The PIK-related kinases intercept conventional signaling pathways

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Early efforts to place the first cloned mammalian PIK-related kinase, FRAP, into a conventional membrane to nuclear pathway met with little success. More recent data suggest that members of the family of PIK-related kinases act as intracellular sensors that govern radial and horizontal pathways. These pathways can impinge upon classical membrane to nuclear pathways, as well as components of the cell-cycle machinery.

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The fascinating story behind the discovery of the phosphatidylinositol kinase (PIK)-related kinases sheds much light on the function of these enzymes. At an early stage, it became clear that loss of one of these kinases would have many biological or medical effects that sometimes appeared to be unrelated. Because of their apparent involvement in many different processes, these kinases were not easily linked together.

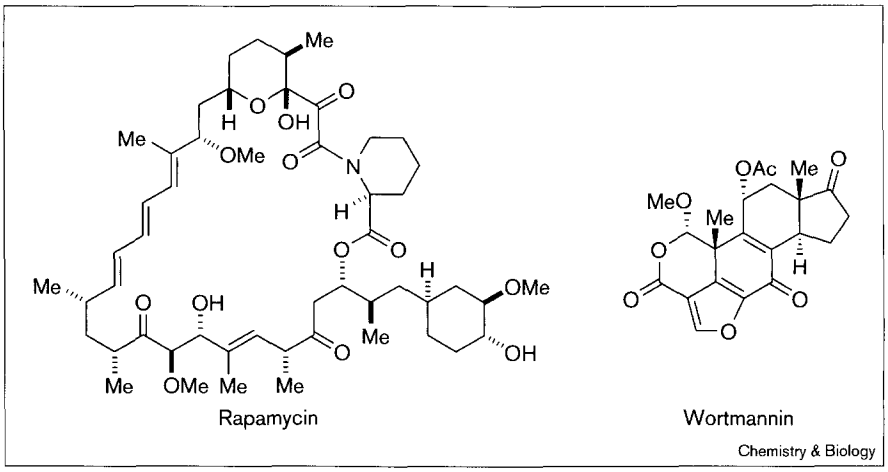
In 1926 a report came from Prague of three ill adolescent siblings who displayed a peculiar involuntary writhing movement [1]. Their movements were so striking that serial photographs were included to try to capture a sense of their abnormal motion. Another report followed in 1941, but it was not until the late 1950s that Boder and Sedgwick [2] codified this report with their own findings and described what they called ataxia-telangiectasia (AT) for two other striking defects that these patients bore: the inability to walk (ataxia), and dilated blood vessels (telangiectasia) often found on the eyes or extremities. They noted highly pleiotropic and multisystem defects in those afflicted with the disease. Sinopulmonary infections were the most common cause of death, and lymphoreticular malignancies followed second. The challenge from the beginning for those studying AT was how to link such pleiotropic defects into a unifying yet reasonable mechanistic proposition.

In a seemingly unrelated field, a team of scientists published a report in 1975 of a novel natural product derived from the microorganism *Streptomyces hygroscopicus* that they isolated on Easter Island in the South Pacific [3,4]. The compound potently inhibited the growth of the fungus *Candida albicans*, faring comparably to other antifungal agents such as amphotericin or nystatin, but it had no effects on bacteria. Because the natives referred to their island as Rapa Nui, the natural product was named rapamycin. Two years later rapamycin was found to have extremely potent immunosuppressive activity, which sparked considerable medical research. Because rapamycin was also found to be a stereochemically complex macrolide and polyketide (Figure 1), it attracted the attention of synthetic organic chemists, as did other immunosuppressive agents such as FK506 [5]. Why a molecule like rapamycin would have antifungal as well as immunosuppressive properties remained a mystery.

In the mid 1990s, a gene that when mutated led to rapamycin resistance in *Saccharomyces cerevisiae* was cloned and termed *TOR2/DRR2* (target of rapamycin/dominant rapamycin-resistant) [6,7]. The sequence was found to code for a very large protein (greater than 250 kDa) that bore strong homology to the lipid kinase phosphatidylinositol-3-kinase (PI3K) in the region of its carboxyl terminus. In mammalian cells, the functionally relevant protein that binds the complex of rapamycin and its immunophilin receptor FKBP12 was discovered and its cDNA was cloned. This protein was named FRAP/RAFT (FKBP–rapamycin associated protein/rapamycin and FKBP target) [8,9]. It was found to be very similar to the protein inferred by the *TOR2* gene, including the region of homology to PI3K. The next year, through a large positional cloning effort, the human gene defective in AT was cloned and termed ATM (ataxia-telangiectasia mutated) [10]. It astonishingly also coded for a very large protein with homology to PI3K at its carboxyl terminus. ATM, FRAP and Tor2p all share an extreme carboxy-terminal tail that is also highly conserved. In yeast, the cDNA of another target of rapamycin, Tor1p, was cloned and found to have the same sequence similarities [11]. These four genes were therefore dubbed members of the PIK-related kinase family [12].

