen activated protein kinase (MAPK) family members, this is exactly true. ERK1 (extracellular signal related kinase) and ERK2 are substrates of MEK1 (MAPK/ERK kinase) and MEK2, which are themselves substrates of Raf (Brott et al., 1993; Crews et al., 1992; Crews and Erikson, 1992; Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992).

However, many kinases are known to phosphorylate their own activation loops and beg the question of how the kinases resolve this contradiction. Accumulating structural and biochemical data for various kinases reveal novel molecular mechanisms for how this first phosphorylation can occur: post-translational transient folding intermediates can lead to *cis*-autophosphorylation while dimeric or multi-protein complexes allow for *trans*-autophosphorylation. These revelations are changing the way we look and think about kinase activity. First, a kinase-substrate relationship involves more distal areas from the enzyme's active site residues and the substrate's few amino acids held in the binding pocket. Second, and perhaps more exciting, the fact that these autophosphorylation events underpin the kinases' very ability to mediate their own activation in addition the activation of their substrates and that this autophosphorylation event can occur through several different mechanisms, a new avenue is being paved that could lead to more kinase-specific inhibitors.

Cis-autophosphorylation

The serine-threonine kinase glycogen synthase kinase 3 (GSK3) plays a role in cell fate, cell division, apoptosis and gluconeogenesis (Cohen and Frame, 2001; Frame and Cohen, 2001; Jope

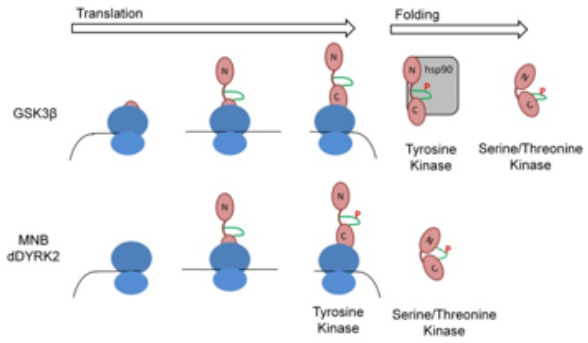

Figure 2. The known *cis*-autophosphorylation mechanisms. The mechanisms for GSK3β (upper) and MNB/dDYRK2 (lower) autophosphorylation are shown. Ribosomes are depicted as blue circles with mRNA as a black line feeding through the ribosomes. The progression of translation is shown as the ribosome is translating the mRNA and partially folded kinase domain is emerging from the top. Kinase lobes are labeled and activation loops are in green. The points in translation or folding that represent tyrosine kinase activity and serine/threonine kinase activity are labeled.

and Johnson, 2004; Kim and Kimmel, 2000) and is a clinically relevant target in diabetes, Alzhiemers, and bipolar disorder (Cohen and Goedert, 2004; Martinez et al., 2002; Meijer et al., 2004) with pharmaceutical companies striving to develop inhibitors (Cohen and Frame, 2001; Martinez et al., 2002; Meijer et al., 2004). In mammals, two separate genes encode GSK3, termed α and β, and both are positively regulated by tyrosine phosphorylation in their highly conserved activation loops (Cohen et al., 1978; Hughes et al., 1993; Wang et al., 1994; Woodgett, 1991). In GSK3β, this residue is the invariant tyrosine at position 216 (Lochhead et al., 2006). Mutation of Y216 to phenylalanine, serine or threonine results in almost no GSK3β enzymatic activity (Lochhead et al., 2006) and mounting evidence implies that GSK3β intramolecularly autophosphorylates its own activation loop (Cole et al., 2004; Lochhead et al., 2006).

