The Death-Associated Protein Kinases: Structure, Function, and Beyond

Shani Bialik and Adi Kimchi

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, 76100 Israel; email: shanib@weizmann.ac.il, adi.kimchi@weizmann.ac.il

Key Words

autophagy, DAP kinase, DRP-1, programmed cell death, ZIP kinase

Abstract

Death-associated protein kinase (DAPk) is the founding member of a newly classified family of Ser/Thr kinases, whose members not only possess significant homology in their catalytic domains, but also share cell death-associated functions. The realization that DAPk is a tumor suppressor gene, whose expression is lost in multiple tumor types, has spurred a flurry of interest in the kinase family and produced an impressive body of literature concerning its function, regulation, and connection to disease. The DAPk family has been linked to several cell death-related signaling pathways, and functions other than cell death have also been proposed. This review presents a thorough structural analysis of the kinases, discusses methods of regulation, clarifies their cellular targets and functions, and shows how these functions are integrated. Although many gaps in our knowledge still remain, the data generated to date can be combined to delineate a place for the DAPk family within the general cell death-signaling network.
DAPk suppresses cellular transformation at early stages of tumor development (4) and also inhibits later metastatic events, as shown in a mouse model of tumor metastasis (3). Interestingly, embryonic fibroblasts derived from DAPk−/− mice have a greater spontaneous immortalization rate than their wild-type (WT) counterparts (H. Berissi, T. Raveh, and A. Kimchi, unpublished data). This indicates that lack of DAPk also interferes with cellular senescence. These data have motivated numerous researchers to examine the status of DAPk in human primary tumors. The majority of these studies found a significant loss of DAPk expression in a large variety of tumor types, mainly owing to DNA methylation.

**Supplementary Table 1** summarizes these findings (follow the Supplemental Material link from the Annual Reviews home page at [http://www.annualreviews.org](http://www.annualreviews.org)). The tumor screens combined with the previously mentioned experimental data on the tumor suppressive functions of DAPk suggest that DAPk may play a causative role in tumor development, and assays of its expression levels may serve as a diagnostic and prognostic tool to evaluate disease severity, progression, metastatic rate, and recurrence. Furthermore, DAPk, which is highly abundant in the brain, has been linked to diseases associated with neuronal injury and may serve as a target for therapeutic intervention in the treatment of neurodegeneration. The reader is referred to a recent review for an in-depth discussion of the contribution that loss and gain of DAPk function has to cancer and neuronal disease, respectively, as well as the potential strategies available to modulate DAPk's function (10).

Over the past few years, several groups have joined the effort to decipher DAPk's cellular function, focusing on areas that include its biochemical properties, regulation, and target substrates. From these studies, it has become apparent that DAPk has multiple functions and is linked to several cell death–related signaling pathways. Furthermore, it is now recognized that DAPk is the prototype

**INTRODUCTION**

Death-associated protein kinase (DAPk) is a Ca²⁺/calmodulin (CaM)-regulated Ser/Thr kinase that mediates cell death. Increased DAPk activity, due to overexpression of the kinase, leads to pronounced death-associated cellular changes, which include membrane blebbing, cell rounding, detachment from extracellular matrix, and the formation of autophagic vesicles (1–8). Furthermore, DAPk activity is necessary for the induction of cell death by multiple death signals, including those generated by death receptors, cytokines, matrix detachment, and oncogene-induced hyperproliferation (1–4, 6, 7, 9).

DAPk acts as a tumor suppressor largely because of its ability to sensitize cells to many of the apoptotic signals that are encountered as a cell undergoes tumorigenesis.
of a family of closely related kinases, all of which have been associated with cell death. The purposes of this review are to provide a comprehensive overview of the recent literature on DAPk and its family members, to resolve some discrepancies that have arisen, and ultimately, to place DAPk in the proper context of cell death signaling.

DEATH IS A FAMILY MATTER: DAPk AND RELATED PROTEINS

DAPk belongs to a family of related death kinases, all of which share significant sequence and functional homology (11–14). The family includes two closely related homologues of DAPk: ZIPk (ZIP kinase, also known as Dlk (DAP-like kinase) or DAPk3) (11, 12) and DRP-1 (DAPk-related protein 1, also known as DAPk2) (13, 14). The human genes share 83% and 80% identity at the amino acid level, respectively, with DAPk's catalytic domain. More distantly related are human DRAK-1 and DRAK-2 (DAPk-related apoptosis-inducing protein kinase-1 and -2), whose kinase domains are only 50% identical to DAPk (15). Phylogenetically, the DAPk family is most closely related to the family of CaM-regulated kinases, in particular to myosin light chain kinase (MLCK), which shares 44% identity within the corresponding catalytic domain. DAPk orthologues exist in rodent (16) and in Caenorhabditis elegans (13) but not in Drosophila or lower organisms. In contrast, ZIPk and DRP-1 are only present in mammals (Y. Shoval and A. Kimchi, unpublished data). An orthologue of DRAK-2, but not DRAK-1, exists in the rodent and is expressed almost exclusively in lymphoid tissue (17). For the most part, the DRAKs have not been well characterized, although a mouse knockout of DRAK-2 suggests that the protein negatively regulates T-cell activation, with no apparent role in apoptosis (17). This review therefore focuses on the three closer death-associated relatives, DAPk, ZIPk, and DRP-1.

Structural Features of DAPk Family Members

DAPk, DRP-1, and ZIPk are grouped together into one kinase subfamily because of the high degree of conservation within their common catalytic domains. However, outside this region, the kinases vary greatly in size and structure. This section compares the various structural domains of the kinase family and discusses how these domains affect the function and localization of each kinase.

The catalytic domain: structure and regulation. Each DAPk family member contains at its N terminus a catalytic domain composed of the typical 11 subdomains found in all Ser/Thr kinases. The X-ray crystal structure of DAPk's catalytic domain has been resolved to an impressive 1.5 Å and has provided structural hints as to DAPk's mechanism of activation, its interactions with substrates, and its potential inhibitor design (18 and reviewed in 10). Of note, the presence of two clusters of acidic amino acids at the proposed substrate-binding site suggests that complementary interactions with basic residues near the substrate's core phosphorylation site may play a role in substrate recognition. In fact, many proposed substrates of the DAPk family possess two to three basic residues just N-terminal to the phosphorylated Ser or Thr (see Table 1), and mutation of such a basic cluster in an optimized DAPk peptide substrate reduced phosphorylation efficiency (19). Another outstanding feature of the structure is a highly ordered, positively charged loop, enriched in basic residues, that is positioned on the upper lobe of the catalytic domain (18). The 12 amino acids (aa 45–57) that comprise this loop are a conserved feature of the DAPk family members and are thus referred to as "the fingerprint" of the family (13). Of note, mutation of the basic residues in the loop did not affect the $K_m$ of a peptide substrate (19), indicating it is not directly involved in substrate binding, but rather may be

ZIPk: ZIP kinase

DRP-1: DAPk-related protein 1

MLCK: myosin light chain kinase
involved in other functions, as detailed below in the section on Mechanisms of Regulation.

The kinase domains of DAPk and DRP-1 are followed by a CaM autoregulatory/binding segment, which serves to suppress catalytic activity by binding to the catalytic cleft, and functioning as a pseudosubstrate (20, 21; for a detailed discussion, also see 10 and 22). In addition, this domain undergoes autophosphorylation at Ser308, an inhibitory event that reduces its affinity to CaM and may further stabilize its docking within the substrate-binding site (20, 21). Hence, activation of DAPk and DRP-1 requires two events: First, as in all CaM-dependent kinases, binding of Ca\(^{2+}\)-activated CaM to the autoregulatory/CaM-binding segment pulls this domain out from the catalytic cleft (1, 13, 14). Second, and unique to DAPk and DRP-1, dephosphorylation of Ser308 increases the affinity for CaM and, as a consequence, promotes catalytic activity at low CaM levels (20, 21). Interestingly, a low level of catalytic activity is detected upon Ser308 dephosphorylation of DAPk even in the absence of CaM (21). In DRP-1’s case, dephosphorylation of Ser308 promotes homodimerization, which is also critical for CaM binding and full functional activation in cells (20). In support of this model, deletion of the CaM-binding domain from either DAPk or DRP-1, or substitution of Ser308 to Ala, generates constitutively active kinases, which exhibit greater Ca\(^{2+}\)-independent catalytic activity in vitro and stronger killing potential in vivo (1, 13, 14, 20, 21).