Studies of mammalian, yeast and fly systems revealed six other PIK-related kinases. All possessed the characteristic PI3K-homologous kinase domain as well as the highly conserved carboxy-terminal tail, although none has yet been demonstrated to have lipid kinase activity. A high-molecular-weight kinase present in cell extracts found to be stimulated *in vitro* by double-stranded DNA was discovered and termed DNA-PK (DNA-dependent protein kinase) [13]. Studies in *Drosophila melanogaster* uncovered an ATM homolog named MEI-41 [14]. In *S. pombe*, loss of the PIK-related kinase RAD3 was discovered to cause severe radiosensitivity [15]. Similarly in *S. cerevisiae*, two groups independently identified a PIK-related kinase

Figure 1



The chemical structures of the natural products rapamycin and wortmannin. Rapamycin binds to FKBP12 and the resulting complex inhibits the protein kinase FRAP. Wortmannin inhibits the lipid kinase PI3K.

called Mec1p (mitosis entry checkpoint) that controls a checkpoint into mitosis, as well as sensitivity to DNA-damaging agents [16,17]. Also in *S. cerevisiae*, a PIK-related kinase named Tel1p was discovered and shown to be important for maintaining telomere length [18,19]. By homology to other PIK-related kinases, a human cDNA was cloned that was first named FRP (FRAP-related protein) but, following functional analysis, later termed ATR (ATM- and RAD3-related) [15,20].

The final and most perplexing addition to the family is the protein TRRAP (transformation/transcription domain associated protein), which was discovered by its ability to bind to the transcription factors c-myc and E2F [21]. The most surprising feature of TRRAP is that, although its sequence clearly resembles that of a PIK-related kinase, it is missing critical residues in the kinase domain, almost certainly rendering the protein devoid of kinase activity. The same protein was found to be present in histone acetyltransferase/transcription regulation complexes in mammalian cells [22] and also in yeast, in which the homologous protein is called Tra1p [23,24].

Altogether 12 proteins from completely different origins were surprisingly linked by their primary sequence homology. Recent investigations have illuminated their functions and revealed similarities in their mechanisms of action. Perhaps the best understood of all the PIK-related kinases are mammalian FRAP and its yeast homologs Tor1p/2p — by understanding these proteins we may gain insight into all the family members.

Rapamycin-sensitive signaling

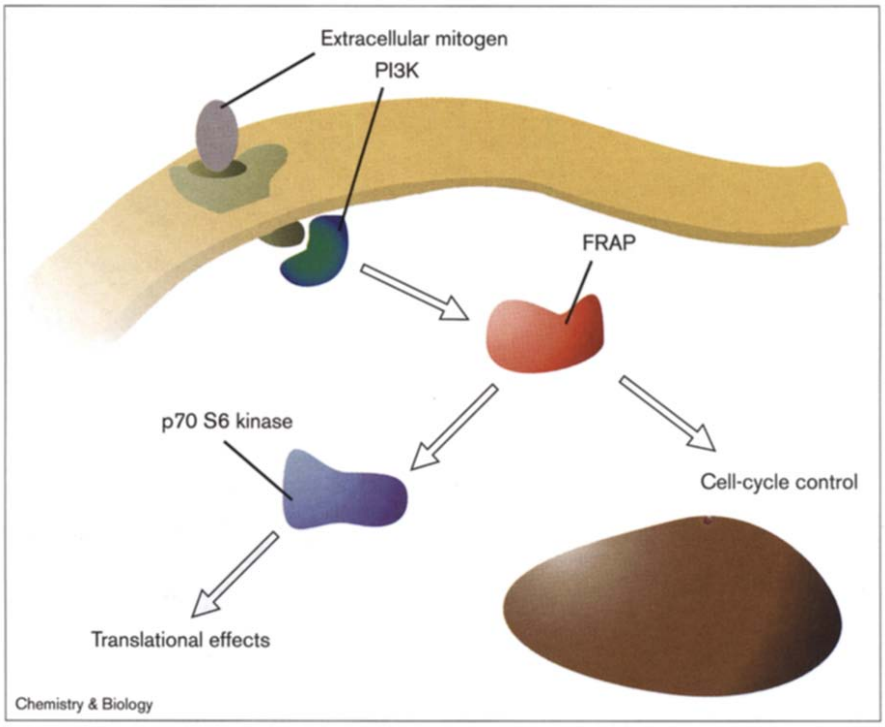
A potential explanation for rapamycin's immunosuppressive effects comes from the observation that rapamycin potently inhibits the proliferation of immune cells such as T lymphocytes grown in cell culture. Rapamycin actually inhibits the growth of a variety of cells in different organisms such as mammalian fibroblasts or unicellular baker's yeast, causing a G1 cell-cycle arrest. Rapamycin inhibits serum's ability to promote the G1 to S transition in certain mammalian cells, therefore also leading to G1 arrest. As serum stimulates transmembrane receptors, initial studies tried to place FRAP downstream of a mitogenic receptor.