The mechanism for this intramolecular or *cis*-autophosphorylation event was described in 2006 by Lochhead et al. (Figure 2). Following translation of GSK3β at the ribosome, heat shock protein 90 (Hsp90), a chaperone protein involved in late folding events (Young et al., 2004), binds to the newly translated GSK3β polypeptide chain to form a chaperone-dependent transitional intermediate that is competent for tyrosine autophosphorylation (Lochhead et al., 2006). Mutating Y216 to a serine resulted in an unphosphorylated, inactive kinase, indicating that this transitional intermediate, unlike the mature kinase, is not competent for serine/threonine phosphorylation (Lochhead et al., 2006). Thus, GSK3β has two distinct kinases activities that are separated by maturity of the polypeptide.

The dual specificity tyrosine-phosphorylation regulated protein kinases (DYRKs) have also been shown to *cis*-autophosphorylate their activation loops in a very similar manner. These evolutionarily conserved serine/threonine kinases are members of the CMGC kinase family (Bahler and Nurse, 2001; Becker and Joost, 1999; Himpel et al., 2000; Kentrup et al., 1996; Lochhead et al., 2003; Miyata and Nishida, 1999). Two subclasses of DYRKs exist, known as Class 1 and Class 2 (Aranda et al., 2011; Lochhead et al., 2005). The *Drosophila* kinase minibrain (MNB), so named because knockout flies have impaired post-embryonic neurogenesis that results in both their brain hemispheres as well as their optic lobes being reduced in size (Tejedor et al., 1995), is a class 1 DYRK. Its human orthologue, DYRK1A, maps to a critical region in Down Syndrome and is found overexpressed in patients bearing this chromosomal condition (Guimera et al., 1999; Lochhead et al., 2005).

A critical tyrosine residue within the activation loop of all DYRKs is autophosphorylated (Bahler and Nurse, 2001; Himpel et al., 2000; Kentrup et al., 1996; Lochhead et al., 2006; Lochhead et al., 2003) but the mechanism, believed to be the same for both class 1 and class 2 DYRKs, remained unknown until 2005 (Bahler and Nurse, 2001; Himpel et al., 2000; Kentrup et al., 1996; Lochhead et al., 2003). Similar to the mechanism described for GSK3β, both Class 2 dDYRK2 (*Drosophila* DYRK2) and MNB autophosphorylate their

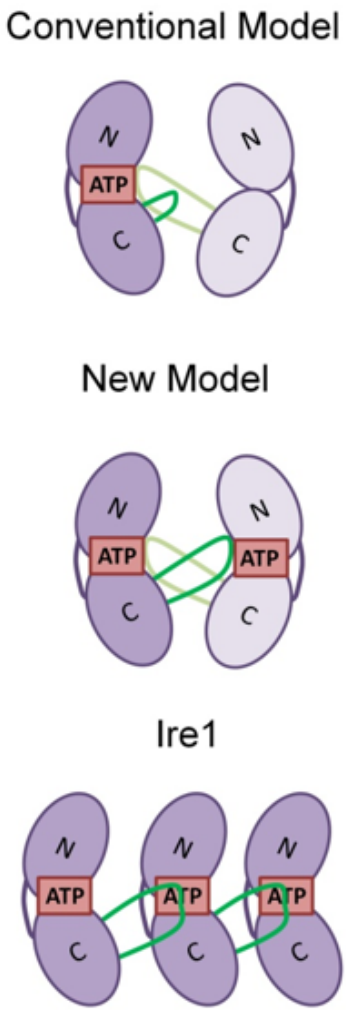

Figure 3. The known *trans*-autophosphorylation mechanisms. Representative diagrams for the conformations of activation loops are shown for each trans-autophosphorylation model. Kinase domains are shown in shades of purple with N- and C-lobes labeled. Activation loops are in shades of green. Domains bearing ATP are labeled. The conventional model (upper) shows the left dimeric subunit acting as an enzyme while the right subunit is acting as a substrate with its activation loop projecting into the enzymatic subunit's active site. The new model (middle) has both subunits bearing ATP and symmetrically exchanged activation loops. Ire1 (bottom) is different than either model. Kinase domains line up and each behaves as both substrate and enzyme. To highlight the activation loop conformations and for clarity, N- and C-lobes are not shown interacting in these depictions, however they do extensively interact in the crystal structures.