The third member, ZIPk, lacks the CaM regulatory domain, and thus its regulation differs substantially from DAPk and DRP-1. Recent work has demonstrated that the catalytic activity of ZIPk may be regulated through positive autophosphorylation. ZIPk undergoes autophosphorylation on multiple sites both in vitro and in vivo, and three of these were mapped by a combination of phosphopeptide analysis and mutagenesis to the catalytic domain (Thr180, Thr225, Thr265) (23). Individual mutation of these residues
Table 1  Substrates of DAPk family members and proposed function of phosphorylation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinase</th>
<th>Sequence</th>
<th>In vivo</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPk</td>
<td>DAPk</td>
<td>FAARKKWQ508VRL</td>
<td>Yes</td>
<td>Inhibits catalytic activity</td>
</tr>
<tr>
<td>DRP-1</td>
<td>DRP-1</td>
<td>QYVRRWKLS108FSI</td>
<td>Yes</td>
<td>Inhibits catalytic activity</td>
</tr>
<tr>
<td>ZIPk</td>
<td>ZIPk</td>
<td>GNEFKNIFGT180PEF PFLGETKQET225LTN VKDPKRRMT261FAQ RKPERRLK299TRL RLKEYT306IKS311SL</td>
<td>Yes</td>
<td>Enhances catalytic activity</td>
</tr>
<tr>
<td>ZIPk</td>
<td>DAPk</td>
<td>RKPELLRLKT299TRL RLKEYTIS109HS311SL PPNN511YADYERFS126K</td>
<td>—</td>
<td>Localization and oligomerization</td>
</tr>
<tr>
<td>MLC</td>
<td>DAPk</td>
<td>KKRPOQAT18S19NVF</td>
<td>Yes</td>
<td>Activates myosin, membrane blebbing</td>
</tr>
<tr>
<td>MLC</td>
<td>ZIPk</td>
<td>—</td>
<td>—</td>
<td>Activates myosin</td>
</tr>
<tr>
<td>MLC</td>
<td>DRP-1</td>
<td>—</td>
<td>—</td>
<td>Activates myosin</td>
</tr>
<tr>
<td>MYPT (MBS)</td>
<td>ZIPk</td>
<td>BQARQSRSTG569QGV</td>
<td>—</td>
<td>Activates myosin</td>
</tr>
<tr>
<td>CPI-17</td>
<td>ZIPk</td>
<td>AQLGKRVL512KLQ GLQKR718VKY</td>
<td>—</td>
<td>Activates myosin</td>
</tr>
<tr>
<td>p21Cip1</td>
<td>ZIPk</td>
<td>DSQGRKRRQT185SMT</td>
<td>Yes</td>
<td>Protein stability</td>
</tr>
<tr>
<td>Mdm2b25</td>
<td>ZIPk</td>
<td>STSSRRRAIS106ETE</td>
<td>—</td>
<td>p53 regulation</td>
</tr>
<tr>
<td>Par-4</td>
<td>ZIPk</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Histone H3</td>
<td>ZIPk</td>
<td>RTKQTARKST311GK</td>
<td>—</td>
<td>Mitosis</td>
</tr>
<tr>
<td>H2A, H4</td>
<td>ZIPk</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ASF/SF2</td>
<td>ZIPk</td>
<td>—</td>
<td>—</td>
<td>Splicing</td>
</tr>
<tr>
<td>Syntaxin-1A</td>
<td>DAPk</td>
<td>SGHMDSSIS188KQA</td>
<td>Yes</td>
<td>Synaptic vesicle/membrane fusion</td>
</tr>
<tr>
<td>α-, β-tubulin</td>
<td>ZIPk</td>
<td>—</td>
<td>—</td>
<td>Cytokinesis</td>
</tr>
<tr>
<td>CaMKK</td>
<td>DAPk</td>
<td>GSRREERLS141APG</td>
<td>Yes</td>
<td>Inhibits activation</td>
</tr>
</tbody>
</table>

aIndicates confirmation of phosphorylation in vivo by exogenous or endogenous kinase.
bPhosphorylated as peptide but not in the context of full-length protein.
cIndirect in vitro proof of in vivo phosphorylation.

to Ala greatly reduced the catalytic activity of ZIPk toward exogenous substrate in vitro and suppressed its ability to induce membrane blebbing and cell rounding in vivo (23). Of note, Thr180 is a conserved residue that lies within the universal kinase activation loop, and phosphorylation of this loop is often an important step in activation of the catalytic activity of many protein kinases (24). Although phosphorylation of Thr180 suggests that ZIPk may be subjected to a regulatory mechanism common to many kinases, such as CaM-regulated kinase I and IV (25), it is not known what signals, if any, regulate ZIPk’s autophosphorylation. Interestingly, all three Thr residues are conserved in DAPk and DRP-1. Yet, there is no evidence to suggest that either DAPk or DRP-1 are positively regulated through phosphorylation of the catalytic domain. Moreover, the crystal structure of DAPk suggests that phosphorylation of the activation loop is unnecessary, because unlike many other kinases which display autoinhibited forms, DAP kinase’s catalytic domain is constitutively in the active “closed” conformation even in the absence of a peptide substrate (18).

The extracatalytic domains. Beyond the common kinase domain, the family members
differ in structure. DAPk, at 160 kDa, contains a large C-terminal extension with multiple functional domains. A series of eight ankyrin repeats follows the catalytic domain, after which is a region that has been shown to direct the kinase to the actin cytoskeleton (1, 26). Two putative P loops of unknown function reside at aa 639–646 and 695–702 of DAPk, respectively, the second of which partially overlaps the cytoskeletal-interacting domain. Studies of exogenous and endogenous DAPk for the most part agree that DAPk is an actin-associated protein kinase. This was demonstrated by imaging live or fixed HeLa cells expressing a GFP-DAPk fusion protein, which localized to actin stress fibers and cortical actin filaments (26). Consistent with this localization, actin coimmunoprecipitates with exogenous DAPk in an almost stoichiometric manner (G. Shohat and A. Kimchi, unpublished data). Endogenous DAPk can be solubilized in mild detergent extraction buffers only upon pretreatment of cells with latrunculin B, an actin-depolymerizing agent (1). Additionally, a recent report indicated that upon induced formation of stress fibers in serum-stimulated NIH3T3 cells, endogenous DAPk localized to the actin stress fibers in the central region of the cell but not at the cell periphery (27).

The ankyrin repeats, a protein-protein interaction motif common to cytoskeletal proteins, are also necessary for the proper localization of the kinase to the actin stress fibers (26). In their absence, DAPk is sequestered to focal adhesions and does not induce cell death morphology. Further evidence in support of an important functional role for the ankyrin repeats is that a peptide from this region (aa 451–498) was isolated as a dominant-interfering fragment of DAPk function (28). Expression of the death domain protected 293 cells from death by tumor necrosis factor (TNF)-α and Fas, and Hep3B cells from transforming growth factor (TGF)-β-induced apoptosis (2, 7). Similarly, ceramide-induced death of primary hippocampal neurons was inhibited by administration of a synthetic peptide corresponding to the Ser-rich tail (9). Deletion of the tail generated a gain-of-function mutant that exhibited a greater killing potential without any effect on in vitro kinase activity (28). Thus, the C-terminal tail negatively regulates the cellular functions of DAPk. In contrast, deletion of the death domain attenuated DAPk’s killing ability in some settings (2, 27). This suggests...
that the death domain competes with the full-length kinase by sequestering a critical death domain-interacting partner.