These FRAP studies were facilitated by investigations of another protein, $p70^{S6k}$, a kinase important in translational regulation [25]. $p70^{S6k}$ is multiply phosphorylated in a complex manner following treatment with serum; this phosphorylation serves to activate its inherent kinase activity. Rapamycin potently inhibits serum-stimulated $p70^{S6k}$ phosphorylation, as does another natural product, wortmannin, an inhibitor of the lipid kinase PI3K (Figure 1). PI3K binds to and is directly activated by transmembrane receptors. The finding that $p70^{S6k}$ phosphorylation is sensitive to wortmannin and rapamycin led to early models in which a transmembrane receptor activates PI3K, which signals through FRAP to $p70^{S6k}$ (Figure 2).

A serious blow to the linear transmembrane receptor-PI3K–FRAP–$p70^{S6k}$ model came from the construction of a mutant $p70^{S6k}$ that was truncated at both its amino and carboxyl termini [26]. It was shown that this modified $p70^{S6k}$ responded appropriately to mitogens and was wortmannin-sensitive. Astonishingly, it was also shown that the doubly truncated $p70^{S6k}$ phosphorylation was rapamycin-insensitive. This result suggests that the modified $p70^{S6k}$ lacks the region required for rapamycin inhibition. As it still signals normally with respect to mitogens, being potently activated by serum, the region required for mitogenic activation must still be present, however. Thus $p70^{S6k}$ is probably receiving two distinct signals from different effectors, one coming from a mitogenic receptor, the other from FRAP. Hence the rapamycin insensitivity suggests that the serum-sensitive

Figure 2

The linear PI3K–FRAP–p70^{S6k} model. In this pathway, PI3K is thought to be upstream of FRAP and lead to its activation by producing 3-phosphoinositides. Active FRAP then promotes the phosphorylation of p70^{S6k}.



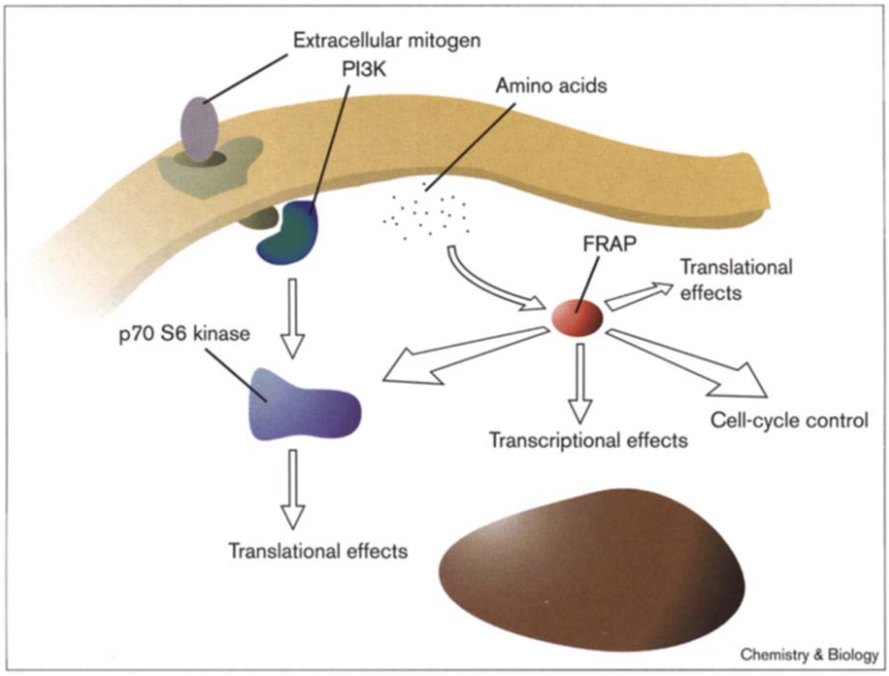
signaling pathway proceeds to p70^{S6k} without passing through FRAP. It is therefore unlikely that FRAP has a role in a linear membrane to p70^{S6k} pathway.