activation loops before folding into their mature states (Figure 2). However, these two proteins do not require a chaperone. Instead, the protein's N terminus folds into a translation intermediate competent for tyrosine phosphorylation. For Class 2 DYRKs, a conserved domain termed the N-terminal autophosphorylation accessory (NAPA) region, is thought to be responsible for the transitory tyrosine phosphorylation capability (Kinstrie et al., 2010). Following release from the ribosome both dDYRK2 and MNB lose their tyrosine-kinase activity and only function as serine/threonine kinases (Lochhead et al., 2005).

Using a similar mechanism, the homeodomain-interacting protein kinase 2 (HIPK2), another member of the CMGC family and a close relation to DYRKs, was also found to *cis*-autophosphorylate its activation loop residues Y354 and S357 to achieve kinase activity (Saul et al., 2012). Of the four HIPK family members, HIPK2 is the most studied and is known to regulate cell proliferation, play a functional role in cancer, as well as an extremely large number of signaling pathways including DNA damage response, p53 activation and Hedgehog signaling (Saul et al., 2012).

Other kinases are speculated to undergo *cis*-authophosphorylation. ERK7 (extracellular signal regulated kinase 7) is a member of the MAPK family, which require phosphorlyation of either tyrosine or threonine in their activation loops for activity (Abe et al., 2001). Unlike other ERKs, ERK7 is constitutively active and its catalytic activity fails to further increase when other MAPKs are activated (Abe et al., 2001). Competition experiments with the catalytically dead K43R ERK7 indicates that autophosphorylation is an intramolecular event and an ERK7 C terminal deletion mutant fails to phosphorylate its activation loop or become catalytically active (Abe et al., 1999). Together, these results suggest that ERK7 may activate itself in a mechanism similar to those described for GSK3β, dDYRK2 and MNB, thus introducing a new and novel class of MAPK family members (Abe et al., 2001). cAMP-activated protein kinase (PKA) is another candidate. The kinase is phosphorylated at T197 within its activation loop and a T197A mutant has virtually no activity in cells (Huggenvik

et al., 1991; Steinberg et al., 1993). Unfortunately, several biochemical studies suggest that T197 phosphorylation is an authophosphorylation event (Cheng et al., 1998; Steinberg et al., 1993; Williams et al., 2000) but others claim it is phosphorylated by separate kinase (Cauthron et al., 1998; Moore et al., 2002). Interestingly, it was shown that phosphorylation of S338, which is not itself necessary for catalytic activity but a required prerequisite for T197 phosphorylation, does proceed through a *cis*-autophosphorylation mechanism (Keshwani et al., 2012).

The mechanism of *cis*-autophosphorylation provides a necessary and key event to yield an active kinase, but has both positive and negative consequences. The kinase is able to form a translational intermediate capable of phosphorylating itself, thus describing an independent event that does not rely on any other kinase. However, phosphatase treatment of these kinases shows that these *cis*-autophosphorylations are difficult to remove indicating that they most likely become buried in the final protein conformation. When quick responses to stimuli are needed, these kinases cannot be turned off by merely removing the phosphate groups that activated them and instead must rely on additional mechanisms to regulate their activity, such as inhibitory phosphorylations, binding of other regulatory proteins or rapid protein turnover (Lochhead et al., 2005).