Two proteins have been shown to independently interact with DAPk's death domain. The first is the extracellular signal-regulated kinase (ERK), which binds a docking site within DAPk's death domain (34). As will be discussed in more detail below, ERK phosphorylates DAPk on Ser735 within the cytoskeletal binding region, a catalytically stimulating modification that presumably depends on the interaction with DAPk's death domain (34). DAPk's death domain also mediates homotypic interactions with the UNC5H2 dependence receptor (35), a death domain-containing protein that induces cell death when unbound to its ligand, netrin-1. Interestingly, the death domains of DAPk and UNC5H2 are more closely related to each other than to other death domains, such as that of Fas (33), which may explain the selectivity of the DAPk/UNC5H2 death domain interaction. Death induced by expression of unliganded UNC5H2 was partially attenuated by the absence of DAPk in cells derived from knockout mice, indicating that the interaction between the two proteins is critical for UNC5H2's death effects (35). Netrin-1 binding to the receptor had no effect on its association with DAPk; however, removal of the ligand led to dephosphorylation of DAPk at Ser308 (35). Thus, the death domain mediates two interactions that serve, in different circumstances, to activate DAPk. Deletion of the death domain from DAPk, or competition with excess death domain, would disrupt these interactions and thus interfere with DAPk activation and cellular function.

Interestingly, an alternatively spliced isoform of DAPk, DAPkβ, which possesses a unique 12-aa extension following the Ser-rich tail, has been identified in the mouse (Figure 1) (36). An antibody raised to this unique C terminus recognizes a protein of the correct molecular weight in HeLa cell extract (37), suggesting that this second isoform exists in humans as well, although it has not been observed in human databases (Y. Shoval and A. Kimchi, unpublished observations). This longer kinase was reported to have anti-apoptotic activity and blocked TNFα-induced killing in HeLa and MDCK cells (36), suggesting that the C-terminal extension augments the inhibitory nature of the tail region. It should be noted, however, that DAPkβ functioned identically to the prodeath human DAPk when tested by independent investigators (S. Bialik and A. Kimchi, unpublished data). Considering that Jin et al. (36) did not see any difference in the two isoforms' in vitro kinase activity, or in their ability to facilitate TNFα-induced phosphorylation of an apoptosis-relevant substrate, myosin II regulatory light chain (MLC), the anti-apoptotic nature of DAPk requires further substantiation.

DRP-1, a 42-kDa protein, contains a 40-aa extension C-terminal to the CaM regulatory domain. This region is essential for homodimerization of the protein, a feature necessary for its functional activity and, in particular, for its ability to be regulated by CaM (13, 20). DRP-1 lacks any of the cytoskeletal features of DAPk, and consistent with this, exogenous DRP-1 was shown to be a soluble, cytoplasmic protein (13). In addition, Flag-tagged DRP-1 accumulated within double-membrane-enclosed autophagic vesicles (5). However, as there is a lack of efficient antibodies that recognize the endogenous protein, it is not known whether the endogenous DRP-1 behaves similarly.

The 52-kDa ZIPk possesses a C-terminal leucine zipper motif (11, 12). This domain mediates homodimerization and interactions between ZIPk and additional leucine zipper-containing proteins, such as ATF4, AATF, and rat CDC5 (12, 20, 23, 38, 39). There are also several regions that resemble nuclear localization signals (NLSs), two of which have been shown to be functional within the exogenous protein (40). Curiously, although ZIPk itself lacks DAPk's death domain and cytoskeletal-interacting region, it binds Par-4, a 38-kDa protein.
death domain-containing protein that directly interacts with actin filaments (41, 42). Thus, the ZIPk/Par-4 complex can be thought of as a bipartite structural mimic of DAPk. These structural features of ZIPk predict both cytoplasmic and nuclear distributions, and in fact, overexpressed ZIPk has been observed either in the cytoplasm, the nucleus, or both (11, 12, 43, 44). In one system, coexpression of Par-4 shifted the localization of ZIPk from the nucleus to the cytoplasm, where the two proteins colocalized to actin filaments (41). The nuclear distribution of ZIPk has been reported as both diffuse and speckled, and associations with PML (promyelocytic leukemia protein) nuclear bodies (44), chromatin (12), centrosomes, centromeres during mitosis, and the contractile ring during cytokinesis have been observed (45, 46). The discrepancies among the data were initially attributed to the inherent problematic nature of studies using overexpressed protein and/or to reflect differences among the tested cell lines. Yet, immunostaining of endogenous rat ZIPk in REF-2A cells revealed both a diffuse nuclear distribution as well as an association with actin stress fibers, indicating that ZIPk can maintain both nuclear and cytoplasmic localizations within a single cell (47). Interestingly, in addition to full-length ZIPk, a smaller-molecular-weight form is present in lysates from these cells. This is similar in size to a 32-kDa ZIPk observed in bovine smooth muscle, which was isolated in association with myofibers (48). This small ZIPk is most likely a cellular proteolytic fragment of the full-length protein because there is no experimental evidence or sequence data to support the presence of alternate splicing (49). The small form was isolated as an active kinase, so it is predicted to terminate just beyond the catalytic domain and to lack the C-terminal portion of the extracatalytic region, including the NLS and the Leu zipper. In fact, it is reminiscent of a previously described artificial C-terminal truncation of the rat protein that localized to the actin cytoskeleton when overexpressed (40). Thus, the dual localization of ZIPk in these cells may reflect alternate behaviors of the two protein forms. Other posttranslational events, such as phosphorylation of ZIPk by DAPk, may also change its intracellular localization, as detailed below in the next section.

Mechanisms of Regulation of the DAPk Family

DAPk, DRP-1, and ZIPk are all ubiquitously expressed in numerous adult mouse and rat tissue (11, 12, 14, 36, 50, 51). DAPk is particularly abundant in the adult and embryonic brain, especially the hippocampus. The constitutive presence of these potentially lethal proteins in normal tissue necessitates tight regulation, which, on the one hand, maintains their silence in growing cells but, on the other hand, facilitates efficient activation in response to death signals. Most of the activation mechanisms characterized so far in DAPk and DRP-1 influence catalytic activity by targeting the CaM autoregulatory/binding segment. As stated above, relief from the inhibition by this domain requires binding of Ca\(^{2+}\)-activated CaM and dephosphorylation of Ser308. In fact, dephosphorylation of both DAPk and DRP-1 at Ser308 has been observed in vivo in response to certain death stimuli, such as C6-ceramide, TNF-\(\alpha\), inhibition of mitochondrial respiration, unliganded UNC5H2, and interferon (IFN)-\(\gamma\) (20, 21, 35, 52; G. Shohat, and A. Kimchi, unpublished data). Thus, two important cellular factors are predicted to activate DAPk and DRP-1: elevation in intracellular Ca\(^{2+}\), which is often observed during different scenarios of programmed cell death, and a death-regulated phosphatase that specifically dephosphorylates Ser308. This combination should confer the required specificity to allow activation of these kinases in limited circumstances and not in response to every local spike in intracellular Ca\(^{2+}\). Clearly, identification of the putative phosphatase and its mode of activation during cell death would greatly contribute to our understanding of how these kinases are regulated in vivo.
Additional phosphorylation events, involving ERK and mitogen-activated protein kinase (MAPK) signaling cascades, have been shown to regulate DAPk activity. As mentioned above, ERK phosphorylates DAPk on Ser735 both in vitro and in vivo (34). Increased phosphorylation on Ser735 in vivo correlated with enhanced phosphorylation of a DAPk substrate, MLC, and increased killing activity by overexpressed DAPk. Interestingly, this was attributed to a direct enhancement of in vitro catalytic activity, although the mechanism by which phosphorylation of a residue within the distant cytoskeletal interacting domain can affect catalysis is not clear. A second study has recently demonstrated that a downstream effector of ERK, the p90 ribosomal S6 kinase (RSK), inhibited exogenous DAPk cellular activity by phosphorylation of Ser289 within the CaM-autoregulatory/binding segment (53). These two studies suggest that the same upstream signals can lead to reverse effects on DAPk activity. It is not known, in fact, whether these phosphorylations occur simultaneously; perhaps they are sequential modifications that act to transiently turn DAPk activity on or off. Alternatively, they may represent different adaptations to subtle variations in the upstream signaling events, which may require alternative cellular responses. Importantly, it is not yet known whether MAPK/ERK signaling modifies endogenous DAPk and whether phosphorylation at either Ser289 or Ser735 serves to regulate DAPk activity in response to physiologic life and death decisions.