Although FRAP is probably not along such a linear pathway, rapamycin rapidly causes p70^{S6k} dephosphorylation, an observation that must be explained. A possible model is that rapamycin treatment activates a phosphatase that in turn dephosphorylates p70^{S6k}. This is consistent with the finding that the protein phosphatase pp2A forms a complex with p70^{S6k} [27]. The idea of a phosphatase being downstream of FRAP is supported by reports of a rapamycin-sensitive association between a phosphatase and a regulatory subunit in yeast [28], although these results do not consistently hold in mammalian systems [29–31]. It is also supported by the observation that cell extracts from rapamycin-treated cells have increased phosphatase activity [32]. Further evidence comes from the observation that, although pp2A stably binds to full-length p70^{S6k}, it does not associate with the doubly truncated mutant p70^{S6k}, potentially explaining its rapamycin resistance [32].

Rapamycin treatment elicits several other important effects besides the dephosphorylation and inhibition of p70^{S6k}. Rapamycin also causes the dephosphorylation of the translational factor 4EBP1 [33]. It might be that the same phosphatase that acts upon p70^{S6k} also dephosphorylates 4EBP1. In yeast, the serine/threonine kinase NPR1 is also dephosphorylated rapidly following treatment with rapamycin [34]. It has been proposed that NPR1 control in yeast is analogous to the control of p70^{S6k}/4EBP1 in mammalian cells. The protein p27^{Kip}, which potently arrests the cell cycle by inhibiting cyclin-dependent kinases, was also shown to be under the control of FRAP [35]. Rapamycin inhibits p27^{Kip}'s downregulation caused by serum in a distinct pathway from that involving p70^{S6k} [36]. In yeast, rapamycin has been reported to cause the degradation of the translational factor eIF4G [37]. Rapamycin has dramatic translational effects in yeast, decreasing translation to 10% of normal levels [38], and also has a variety of effects on transcription [39]. Thus FRAP and Tor1p/2p control aspects of several diverse cellular functions, including post-translational modification, protein stability, translation and transcription.

Rapamycin's effects on so many biological processes might be explained by a late cell-cycle effect. One could argue that cells respond to rapamycin treatment by arresting the cell cycle and then secondarily modulating the pathways described above. The kinetics of rapamycin-induced effects point to FRAP and Tor1p/2p having more direct roles in these signaling pathways, however. Translational and transcriptional inhibition, along with eIF-4G degradation, occur in the time scale of an hour, whereas p70^{S6k} dephosphorylation occurs with a $t_{1/2}$ of 2 minutes [40]. In yeast, NPR1 is completely dephosphorylated on rapamycin treatment within 5 minutes. These kinetics indicate that FRAP and Tor1/2p are closely positioned to these processes.

Figure 3



A current model of FRAP function. Results have suggested that FRAP sits outside the $p70^{S6k}$ pathway, downstream of amino acids, and regulates $p70^{S6k}$ by intercepting the PI3K pathway. This model thus conveys the idea of PIK-related kinase horizontal signal transduction.

Horizontal signal transduction

Like a transmembrane receptor, FRAP activates multiple and distinct signaling cascades in a kinetically rapid fashion. But instead of controlling its own pathways uniquely, FRAP controls pathways that transmembrane receptors activate. For example, FRAP regulates $p70^{S6k}$, even though it is under the control of mitogenic receptors. The situation is similar for translation in which FRAP and Tor1p/2p dominantly regulate key components of the translational machinery. In both cases, FRAP is controlling effectors that impinge upon core cellular processes or components of linear membrane to nuclear pathways (Figure 3).