***Trans*-autophosphorylation**

The conventional model of *trans*-autophosphorylation is that activation loop phosphorylation is mediated by one kinase transiently adopting an active conformation, thereby phosphorylating its neighbor (Figure 3). The activated neighbor then becomes locked in the more permanent active conformation and reciprocally phosphorylates the first (Bae and Schlessinger, 2010; Oliver et al., 2007). However, this mechanism has inherent inefficiency due to the conformational instability of an unphosphorylated activation loop (Oliver et al., 2007). It also assumes that the activation loop amino acids surrounding the important residue for phosphorylation match the kinase's consensus sequence for substrate phosphorylation, which is not always the case (Oliver et al., 2007; Oliver et al., 2006; Pike et al., 2008). In light of this, a new model of *trans*-autophosphorlyation was crafted following the publication of several kinase dimeric structures depicting catalytic domains with swapped activation loops (Oliver et al., 2007; Oliver et al., 2006; Pike et al., 2008; Wu et al., 1008).

Chk2 is a serine/threonine kinase that responds to serious DNA damage caused by ionizing radiation, genotoxic chemicals or collapsed replication forks (Oliver et al., 2006). Its function is mediated by ataxia telangictaria mutated (ATM) kinase, which phosphorylates Chk2 at T168 and causes the formation of a symmetrical, face to face dimer via the FHA domain (Ahn et al., 2000; Oliver et al., 2006). Following subsequent autophosphorylation at T383 and T387 within both subunits' activation loops, Chk2 becomes active and phosphorylates many downstream targets such as cdc25A, cdc25C, BRCA1 and p53, which will lead to cell cycle arrest or apoptosis (Bartek and Lukas, 2003).

The exact mechanism leading to autophosphorylation was unknown until 2006 when Oliver et al. published the X-ray crystal structure of a dimerized Chk2 catalytic domain where each subunit was bound to Mg-ADP and the ATP-competitive inhibitor debromohymenialdisine (DBQ). Interestingly, the domains have exchanged T loops (Oliver et al., 2006). The activation loop makes many hydrophobic contacts to residues of the opposite catalytic domain and buries a total of 2688 Å2 of surface area, but the activation loop must rearrange slightly to place T383 in proper orientation for phosphoryl transfer (Oliver et al., 2006; Wu et al., 1008). The structure of each subunit is considered active because all the necessary residues are spatially arranged for catalysis, the nucleotide binding site is accessible, the relative orientation of the N- and C-lobes are consistent with previous active structures and the activation loop is fully ordered (Oliver et al., 2006). Whether Chk2 remains with swapped T loops after autophosphorylation or reverts back to activation

Kinase Name	Kinase Family	Autophosphorylation Mechanism	Species Studied	Key Activation Loop Residue(s)
GSK3β	CMGC*	cis	Rat	Y216
MNB	CMGC*	cis	Drosophila	Y326
dDYRK	CMGC*	cis	Drosophila	Y358
Chk2	CAMK	trans, new	Human	T383, T387
SLK	STE	trans, new	Human	T183, S189
LOK	STE	trans, new	Human	T185
DAPK3	CAMK	trans, new	Human	T180
1GF1RK	TK	trans, new	Human	Y1135, Y1131, Y1136
Pak1	STE	trans, conventional	Human	T423
Ire1	OTHER*	trans, other	Yeast	S840, S841, T844

Table 1. Key information for reviewed kinases. Each kinase discussed in this review is listed with the species studied and autophosphorylation mechanism listed. The necessary residues in the activation loop that need to be phosphorylated for kinase activation are listed. For those with more than one, the residues are listed in their sequential order. The human kinase group each studied kinase represents is also listed. For those studied in other organisms, the group corresponding to the human homologue is listed. For Ire1, yeast refers to Saccharomyces cerevisiae.

loops folded upon their own domains as seen in other active kinase structures remains unclear, however the authors note that phosphorylation of T168 that drives the dimerization places the catalytic domains in close contact with each other and allows for activation loops that do not match substrate consensus sequences to arrive in the active site, become autophosphorylated in *trans*, and activate the kinase (Oliver et al., 2006). This is a process that is distinct from the method of true substrate phosphorylation (Oliver et al., 2006).