Under certain circumstances, particularly in cells with minimal DAPk protein expression, transcriptional and/or translational mechanisms may be an important means of activation of the kinase. DAPk gene expression increases in response to TGFβ (7) and to stimuli that activate p53, such as DNA-damaging agents and oncogene expression (54). In fact, the DAPk promoter contains both a TGFβ-response element, which is activated, at least in part, by the Smad transcription factor family (7) and a p53-binding element (54). Other death triggers, such as IFN, C2-ceramide, and expression of transforming oncogenes, induce DAPk expression as well (4, 9, 55).

Less is known about the mechanisms that activate ZIPk in response to stress signals. Recent work, however, has suggested that the cellular localization of ZIPk may be a critical determinant in ZIPk’s cell death–promoting activity. Initial studies of ZIPk subcellular distribution, described in detail above, correlated ZIPk’s killing activity with its presence in the cytosol (11, 12, 40, 43). The cytoplasmic distribution may be regulated through phosphorylation of ZIPk by DAPk. DAPk and ZIPk form a complex, which enables phosphorylation of DAPk on ZIPk, but not vice versa (43). Importantly, the interaction has been confirmed at the level of the endogenous kinases. Mutational analysis of the interacting domains indicated that it is mediated by the catalytic domains, with the fingerprint basic loop contributing to the heterodimerization (43). DAPk phosphorylates ZIPk on multiple sites, including Thr299, along with five serines clustered within the region spanning aa 309–326. Significantly, a ZIPk phosphorylation mimetic, in which the target residues were mutated to Asp, was preferentially retained in the cytosol and displayed higher cell-killing activity (23, 43). Interestingly, Thr299 is also a site of ZIPk autophosphorylation (23). This ZIPk/DAPk interaction suggests a means by which a death signal can be transferred from one kinase to another in a catalytic amplification loop.

**DOWNSTREAM OF DAPk: A CLOSE LOOK AT DAPk FUNCTION**

DAPk’s primary function is, as its name suggests, to regulate cell death. There is, in fact, a strong body of evidence that shows that DAPk is an essential component of different cell death signaling pathways. As discussed above, endogenous DAPk undergoes activation in response to various death stimuli, and death...
Type II
(autophagic) cell death: the result of autodigestion of intracellular organelles, distinguished by the accumulation of double-membrane-enclosed autophagocytic vesicles

MEF (or REF): mouse (or rat) embryonic fibroblast

Stimuli have been associated with an increase in DAPk catalytic activity (6, 52). ZIPk and DRP-1 are mostly guilty by association with their more famous relative DAPk. The evidence to support ZIPk’s and DRP-1’s roles as cell killers is rather circumstantial and is based mainly on their ability to promote cell death–related morphologies when overexpressed (5, 11, 13, 14, 23, 40, 41, 43, 56). Further investigation into their functions at the level of the endogenous kinases is required. This is especially demanded for ZIPk, which has been additionally shown to interact with and/or phosphorylate several nuclear factors, such as histone H3 and p21WAF1 (see Table 1), suggesting potential roles in transcription, splicing, chromatin modification, and cell cycle control (11, 38, 39, 45, 46, 57). Several functions unrelated to cell death have also been ascribed to DAPk. The sections below describe and evaluate the various functional arms of the kinases, focusing mainly on DAPk, for which there is more substantial data.

Death-Associated Protein Kinases: How DAPk Earned Its Name

DAPk was originally isolated in an unbiased antisense-based genetic screen for genes whose protein products were necessary for IFN-γ-induced death in HeLa cells (58). IFNs are multifunctional cytokines, which can, depending on the cell setting, exhibit antiproliferative, immunomodulatory, differentiation- or death-promoting properties (55). In the HeLa cell system used in the screen, IFN-γ led to a slow but very efficient cell death, classified as Type II (see below) (5, 58). The fact that a reduction in DAPk expression led to increased cell survival in long-term clonal viability assays was the first clue that DAPk is a death-promoting kinase. These DAPk antisense-expressing HeLa cells were also resistant to cell death induced by Fas (2). Numerous studies have since demonstrated that the dominant-negative DAPk K42A mutant, or interfering fragments such as the death domain or C-terminal tail segment, reduced the extent of cell death in response to multiple triggers, such as Fas and TNFα (2), ceramide signaling (6, 9), TGF-β (7), and expression of the UNC5H2 dependence receptor in the absence of its ligand (35). More elegant approaches to elimination of DAPk function involved generation of a mouse deleted of DAPk through gene targeting (4, and D. Gozuacik, T. Raveh, and A. Kimchi, unpublished data). Although no defects were observed in developmental cell death, DAPk’s necessity for cell death was observed when primary cells derived from the knockout mouse were subjected to external stresses. For example, DAPk −/− hippocampal neurons were much more resistant than their WT counterparts to ceramide-induced apoptosis, triggered either directly by administration of C6-ceramide or indirectly by activation of p75 neurotrophin receptors with nerve growth factor (NGF) (9). In addition, mouse embryonic fibroblasts (MEFs) derived from DAPk −/− mice showed decreased induction of p53 and p19ARF and, consequently, decreased levels of apoptosis in response to the hyperproliferative signals generated by forced expression of oncogenes such as c-myc and E2F (4). Likewise, UNC5H2-mediated cell death was partially attenuated in immortalized DAPk −/− MEFs (35). In the intact animal, retinal ganglion cells from DAPk −/− mice showed increased survival (79% vs 56% in matched controls) following administration of glutamate (59). Furthermore, renal tubular cells in which the DAPk catalytic domain was deleted by homologous recombination were more resistant to apoptosis in a mouse model of chronic obstructive uropathy (60). All of these studies prove independently the necessity of DAPk for cell death.

Still further evidence emerges from the cellular phenotype obtained by ectopic expression of DAPk, which has shown that DAPk is sufficient to induce cell death in various cell lines (1, 2, 4, 5, 7, 8). Furthermore, expression of DAPk enhanced ceramide- and UNC5H2-induced death (6, 35). Although constitutively active forms
of the kinase (e.g., DAPk lacking the CaM-autoregulatory/binding segment) were used in many of these studies, the WT form of the kinase is also capable of inducing cell death, albeit to lower levels (1). In fact, the degree of cell death correlates with the levels of protein expressed; low levels, such as those achieved upon stable, inducible expression of DAPk, do not lead to an apparent cell death phenotype (36; O. Cohen and A. Kimchi, unpublished observations). Such low amounts of protein, albeit more accurately reflecting the physiologic situation, are more likely to be subject to the same regulatory control that keeps endogenous DAPk inactive in the absence of an activating stimulus. Expression of particularly high levels of DAPk, however, may bypass the inhibitory regulation and result in the production of active kinase that is capable of inducing cell death. Thus, although such experiments deal with nonphysiologic protein levels, they very likely reflect the behavior of the endogenous kinase upon activation by a death signal.

Molecular Dissection of the Death Pathways Induced by the DAPk Family

Closer inspection of the molecular and cellular events that occur as a consequence of DAPk activation or that are blocked by loss of DAPk function have linked DAPk to both caspase-dependent (Type I) and caspase-independent cell death (4, 5, 7). ZIPk, too, was shown to induce two distinct morphologic stages of cell death within one cell type: The first stage was not blocked by caspase inhibitors or Bcl-xL, but a later terminal stage had all the features of Type I apoptosis (61). The emerging model is that the DAPk family members can be linked to various molecular pathways depending on the cellular setting, which culminate in different forms of cell death. Importantly, the functional death effects of DAPk and its family members require catalytic activity; catalytically inactive K42A mutants of DAPk, DRP-1, or ZIPk fail to induce a death phenotype (1, 11, 13, 14). Thus, phosphorylation of some specific substrate(s) is required for the death-inducing effects of the DAPk family (see Table 1). The main challenges now are to dissect the death pathways at the molecular level, to elucidate the specific substrates within each pathway, and to determine the factors that enable one pathway to predominate over another.