Ordinarily one thinks of signal transduction as occurring 'vertically', from membrane to nucleus. This view of FRAP signaling, in which FRAP intercepts the more conventional pathways or cell-cycle proteins, resembles what might be called 'horizontal' signal transduction in two dimensions or 'radial' in three.

Although less is known about other PIK-related kinases, a similar picture emerges with other family members. There is evidence that ATM directly phosphorylates the transcription factor p53 in response to ionizing radiation [41,42]. p53, widely known as the most commonly mutated gene in cancer, can have many effects, including the induction of cell-cycle arrest or apoptosis [43]. ATR has also been shown to play an essential role in the phosphorylation and activation of p53 in response to both ionizing radiation and ultraviolet light [44]. A third PIK-related kinase, DNA-PK, has also been demonstrated to be upstream of p53 [45]. By regulating p53, which itself controls many processes, ATM, ATR and DNA-PK therefore gain influence over many aspects of cell signaling.

Like FRAP, other PIK-related kinases have multiple and distinct downstream pathways. In addition to its role in regulating p53, ATM has been shown to play an important role in the control of the nonreceptor tyrosine kinase Abl [46]. Abl function is poorly understood although it was initially characterized as a viral oncogene, implicating the kinase in cell-cycle function [47]. In *S. pombe*, the PIK-related kinase RAD3 is upstream of the protein kinase Chk1 [48]. When activated, Chk1 can inhibit cdc25, which prevents cdc25 from dephosphorylating cdc2, thus causing cell-cycle arrest [49]. Such an intervention is an excellent example of a PIK-related kinase dominantly impinging on the cell-cycle machinery, as cdc2 is the cyclin-dependent kinase responsible for mitotic entry.

An emerging theme to PIK-related-kinase transduction is the involvement of phosphatases. As discussed earlier, a phosphatase is likely to be downstream of FRAP/Tor1/2p in the control of $p70^{S6k}$/4EBP1/NPR1. The importance of a phosphatase is demonstrated in yeast, in which a mutation in the phosphatase regulatory subunit TAP42 confers rapamycin resistance [28]. Another family member, ATM, also appears to be linked to a downstream phosphatase. p53 undergoes an ATM-dependent dephosphorylation step after ionizing radiation, which in turn leads to 14-3-3 protein

binding to the resulting monophosphorylated protein [50]. 14-3-3 proteins, which bind certain elements in proteins containing phosphoserine and phosphothreonine residues, have been genetically linked to the Tor1p/2p signaling pathway in yeast, suggesting common features in the Tor1p/2p and ATM mechanisms [51].

Indirect support for FRAP and Tor1p/2p themselves binding to a phosphatase comes from the identification of a protein motif known as the HEAT repeat [52]. The HEAT repeat occurs 15 times in tandem and comprises the entire sequence of the A subunit of the phosphatase pp2A. The A subunit directly binds to the catalytic, or C, subunit of the phosphatase. The structure of the A subunit of pp2A has been determined to consist of elongated and repeating pairs of antiparallel α helices [53]. The HEAT repeat occurs approximately 20 times in TOR1p/2p and FRAP. Given the sequence homology, FRAP and Tor1p/2p might bind directly to the catalytic subunit of pp2A through their own HEAT repeats. Indeed, although it has not yet been established *in vivo*, it has been demonstrated that FRAP can directly phosphorylate the catalytic subunit of pp2A *in vitro* [32].

What are the regulators of the PIK-related kinases? If FRAP is not under the control of mitogenic receptors, then what is the signal that modulates its activity? And what might be regulating ATM, ATR and DNA-PK functions?

Regulating the PIK-related kinases

Recent studies of $p70^{S6k}$ have shed light on FRAP regulation. It was discovered that amino acids have even more potent control over $p70^{S6k}$ than does serum. Amino-acid withdrawal results in the rapid dephosphorylation of $p70^{S6k}$, and without amino acids in the media, serum stimulation of $p70^{S6k}$ is ineffective [54,55]. One of the most intriguing observations about amino-acid withdrawal is that the doubly truncated $p70^{S6k}$ is resistant to the dephosphorylation induced by amino-acid withdrawal, as it is with rapamycin [55]. Although this is only correlative evidence implicating FRAP, it is highly suggestive that FRAP might be sensing amino acids.