Several dimeric kinase structures representing *trans*-autophosphorylation followed Chk2 in the next two years. Pike et al. reported on the structures of Ste20-like kinase (SLK), lymphocyte-originated kinase (LOK, also known as serine/threonine kinase 10 or STK10), and death associated protein kinase 3 (DAPK3, also known as ZIPK) (Oliver et al., 2007; Pike et al., 2008). All of these kinases are known to have crucial roles in cellular health with LOK implicated in testicular germ cell tumors and DAPK3 linked to misregulation of oncogenes (Gozuacik and Kimchi, 2006; Pike et al., 2008). Four similar structures were described for SLK while one each was solved for LOK and DAPK3. Symmetric dimerization of catalytic domains, each burying 1730 to 2030 Å2 of surface area, with swapped activation loops and approximate orientations for phosphoryl transfer was observed for each kinase (Pike et al., 2008). In 2008, Wu et al. claimed the first reported structure capturing a true *trans*-autophosphorylation moment with their dimeric structure of unphosphorylated IGF1R (insulin-like growth factor-1 receptor) kinase domain. Activation loops are swapped between subunits and the first of three activation loop tyrosines to be phosphorylated (Y1135) is correctly oriented in the active site (Wu et al., 1008). IGF1R is a transmembrane receptor tyrosine kinase that binds to IGF1 (insulin-like growth factor 1) or IGF2 and regulates growth, cell survival and differentiation (Adams et al., 2000; Baserga et al., 1997; Butler et al., 1998; Wu et al., 1008). This kinase is important in prenatal development and is found overexpressed in human lung cancer, colon carcinoma, and central nervous system tumors (Baserga et al., 1997; LeRoith and Roberts, 2003; Valentinis and Baserga, 2001; Wu et al., 1008).

These dimer structures suggest a mechanism of symmetrically swapped activation loops as a new model for *trans*-autophosphorylation (Figure 3). Oliver speculates that it might be a common mechanism among kinases that rely on autophosphorylation, that dimerize either transiently or constitutively, and have amino acid sequences surrounding the crucial activation loop residue requiring phosphorylation that do not match the kinase's consensus sequence (Oliver et al., 2007)

However, in 2011, a structure published by the laboratory of Zhi-Xin Wang suggested that Pak1's catalytic-activating autophosphorylation at T423 in its activation loop occurs via the conventional model (Wang et al., 2011). Pak1 is a serine/threonine kinase that exists as an inactive homodimer whose steric inhibition is released upon binding of the activated GTPases Rac1 or cdc42 and the phospholipid phosphatidylinositol 4,5-bisphosphate to the N terminal GTPase binding region and a basic motif, respectively. This kinase is known to play key roles in cytoskeletal dynamics (Arias-Romero and Chernoff, 2008;

Bokoch, 2003; Strochlic et al., 2010) and is overexpressed in several cancers, including breast cancer (Arias-Romero and Chernoff, 2010; Arias-Romero et al., 2010) and melanoma (Jung et al., 2004; Pavey et al., 2006). The authors report that the truly catalytically dead Pak1 (K299R/D389N) crystallized in the presence of the nonhydrolyzable ATP-analog AMP-PNP in a dimeric arrangement where one subunit adopts the well-established "active" conformation (Wang et al., 2011) while the other resembles an autoinhibited Pak1 kinase domain (Lei et al., 2000) with an extended, somewhat disordered activation loop in the "active" subunit's active site. This structure represents a genuine enzyme-substrate complex and fits more nicely with the conventional model of *trans*-autophosphorlyation.