One cellular setting in which DAPk was linked to Type I (apoptotic) cell death is primary embryonic fibroblasts with a functional p53-signaling pathway. In WT, but not p53-null primary REFs and MEFs, activated DAPk triggered caspase-dependent Type I apoptosis (4). Furthermore, in the absence of DAPk, p53 was only partially upregulated in response to proliferative signals generated by oncogene expression, indicating that DAPk is an upstream activator of p53 along this particular pathway (4). In fact, DAPk expression induced p53 and its response genes in both transformed and primary fibroblasts (4, 8). This is particularly interesting, considering that DAPk itself is a transcriptional target of p53 (54), which indicates a signaling feedback loop in which DAPk and p53 can activate each other. These experiments show that p53 activation molecularly links DAPk to the classical death pathway involving mitochondrial-based activation of the caspase cascade.

DAPk's upregulation of p53 and subsequent induction of Type I cell death require the presence of p19ARF (4), an inhibitor of Mdm2, which normally promotes the ubiquitin-dependent degradation of p53 (62). DAPk can phosphorylate p19ARF in vitro, although this phosphorylation is not efficient (T. Raveh, S. Kahan-Reef, and A. Kimchi, unpublished observations). Intriguingly, ZIPk was shown to interact directly with Mdm2 and phosphorylated peptide derivatives of Mdm2 on Ser166 (57). Phosphorylation of the full-length protein, however, was extremely weak, and no kinetic parameters were presented to evaluate its efficiency. Thus, although p19ARF and Mdm2

---

**Type I (apoptotic) cell death:** involves caspase-mediated dismantling of the cell and nucleus into residual apoptotic bodies that are engulfed by neighboring cells.
THE DUAL NATURE OF AUTOPHAGY

The manner in which autophagy contributes to the overall death outcome is controversial. At times, both apoptotic and autophagic processes contribute to cell death and may even be regulated by the same signaling molecules (64, 80, 81); although with some signals, Type II cell death is only observed when the more predominant apoptotic pathway is blocked (82). Autophagy, however, is also used by the cell to maintain homeostasis. The controlled breakdown of cellular components provides an emergency nutrient supply during times of cell stress, and removal of damaged organelles, such as mitochondria with perturbed membrane potential, can fend off more serious damage (63, 83). In these conditions, inhibition of autophagy actually enhances apoptotic cell death (84–86). Thus, the accumulation of autophagic vesicles in any death scenario may be causative to cell lethality or may be a futile attempt at rescue. The dual nature of autophagy may, in fact, explain why the death-inducing DAPk has occasionally shown anti-apoptotic behavior (36, 37). It is unclear when and how autophagy shifts from its cytoprotective role to a “point of no return.” DAPk may be critical in this decision or may be an intrinsic component of the autophagic machinery in both circumstances.

At times, DAPk can be connected to the classical apoptotic route in a p53-independent manner. For example, treatment of p53-null Hep3B hepatoma cells with TGF-β led to induction of DAPk expression and subsequent mitochondrial-dependent apoptosis, which was significantly attenuated by DAPk inactivation (7). Ectopic expression of DAPk in these cells to levels similar to those induced by TGFβ triggered Type I apoptosis, including DNA fragmentation. Thus, several roads link DAPk to mitochondrial-based caspase activation processes.

In other cellular settings DAPk, DRP-1, and ZIPk induce an alternate type of programmed cell death, referred to as Type II or autophagic cell death, which occurs independently of caspases (5, 43). The most prominent feature of this type of cell death is the formation of double-membrane-enclosed, intracellular autophagic vesicles that consume organelles and other cytoplasmic components (63). In fact, overexpression of the DAPk family members leads, in some cells, to the appearance of autophagic vesicles, extensive cell rounding and membrane blebbing, nuclear condensation without DNA degradation, and no measurable loss of mitochondrial membrane potential or release of cytochrome c (5). Furthermore, knockdown of DAPk by antisense RNA and inactivation of DRP-1 function by use of a dominant-negative kinase have demonstrated that the two proteins are necessary for autophagy, induced either by IFN-γ in HeLa cells or by steroid withdrawal and amino acid starvation of MCF-7 cells, respectively (5). The involvement of DAPk in Type II cell death may explain why some studies failed to see DAPk’s death-inducing function; these studies relied on assays for activation of caspases and caspase-dependent apoptotic events, such as DNA fragmentation, and would have missed the autophagic component of DAPk expression (36).

The studies discussed in this section indicate that DAPk is capable of regulating both Type I apoptotic and Type II autophagic cell deaths, depending on the cell system and
specific death signal. The extent to which DAPk contributes to Type I death, especially, depends on other signaling pathways, such as p53. Thus, in Hep3B cells, Type I death induced by TGF-β, but not by UV irradiation, required functional DAPk (7). Other studies showed that the presence of DAPk was necessary for Type II death by IFN-γ but not for Type I apoptotic death by TNFα (5, 37).

Interestingly, it is now recognized that some classical apoptotic stimuli, such as etoposide and ceramide, can lead to autophagy (64, 65). DAPk may be particularly important for these signals. Considering this, it is important when analyzing DAPk-mediated death to assess the type of death with specific markers for individual death-associated events, such as autophagosome formation, mitochondrial depolarization and permeabilization, caspase activation, and DNA fragmentation, rather than rely on measurements of overall cell viability or morphologic changes that are common to both pathways.

Death-Related Effects on the Cytoskeleton

One of the most prominent features of DAPk family-induced death is the effect on the cytoskeleton. This is manifested in multiple ways, including cell rounding, loss of matrix attachment, and membrane blebbing. Expression of DAPk, DRP-1, or ZIPk leads to cell rounding and the formation of spherical membrane blebs at the cell surface, with ultimate loss of adherence to the matrix (1, 5, 8, 11, 13, 14). In more adherent cell lines, DAPk and ZIPk have been shown to induce the extrusion of lamellipodia-like protrusions and remodeling of actin stress fibers into ring-like structures at the periphery of the cell body and the base of the blebs (26), and can bind to recombinant GST-MLC in vitro (27). As stated above, ZIPk, under certain conditions, can localize to actin filaments (40, 41, 47), where it encounters myosin and leads to enhanced MLC phosphorylation (42). Significantly, expression of mouse DAPk in TNFα-treated MDCK cells led to enhanced MLC phosphorylation (42). Furthermore, in 293T, HeLa, and NIH3T3 cells, expression of DAPk alone was sufficient to phosphorylate endogenous MLC (36). DAPk- and ZIPk-mediated phosphorylation of MLC was not affected by the presence of Y27632, an inhibitor of the Rho-activated kinase (ROCK) (26, 47), indicating that MLC phosphorylation is not

Membrane blebbing is a common morphologic feature of apoptosis, brought about by increased myosin contractility, resulting from phosphorylation of MLC, in conjunction with weakening of the structural integrity of the cortical actin network owing to proteolysis of many of its components (66–68). Consistent with their high homology to MLCK, DAPk and ZIPk are capable of phosphorylating MLC in vitro, both in isolation and as part of the intact myosin II molecule (1, 12, 26, 36, 69, 70). For DAPk, this phosphorylation occurs mainly on Ser19, which is critical for myosin II activation, and to a lesser degree on Thr18 (26, 27, 36), whereas ZIPk phosphorylates both sites efficiently (69, 70). DRP-1 also possesses MLC kinase activity, although this has so far been demonstrated only in vitro with isolated myosin light chain (13, 14).