The notion of FRAP as an amino-acid sensor is supported by other reports in the literature. It has been noted that rapamycin treatment causes yeast to enter a state that resembles the starvation response [38]. Indeed, *tor2* kinase-dead overexpression also causes yeast to appear starved, both with regard to morphology and gene expression. More recent data also indicate that Tor1p/2p control autophagocytosis, the process by which the cell's own organelles and free proteins are digested and recycled, thus providing an internal source of amino acids and other metabolites [56]. Tor1p/2p were demonstrated to be genetically upstream of every member of a panel of autophagic mutants, suggesting an upstream position in the control of autophagy. It is interesting to note that autophagocytosis is potently inhibited by amino acids. Rapamycin's induction of autophagocytosis occurs even if cells are arrested, irrespective of which phase of the cell cycle the arrest occurs, again implying a cell-cycle-independent and direct connection into the pathway. Together these additional observations support FRAP and TOR1p/2p having an early role in amino-acid sensing.

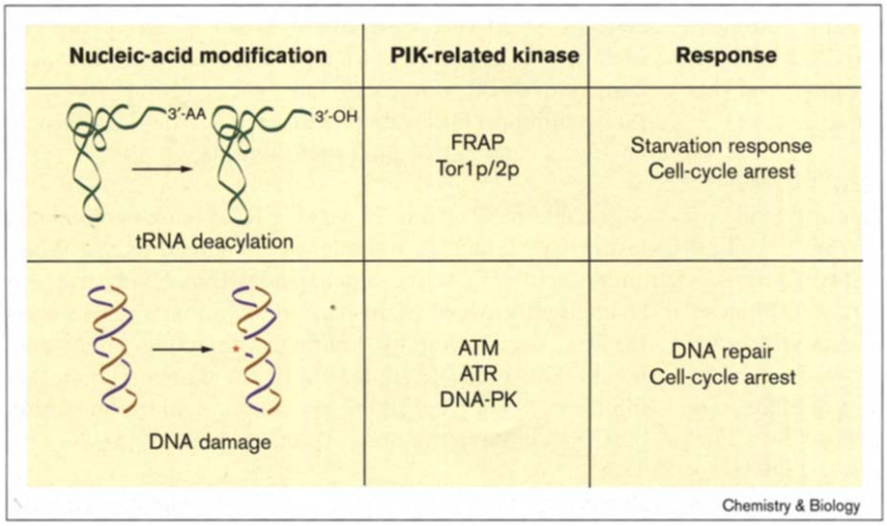
A possible mechanism by which FRAP senses amino-acid levels is to detect the abundance of charged tRNA. When amino acids are scarce, uncharged tRNA levels rise and charged tRNA levels fall. It is possible that FRAP senses these changes, thereby indirectly detecting amino-acid levels. This model is supported by the demonstration that inhibitors of tRNA charging lead to the dephosphorylation of $p70^{S6k}$, even in the presence of amino acids [57].

Regarding ataxia-telangiectasia, ATM knockout mice recapitulate the human disorder and are afflicted by lymphomas, radiosensitivity, immune defects, sterility and growth abnormalities [58,59]. One of the most striking defects in ATM knockout mice is their exquisite sensitivity to ionizing radiation. As mentioned earlier, $ATM^{-/-}$ cells are also defective in their p53 response to ionizing radiation. With the PIK-related kinase ATR, overexpression of a kinase-dead allele of ATR causes similar sensitivity to ionizing radiation to that found in $ATM^{-/-}$ cells, along with sensitivity to genotoxic agents such as cisplatinum or methyl methanesulfonate [60]. These results strongly suggest that ATM and ATR are both involved in sensing DNA damage.

Roles for ATM and ATR in meiosis were suggested on the basis of immunofluorescence studies that identified both proteins on meiotic chromosomes [61]. Their localization suggests they have a role in responding to the normal strand breaks that occur in meiosis as chromosomes break and join with their homologous partner. Given the meiotic defects of the ATM knockout mouse as well as the impairment of meiosis in *mec1* mutant yeast, it is apparent that ATM and other PIK-related kinases are playing a fundamental role in meiosis. As ATM plays a role in processing the normal strand breaks that occur in meiosis, it is probably playing a similar role with the non-natural strand breaks that occur with ionizing radiation.