One other structure of *trans*-autophosphorylation was published in 2009 and, while not fitting either the conventional or new model, it does offer a sense of how variable the mechanisms can become and why two models may not fully explain all possibilities (Figure 3) (Korennykh et al., 2009). The endoplasmic reticulum (ER) resident kinase/RNase Ire1 (inositol-requiring enzyme 1) is activated by misfolded proteins whereby it initiates a comprehensive transcriptional program to change the protein folding capacity of the ER (Korennykh et al., 2009). This entire process is upregulated in several diseases, including various forms of cancer (Koong et al., 2006; Ma and Hendershot, 2004) and protein-folding diseases (Bartoszewski et al., 2008; Kudo et al., 2002). To activate its kinase activity, Ire1 autophosphorylates residues S840, S841 and T844 within its activation loop. This protein is also known to oligomerize. The reported crystal structure shows each subunit in the oligomer offering its activation loop to the preceding subunit's active site. Fascinatingly, each subunit acts both as enzyme and substrate.

As more kinase structural information becomes available, further unpredicted methods for enabling kinase autophosphorylation may be identified. Interestingly, even within those kinases that symmetrically exchange activation loops, their subunit interactions vary considerably. For example, the DAPK3 dimerization subunits are less rotated with respect to each other than the subunits reported in IGF1R (Malecka and Peterson, 2011). Kinases from several different branches of the human kinome undergo some form of activation loop exchange (either symmetric or asymmetric), which suggests that these broad models of *trans*-autophosphorylation may be common activation mechanisms (Table 1) (Oliver et al., 2007). Important questions remain about this type of autophosphorylation, however, including how the intermediates are resolved after phosphoryl transfer and what are the distinct functional roles of symmetric versus asymmetric *trans*-autophosphorylation (Malecka and Peterson, 2011).

Inhibition of Kinase Activation

The importance of kinase inhibitors as therapeutic tools has long been established (Duong-Ly and Peterson, 2012; Fedorov et al., 2007; Lahiry et al., 2010; Zhang et al., 2010). It is believed that close to one third of all pharmaceutical drug discovery projects are focused on kinase inhibitors, but still only a small number remain approved by the US Food and Drug Administration (Duong-Ly and Peterson, 2012; Matthews and Gerritsen, 2010). Beyond therapeutics, kinase inhibitors as research tools would be invaluable to scientists striving to understand the function particular kinases play in the cell (Duong-Ly and Peterson, 2012).

Traditionally, inhibitors have been identified through the use of high throughput assays where small molecules are incubated with purified kinases (Anastassiadis et al., 2011). The vast majority of inhibitors exploit the highly conserved ATP binding region (Figure 1C), but this leads to variable selectivity among all the cellular kinases (Anastassiadis et al., 2011; Davis et al., 2011). These types of assays also often overlook the importance of mono or diphosphorylated domains in inhibitor sensitivity differences (Table 1) (Anastassiadis et al., 2011; Davis et al., 2011; Wu et al., 1008). To counteract these problems, researchers have strived to take advantage of the less conserved pockets adjacent to the ATP binding pocket as a way to boost selectivity (Duong-Ly and Peterson, 2012; Maksimoska et al., 2008). Also,

some non-ATP competitive inhibitors, which are often more selective, have been identified, such as the Pak1 covalent inhibitor IPA-3 (Viaud and Peterson, 2009), rapamycin (Geda et al., 2008) and GNF-2 (Adrian et al., 2006). Nonetheless, achieving highly selective inhibition of kinases remains a difficult task and innovative ideas are needed (Anastassiadis et al., 2011). However, these self-activation mechanisms offer a new way to consider kinase inhibitor development.