Importantly, both DAPk and ZIPk are well positioned within the cell to encounter MLC as a substrate. DAPk, in particular, is found in close proximity to myosin within the blebbing cell, including a ring-like structure at the periphery of the cell body and the base of the blebs (26), and can bind to recombinant GST-MLC in vitro (27). As stated above, ZIPk, under certain conditions, can localize to actin filaments (40, 41, 47), where it encounters myosin and leads to enhanced MLC phosphorylation (42). Significantly, expression of mouse DAPk in TNFα-treated MDCK cells led to enhanced phosphorylation of endogenous MLC (36). Furthermore, in 293T, HeLa, and NIH3T3 cells, expression of DAPk alone was sufficient to phosphorylate endogenous MLC, whereas expression of ZIPk in HeLa cells induced its diphosphorylation, implying that both DAPk and ZIPk are capable of phosphorylating the light chain in vivo (26, 27, 36). DAPk- and ZIPk-mediated phosphorylation of MLC was not affected by the presence of Y27632, an inhibitor of the Rho-activated kinase (ROCK) (26, 47), indicating that MLC phosphorylation is not
an indirect result of activation of ROCK by these kinases. At least in the case of ZIPk, the morphologic changes and rearrangements of the actin cytoskeleton that accompany its overexpression were directly attributed to myosin phosphorylation because they were blocked by expression of a form of MLC that can not be phosphorylated (56). Conversely, expression of the dominant-negative DAPk K42A or siRNA to ZIPk led to reductions in the basal levels of MLC phosphorylation (27, 47), indicating that the endogenous kinases also phosphorylate myosin. Thus, although not excluding the existence of additional substrates, phosphorylation of MLC and activation of myosin at the stress fibers may account for the contractile forces that result in membrane blebbing and/or protrusion formation.

ZIPk, like ROCK, can also negatively regulate the myosin phosphatase responsible for reverting MLC to its inactive, dephosphorylated form. In smooth muscle, ZIPk (in its full-length or truncated form) interacts with and phosphorylates the myosin-binding subunit (MBS) MYPT1 of the smooth muscle myosin phosphatase (48, 49), although not nearly as efficiently as it phosphorylates MLC (70, 71). This phosphorylation, which occurs on Thr697, inactivates the phosphatase. ZIPk can also phosphorylate CPI-17, an inhibitor of the phosphatase whose function is enhanced by phosphorylation (72). Thus, ZIPk can lead to increased phosphorylation of MLC, either through direct phosphorylation on Ser19 and Thr18 or indirectly by inhibiting the activity of the myosin phosphatase. It should be noted, however, that in vivo the direct route may be more prominent because knockdown of ZIPk in NIH3T3 cells by siRNA, which effectively reduced MLC phosphorylation, had little effect on the phosphorylation of the MBS (47).

DAPk and ZIPk can now be added to a growing list of kinases that phosphorylate MLC, which includes MLCK and ROCK, both of which have been implicated in apoptotic membrane blebbing (66, 67, 73). From kinetic analysis alone, MLCK is the best candidate (\(K_{\text{m}}\), 5–12 \(\mu\)M; \(K_{\text{cat}}\), 21–62 s\(^{-1}\)). ZIPk, too, is an adequate MLC kinase, with a less effective \(K_{\text{cat}}\) (0.74 s\(^{-1}\)), but a lower \(K_{\text{m}}\) (1.3 \(\mu\)M) (56). DAPk, in contrast, is much less efficient at phosphorylating either the intact MLC or a peptide derived from the phosphorylation site (19, 34, 36). ROCK is also a relatively inefficient MLC kinase, and it is believed to exert its effects on MLC phosphorylation preferentially through phosphorylation of the myosin phosphatase (74). Yet, in vivo the cell may purposefully utilize the different kinetic properties of the various MLC kinases to achieve different contractile activities (75). For example, ROCK and MLCK were shown to have differential spatial roles in 3T3 cells, with both kinases directly phosphorylating MLC in different regions of the actin network (75). Furthermore, ZIPk is unique in that it efficiently produces diphosphorylated MLC (69, 70), which is differentially distributed within the cell and may have different effects on myosin assembly and contractility, compared to monophosphorylated MLC (47). In motile fibroblasts, ZIPk was shown to be responsible for a major part of the MLC diphosphorylation, particularly at central stress fibers, whereas ROCK activity appeared to be primarily directed at regulation of the myosin phosphatase (47). Furthermore, in vivo local concentrations of substrate, kinase, and other regulators/activators may influence overall kinetics. For example, phosphorylation of DAPk by ERK reduces DAPk's \(K_{\text{m}}\) toward MLC to a very respectable 10–17 \(\mu\)M (34). Thus, it remains plausible that DAPk and ZIPk act as direct MLC kinases in vivo.

In addition to its effects on myosin contraction, DAPk also affects adhesion of the cells to the extracellular matrix (ECM) (8). This may be a result of indirect changes in the contractile nature of the actin cytoskeleton or, perhaps, of direct phosphorylation of a novel substrate involved in adhesion. DAPk-expressing 293T, NIH3T3, or MCF10A mammary epithelial cells exhibited decreased adherence to fibronectin or...
laminin; conversely, the dominant-negative K42A mutant led to enhanced spreading and attachment (8). The loss of adherence was attributed to inhibition of integrin signaling; decreased Tyr phosphorylation of Fak and paxillin was observed upon DAPk expression, and the anti-adhesion effects could be blocked by forcibly activating β1 integrins (8). For cells that are dependent on ECM signaling for viability, DAPk's ability to interfere with integrin function may directly contribute to cell death. In this regard, the anti-adhesion property of DAPk correlated with its ability to induce an anoikis-like apoptotic death in NIH3T3 or MCF10A cells plated on fibronectin in the absence of serum and/or growth factors, and forced activation of integrin signaling attenuated DAPk's apoptotic-inducing properties in these cells (8). Conversely, for cells that are capable of anchorage-independent growth, DAPk's effects on integrin activity may not be a direct cause of death but may account for the reduced adhesion and cell rounding observed upon activation of DAPk (26).

Cross Talk with Other Kinases

In addition to the direct consequences of DAPk activity on cell morphology, DAPk can also regulate several kinase cascades that may impact cell viability. DAPk interacts with CaM-regulated kinase kinase (CaMKK)β and phosphorylates it on Ser511 with an impressive Km of 3 μM (76). Significantly, expression of the DAPk catalytic domain in neuroblastoma cells led to enhanced phosphorylation of endogenous CaMKK on Ser511. This phosphorylation site is located adjacent to the CaM regulatory domain, and preincubation with DAPk attenuated CaMKK's ability to undergo CaM-dependent autophosphorylation in vitro, which is important for activation of the kinase. Although this suggests a mechanism by which DAPk can inactivate CaMKK and, consequently, the survival pathways that lie downstream, there is no physiological evidence that DAPk regulates CaMKK signal transduction in vivo nor that such cross talk is critical to DAPk's cell-killing functions.

More substantial evidence supports a role for DAPk as a negative regulator of the pro-survival signals from the MAPK/ERK pathway. In addition to ERK's ability to phosphorylate and activate DAPk, DAPk can exert its influence on ERK by promoting its cytoplasmic retention, through some unknown mechanism, effectively sequestering it away from nuclear substrates that may be critical for cell survival (34). In this manner, DAPk and ERK cooperate in a feedback mechanism whereby ERK activates DAPk, which then promotes death by shutting down the ERK survival pathway. As an example, phorbol-12-myristate-13-acetate (PMA) treatment of D2 erythroblasts in suspension, but not adherent cells, led to cell death. In the nonadherent population, the DAPk/ERK interaction predominated, resulting in cytoplasmic accumulation of ERK, whereas DAPk and ERK did not interact to any appreciable degree in the adherent cells. It is not known what secondary signals, such as those generated by cell detachment, promote the DAPk/ERK interaction. Furthermore, it remains to be determined whether inactivation of the ERK survival pathway directly contributes to DAPk's death effects or whether it serves as a means to silence the opposition so that DAPk can execute the cell through direct phosphorylation of its substrates in the various functional arms described above.