Such a precedent is mimicked with DNA-PK. It was found that mice defective in DNA-PK are immunodeficient and sensitive to the DNA damage caused by X rays [62,63]. Cells deficient in functional DNA-PK are also defective in V(D)J recombination, the process by which antibody genes are cut and joined, thus rearranging their coding sequences in order to generate diversity. Like ATM and ATR, DNA-PK probably processes the normal DNA breaks of V(D)J recombination as well as the non-natural double-stranded breaks caused by X rays. Both would require cell-cycle arrest

Figure 4



A possible model for PIK-related kinase sensing. In this model, the common feature of PIK-related kinase sensing is their detection of nucleic-acid modifications. FRAP and Tor1p/2p would detect the increased tRNA deacylation that occurs when amino acids are scarce whereas ATM, ATR, and DNA-PK would detect DNA damage after genotoxic stress.

and repair until the break was corrected. In this way, the loss of DNA-PK causing immunodeficiency as well as sensitivity to radiation-induced strand breaks might be explained.

Using electron crystallography, a structure of DNA-PK with 22 Å resolution was obtained [64]. Its structure is highly sculpted and contains multiple channels and grooves where single- and double-stranded DNA may bind. Using both structural and biochemical data an elegant molecular mechanism has been proposed that explains how the kinase activity of DNA-PK might be modulated by the presence of double-stranded breaks [64].

As ATM, ATR and DNA-PK all respond to DNA damage, whereas FRAP and Tor1p/2p possibly detect tRNA charging status, it is tempting to suggest that perhaps the PIK-related kinases are unified through a role of sensing nucleic acids, whether it be DNA or RNA (Figure 4). Such a model does not exclude the possibility of other proteins being involved in the sensing mechanism, as has already been demonstrated with the Ku proteins and DNA-PK [65].

These kinases also have functions not modulated by their canonical sensor activity. In some cases, these additional functions are essential. In yeast, *TOR2* deletion is lethal, attributable to a cytoskeletal defect in actin organization [66]. This function of Tor2p is amino-acid- and rapamycin-insensitive, consistent with its kinase domain being differentially regulated [67]. In $ATM^{-/-}$ cells, serum is inefficient in promoting the G1 to S transition [68]. $ATM^{-/-}$ cells also senesce prematurely, dying only after a few passages in culture. ATM therefore performs additional functions that are not responses to sensing DNA damage. Similarly, Mec1p in yeast has an essential function which is related to the maintenance of the free deoxynucleotide pool [69].

Prospects

The last five years have witnessed many leaps in our understanding of the PIK-related kinases. We are beginning to understand how deeply embedded they are in such processes as the control of transcription, translation, growth, the cytoskeleton, as well as the response to DNA damage and starvation. But despite our advances, the mechanisms of their actions remain mysterious. The mystery of the homology to lipid kinases is unclear, especially as no lipid substrates have been found. Although the discovery of protein substrates like p53 is exciting, the existence of family members like TRRAP/Tra1p that appear to be devoid of kinase activity suggests that there may be more to downstream function than simple phosphorylation. There is no known function of the highly conserved tail possessed by all family members and the reason for their extraordinarily large size is also not understood. With the exception of the FKBP–rapamycin binding (FRB) domain of FRAP [70], there is no atomic-level structural information for any of the domains of these proteins, most notably lacking in the kinase and tail regions.

Although members of this family of proteins act as sensors of factors critical to the cell, how they are able to sense these factors is unknown. It will no doubt be fascinating to understand in rigorous molecular detail how these kinases behave as sensors, whether it be in DNA-damage detection, meiotic strand-break sensing, or nutrient-status monitoring. Surveillance of genomic integrity alone is one of the most important tasks a cell has, and the PIK-related kinases are central to this process. They may even be more accurately termed the 'guardians of the genome', a term famously applied to p53, because the PIK-related kinases operate in unicellular organisms that lack p53. In mammalian cells, p53 is downstream of three PIK-related

kinases — ATM, ATR and DNA-PK. Indeed p53 may be simply the PIK-related kinase effector into some of pathways that the family controls in mammalian cells.

So far, the PIK-related kinases have been recalcitrant to placement in traditional pathways. It will be important to understand the nature of the signals propagating from these intracellular receptors in order to understand the family's relationship to conventional cell signaling. How does the program of cell-cycle arrest, entering starvation or invoking DNA repair actually occur? There are at present no satisfying answers to these questions.

Given the highly conserved nature of the PIK-related kinases in plants, fungi, nematodes and mammals, it is clear that they play central roles in many diverse species. Understanding these mysterious kinases will provide insights into immunology, the cytoskeleton, nutrient sensing, apoptosis and cancer that will undoubtedly lead to important advances in medicine and in understanding eukaryotic life itself.

Acknowledgements

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