Purvanol A and TBB are known to inhibit DYRK1a and dDYRK2 serine/threonine kinase activity (Bain et al., 2003; Lochhead et al., 2005; Sarno et al., 2003), but only purvanol A is able to block dDYRK2 tyrosine autophosphorlyation following expression in rabbit reticulocyte lysate (Lochhead et al., 2005). Similarly, a panel of known inhibitors of GSK3β's serine/threonine activity was screened and only a handful are found to block the intermediate, activating autophosphorylation of Y216 (Lochhead et al., 2006). Thus, these kinases have two distinct catalytic activities that are separated not only by time but also by drug sensitivities (Lochhead et al., 2006). Interestingly, after maturation of dDYRK2 and GSK3β in the presence of the translational intermediate inhibitors, the crucial tyrosine in the activation loop could not be phosphorylated, indicating that these inhibitors act irreversibly. Part of the reason that PKA was identified as a possible *cis*-autophosphorylating enzyme was because when the protein is expressed in the presence of its inhibitor H-89, catalytic domains are not phosphorylated at T197 and are unable to be phosphorylated later (Steinberg et al., 1993). Not only does this work show that inhibition of the catalytic intermediates are possible and can be further studied, but also suggests that these kinases have inhibitors that are being missed by mining small molecule libraries with traditional highthroughput screening methods (Lochhead et al., 2006).

Just as *cis*-autophosphorylation offers inventive inhibitor development ideas, so does *trans*-autophosphorylation. The dimeric and often face-to-face structures bring the ATP binding pockets in close proximity (Oliver et al., 2007; Oliver et al., 2006). Double-headed, ATP-competitive inhibitors could be developed such that a linker would allow for both ATP binding pockets to be occupied simultaneously, thus increasing specificity of inhibitors. Such molecules have not been explored yet, but the theory has worked for ansamycin, which is a double-headed antiobiotic targeting two close ATP-binding domains in Hsp90 (Ali et al., 2006; Pearl and Prodromou, 2006), and coumermycin A, a natural ATP-competitive antibiotic that binds and inactivates DNA gyrase B (Ali et al., 1993).

Conclusion

The emerging mechanisms of *cis*- and *trans*-autophosphorylation begin answering the perplexing question of how the catalytically activating first phosphorylation can occur. Described here are two general methods for initial phosphorylating events among kinases that require autophosphorylated residues in their activation loops for catalytic activity. Interestingly, those kinases that subscribe to *cis*-autophosphorylation carry out their mechanism in distinctly different ways: GSK3β requires Hsp90 while dDYRK2 and MNB do not (Figure 2). The same variability is also noted among the *trans*-autophosphorylation kinases where Pak1 follows the conventional model while Chk2 conforms to the newer model. Even Chk2, SLK, LOK, DAPK3 and IGF1R, which all carry out the new model for *trans*-autophosphorylation, involve different interacting regions between the dimerized subunits (Figure 3) (Malecka and Peterson, 2011). Clearly no one-size-fits-all mechanism works for all kinases and it is doubtless that as more mechanisms are understood so will they uniquely fit into either of these two categories.

Few examples exist of crystallized protein kinase/substrate complexes where substrates are longer than a few amino acids (Malecka and Peterson, 2011). Here, the *trans*-autophosphorylated kinases allow us to see that a variety of regions, including those distant from the active site, are involved in creating a stable complex for phosphorylation. For example, the Pak1 *trans*-autophosphorylation dimer structure that most resembles a traditional kinase/substrate

relationship buries a total of 1600 Å2 with half of the interacting regions outside the enzyme's active site (Wang et al., 2011). Even more interesting are the speculations to be made about how GSK3β, dDYRK2 and MNB fold up to have distinct phosphorylation activity separate from the final mature enzyme. The catalytic aspartate is known to be important to these three kinases (Lochhead et al., 2006; Lochhead et al., 2005), but what other regions in the N and C terminal lobes, aside from the identified NAPA domain, are also necessary are still unknown as is what the actual intermediately folded kinase structurally looks like and how it differs from the properly folded ePK domain. Together, these mechanisms force us to step back from the kinase active site and consider the entire domain's involvement in initial activation as well as substrate phosphorylation.

All serine/threonine kinases and many tyrosine kinases fall within the RD kinase category, whose members often require the paradoxical activating phosphorylations within their activation loops for full catalytic activity. So far, only a handful protein kinases have their autophosphorylation mechanisms understood, but more will undoubtedly be studied and added to this growing field of kinase study.

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