Nonapoptotic Functions

Until now, DAPk and its relatives have been presented as death-promoting kinases. However, events such as membrane blebbing and loss of adhesion, although often associated with death, are not restricted to dead-end paths. For example, blebbing can occur in such diverse processes as mitosis, cell spreading, and differentiation of primary neurons (77). Furthermore, DAPk's ability to mediate MLC phosphorylation may impact additional myosin-dependent events, such as...
cytokinesis. In support of this, DAPk-expressing MEFs and D122 Lewis lung carcinoma cells that did not die exhibited defects in cytokinesis, which resulted in multinucleation (4, 78). Also, exogenous ZIPk colocalized with phosphorylated MLC to the contractile ring during cytokinesis (45). Enhanced myosin contractility due to phosphorylation of MLC by DAPk was also shown to affect stress fiber formation. Expression of DAPk in serum-starved NIH3T3 cells promoted stress fiber formation, blocking the dissolution of stress fibers that occurs in response to serum starvation (27). This was attributed to MLC phosphorylation, as an inhibitor of myosin, and a MLC mutated at Ser19 blocked this phenotype. Conversely, DAPk was shown to be necessary for serum-induced stress fiber formation in NIH3T3 cells, and increases in DAPk catalytic activity were observed following serum stimulation (27). Similarly, depletion of ZIPk by siRNA in NIH3T3 fibroblasts perturbed stress fiber formation and also led to a reduction in focal adhesions (47). This actually contrasted with DAPk, whose overexpression in serum-stimulated cells, rather than depletion, led to focal adhesion disassembly (27). The combination of these effects on contractility, stress fibers, and adhesion may also influence cell migration. Fibroblasts with reduced ZIPk expression exhibited impaired migration toward a chemotactic factor on fibronectin-coated surfaces, a phenotype attributed to the altered stress fiber structure resulting from decreased myosin phosphorylation (47). Thus, the consequences of DAPk/ZIPk's cytoskeletal activities are important in a more global sense, and the DAPk family members may display more general cytoskeletal phenomena, some of which are specifically recruited during cell death.

In this regard, the DAPk family has been shown to phosphorylate substrates that are not a priori linked to apoptosis. DAPk interacts via its C terminus with syntaxin-1A, a component of the SNARE complex that mediates docking and fusion of synaptic vesicles with the plasma membrane (51). Significantly, DAPk phosphorylates syntaxin-1A in a Ca\(^{2+}\)–dependent manner on Ser188. Identification of a nonapoptotic substrate involved in neuronal synaptic transmission may account for DAPk's abundance in adult hippocampal neurons. Phosphorylated syntaxin-1A had reduced binding to Munc18-1, a regulator that prevents formation of the SNARE complex (51). Although this could be a possible mechanism by which DAPk regulates vesicle docking or fusion, no effect on the assembly or stability of the SNARE complex was seen, and no functional relevance of this phosphorylation was demonstrated. However, an RNA-interference-based screen of the human kinome for regulators of endocytosis identified DAPk and DRP-1 as kinases that were necessary for clathrin-mediated endocytosis (79). Knockdown of either gene resulted in the accumulation of early/late endosomes underneath the cell membrane. At the same time, caveolae-mediated and lipid raft-mediated endocytosis was enhanced. This study functionally links DAPk to vesicular trafficking, although it does not address the mechanism or specific stage involved. The two studies together suggest that DAPk may be a general regulator of vesicle fusion. Furthermore, under the right circumstances, DAPk's ability to modulate membrane trafficking may be recruited to mediate related death processes such as autophagy, which involves numerous membrane fusion events, from the starting point when membrane components are recruited to form vesicles to the final stages in which early autophagosomes fuse with lysosomes (63). Thus, the physical and functional interactions of DAPk with components of the endocytotic and exocytotic pathways may be an enlightening line of research into multiple DAPk functions.

The DAPk Signaling Network, an Overview

Although gaps remain in our understanding of the DAPk family signaling network, and
certain models await further verification, a schematic map can now be assembled, which includes both upstream regulators and functional arms that emanate from the active kinases (Figure 2). For simplicity’s sake, we will refer to DAPk as the representative of the family. DAPk can be activated by several mechanisms (Figure 2a), including binding of Ca\(^{2+}\)–activated CaM, phosphorylation of Ser735 by ERK, and dephosphorylation of Ser308 by an unknown phosphatase, which can be activated by several death signals. Other triggers lead to increases in DAPk expression through p53- and/or Smad-mediated transcriptional upregulation of the DAPk gene. Once activated, DAPk can trigger a range of death responses leading to multiple phenotypes (Figure 2b, I–V). DAPk predominantly triggers the formation of autophagic vesicles, which results in a slow Type II death (I). The substrates responsible for this phenotype are unknown, although syntaxin or other related proteins that are involved in vesicle fusion are possible candidates. Autophagy can be accompanied by cytoskeletal rearrangements and global contractility (II), most likely caused by direct phosphorylation of cytoskeletal substrates, including, but not necessarily restricted to, MLC. Through these contractile changes, and/or phosphorylation of unknown substrates, DAPk inactivates integrins and blocks their signaling, leading to decreased adhesion to cell matrix and, consequently, cell rounding (III). In the proper signaling context, alternate pathways can also be initiated. For example, in cells where ECM provides an essential survival signal and p53 is present, inhibition of integrins can additionally lead to anoikis, a caspase-dependent apoptotic pathway initiated by loss of matrix adherence (IV). p53 also serves to link DAPk to the caspase-dependent, mitochondrial-based death pathway through the p19ARF regulatory loop, although the identity of the exact in vivo substrate in this pathway is not yet known. Once activated, p53 triggers a caspase-dependent Type I death process involving the hallmarks of apoptosis, such as DNA degradation, and cellular fragmentation, as well as membrane blebbing and cell rounding (V). This pathway can also be activated by DAPk independently of p53. In addition, DAPk can potentially promote death by shutting off survival pathways that function in parallel, such as those activated by ERK and CaMKK, the former through cytoplasmic retention of ERK, and the latter through direct phosphorylation of CaMKK. The choice of which of these pathways will be followed will ultimately depend on the cell context, the initial substrates that DAPk encounters, and the presence or abundance of factors that interact with or modulate these substrates and the signaling pathways that they regulate. Certainly, many additional layers of cross interaction among the functional arms are still to be uncovered.

In addition to the multiple signaling pathways emanating from DAPk, an additional level of complexity arises from the presence of multiple family members. There seems to be cross talk among the family members because DAPk, DRP-1, and ZIPk are all capable of interacting through their respective kinase domains and because DAPk can trans phosphorlylate ZIPk (43; G. Shani, L. Marash, H. Berissi, and A. Kimchi, unpublished data). Furthermore, the levels of killing achieved by coexpression of low, nonfatal quantities of DAPk and WT ZIPk, but not the nonphosphorylatable ZIPk mutant, were much greater than the additive effect of expressing either one alone, indicating that the two synergize to induce cell killing (43). This suggests a hierarchical relationship among the kinases, with perhaps the most downstream kinase, ZIPk, acting as the effector kinase. Alternatively, the family members may collaborate in parallel to activate a common pathway, mediated by phosphorylation of shared substrates. In this case, the cross talk between DAPk and ZIPk may reflect an internal amplification loop within the overall network to ensure maximal phosphorylation of the common
Figure 2

(a) Regulation of DAPk. DAPk is regulated by multiple signals, either at the level of transcription or at the protein level. Transcriptional activators include Smad and p53, which are each activated by death signals. Other signals lead to changes in the phosphorylation status of Ser308 and Ser735 or to increases in Ca\textsuperscript{2+}, which enables binding of CaM, serving to activate DAPk’s catalytic activity. (b) The DAPk death signaling network. DAPk regulates numerous functional arms through direct phosphorylation of multiple substrates (blue arrows). These pathways result in different phenotypes (red arrows, I–V), culminating in cell death. Additional signaling events (yellow arrows) and survival pathways (green arrows) are indicated. Caspases can also contribute to the membrane blebbing and cell rounding phenotypes through cleavage of cytoskeletal proteins and activation of the MLC kinase, ROCK. See the text for further details.
substrates. A third possibility is that each kinase phosphorylates a unique set of substrates, leading to different cellular outcomes, with coordinated activity among the three kinases to ensure the complete global phenotype. These issues, as well as the complete in vivo substrate profile of each kinase, still await resolution.

**SUMMARY POINTS**

1. DAPk shares structural and functional homology with a family of death-related kinases, including DRP-1 and ZIPk.
2. The DAPk family members regulate both Type II autophagic cell death and Type I apoptotic death, depending on the cellular setting.
3. DAPk activity leads to multiple phenotypes, including membrane blebbing, autophagosome formation, and loss of adhesion, which results from phosphorylation of specific substrates, such as MLC.
4. Functions and substrates unrelated to cell death have also been identified, underscoring the diversity of the functional arms that this kinase family mediates.

**FUTURE ISSUES TO BE RESOLVED**

1. Elucidation of the complete substrate profiles and specificities for DAPk, DRP-1, and ZIPk is needed. Although attempts have been made to predict DAPk substrates using a positional-scanning peptide library (19), the optimal sequence obtained actually does not match the phosphorylation sites of some of the known DAPk substrates, and thus this method is a less than adequate tool. New approaches to substrate identification are necessary, and these need to be followed by rigorous assessment of the functional consequences of substrate phosphorylation.
2. The cross talk among the DAPk family members requires further elucidation, as does the assignment of specific roles to each kinase.
3. It is essential to develop methods of gene deletion and/or knockdown, individually and in combination, in order to assess the function and regulation of the endogenous kinases and their substrates in vivo.
4. An understanding of how each kinase contributes to the development of diseases such as cancer and neurodegeneration is necessary. Although this has been the subject of much interest for DAPk, the contribution of DRP-1 and ZIPk to tumorigenesis, or to other pathological disorders, is not known, and analysis of epigenetic modifications of their loci has not yet been attempted.

**ACKNOWLEDGMENTS**

This work was supported by grants from the European Union (LSHB-CT-2004-511983) and by the Center of Excellence grant from the Flight Attendant Medical Research Institute (FAMRI). A.K. is the incumbent of Helena Rubinstein Chair of Cancer Research.
LITERATURE CITED
Contents

Wanderings of a DNA Enzymologist: From DNA Polymerase to Viral Latency
I. Robert Lehman ................................................................. 1

Signaling Pathways in Skeletal Muscle Remodeling
Rhonda Bassel-Duby and Eric N. Olson ........................................ 19

Biosynthesis and Assembly of Capsular Polysaccharides in Escherichia coli
Chris Whitfield ................................................................. 39

Energy Converting NADH:Quinone Oxidoreductase (Complex I)
Ulrich Brandt ................................................................. 69

Tyrphostins and Other Tyrosine Kinase Inhibitors
Alexander Levitzki and Eyal Misbani ........................................ 93

Break-Induced Replication and Recombinational Telomere Elongation
in Yeast
Michael J. McEachern and James E. Haber .................................. 111

LKB1-Dependent Signaling Pathways
Dario R. Alessi, Kei Sakamoto, and Jose R. Bayascas ...................... 137

Energy Transduction: Proton Transfer Through the Respiratory Complexes
Jonathan P. Hosler, Shelagh Ferguson-Miller, and Denise A. Mills ............ 165

The Death-Associated Protein Kinases: Structure, Function, and Beyond
Shani Bialik and Adi Kimchi .............................................. 189

Mechanisms for Chromosome and Plasmid Segregation
Santanu Kumar Ghosh, Sujata Hajra, Andrew Paek,
and Makkuni Jayaram ......................................................... 211

Chromatin Modifications by Methylation and Ubiquitination:
Implications in the Regulation of Gene Expression
Ali Shilatifard ................................................................. 243
<table>
<thead>
<tr>
<th>Title</th>
<th>Authors</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure and Mechanism of the Hsp90 Molecular Chaperone Machinery</td>
<td>Laurence H. Pearl and Christos Prodromou</td>
<td>271</td>
</tr>
<tr>
<td>Biochemistry of Mammalian Peroxisomes Revisited</td>
<td>Ronald J.A. Wanders and Hans R. Waterham</td>
<td>295</td>
</tr>
<tr>
<td>Protein Misfolding, Functional Amyloid, and Human Disease</td>
<td>Fabrizio Chiti and Christopher M. Dobson</td>
<td>333</td>
</tr>
<tr>
<td>Obesity-Related Derangements in Metabolic Regulation</td>
<td>Deborah M. Muoio and Christopher B. Newgard</td>
<td>367</td>
</tr>
<tr>
<td>Cold-Adapted Enzymes</td>
<td>Khawar Sobail Siddiqui and Ricardo Cavicchioli</td>
<td>403</td>
</tr>
<tr>
<td>The Biochemistry of Sirtuins</td>
<td>Anthony A. Sauve, Cynthia Wolberger, Vern L. Schramm, and Jef D. Boeke</td>
<td>435</td>
</tr>
<tr>
<td>Dynamic Filaments of the Bacterial Cytoskeleton</td>
<td>Katharine A. Michie and Jan Löwe</td>
<td>467</td>
</tr>
<tr>
<td>The Structure and Function of Telomerase Reverse Transcriptase</td>
<td>Chantal Autexier and Neal F. Lue</td>
<td>493</td>
</tr>
<tr>
<td>Relating Protein Motion to Catalysis</td>
<td>Sharon Hammes-Schiffer and Stephen J. Benkovic</td>
<td>519</td>
</tr>
<tr>
<td>Animal Cytokinesis: From Parts List to Mechanisms</td>
<td>Ulrike S. Eggert, Timothy J. Mitchison, and Christine M. Field</td>
<td>543</td>
</tr>
<tr>
<td>Mechanisms of Site-Specific Recombination</td>
<td>Nigel D.F. Grindley, Katrine L. Whiteson, and Phoebe A. Rice</td>
<td>567</td>
</tr>
<tr>
<td>Axonal Transport and Alzheimer’s Disease</td>
<td>Gorazd B. Stokin and Lawrence S.B. Goldstein</td>
<td>607</td>
</tr>
<tr>
<td>Asparagine Synthetase Chemotherapy</td>
<td>Nigel G.J. Richards and Michael S. Kilberg</td>
<td>629</td>
</tr>
<tr>
<td>Domains, Motifs, and Scaffolds: The Role of Modular Interactions in</td>
<td>Roby P. Bhattacharyya, Attila Reményi, Brian J. Yeh, and Wendell A. Lim</td>
<td>655</td>
</tr>
<tr>
<td>Ribonucleotide Reductases</td>
<td>Pär Nordlund and Peter Reichard</td>
<td>681</td>
</tr>
<tr>
<td>Introduction to the Membrane Protein Reviews: The Interplay of</td>
<td>Jonathan N. Sachs and Donald M. Engelman</td>
<td>707</td>
</tr>
<tr>
<td>Structure, Dynamics, and Environment in Membrane Protein Function</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Relations Between Structure and Function of the Mitochondrial ADP/ATP Carrier
H. Nury, C. Dahout-Gonzalez, V. Trézéguet, G. J. M. Lauquin, G. Brandolin, and E. Pébay-Peyroula ................................................................. 713

G Protein–Coupled Receptor Rhodopsin
Krzysztof Palczewski .................................................................................... 743

Transmembrane Traffic in the Cytochrome $b_{6}f$ Complex
William A. Cramer, Huamin Zhang, Jiusheng Yan, Genji Kurisu,
and Janet L. Smith ...................................................................................... 769

INDEXES

Subject Index ............................................................................................. 791

Author Index ............................................................................................ 825

ERRATA

An online log of corrections to Annual Review of Biochemistry chapters (if any, 1977 to the present) may be found at http://biochem.annualreviews.org/errata.shtml