Programmed cell death: From novel gene discovery to studies on network connectivity and emerging biomedical implications

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1. Introduction

During the early 1990s it was believed that programmed cell death is mainly driven by proteolytic cascades activated and executed by members of the caspase family of proteases. The caspases were mapped along two major pathways, the so called ‘extrinsic’ and the mitochondrial-based ‘intrinsic’ pathways. This simple view was based on the initial genetic screens in *C. elegans* and the subsequent intensive search for the corresponding mammalian orthologues and interacting proteins. Yet, being aware of the phenotypic complexity of the death process in mammals, we hypothesized many years ago that the molecular basis of programmed cell death may extend beyond such simplistic linear pathways and a more complicated network model should be constructed. To address this working hypothesis, which was against the consensus at that time, we embarked upon an ambitious direction aimed at performing high throughput genome wide screens in mammalian cell cultures exposed in vitro to a death signal (e.g., a killing cytokine; interferon-γ was finally chosen due to several technical advantages). To this end, we first developed a strategy of random knock down of gene expression (TKO) using anti-sense cDNA libraries carried by episomal vectors ([1,2]; reviewed by [3,4]). We assumed that the selective reduction in the expression of a gene which positively contributes to the final system performance should attenuate the cell death responses in cells which are continuously exposed to the initial death signal, and thus should provide a forward selection to rescue the relevant genes. In retrospect, the large number of novel death promoting genes that we identified by this function-based strategy confirmed our initial hypothesis on the network’s complexity. In addition, the technique was elaborated in a way that we could eventually select individual anti-sense cDNA fragments which displayed different degrees of death protection, suggesting for the first time that different components of the death machinery differ in their impact on system performance (i.e., differ in their ‘functional weight’—a theme which was first discussed in [5]).

We pioneered this function-based genetic screen in a very successful manner as detailed below. Later on the technology was adapted by many research groups and currently it provides one of the most powerful strategies in the developing field of ‘functional genomics’ in mammalian cells (reviewed by [4]; see also [6]). In recent years, once small interfering RNAs were introduced into mammalian cell studies, functional screens with siRNA or shRNA whole genome libraries also started to be conducted based on the same main TKO principles discussed above. Here I wish to detail step by step how the use of this high throughput function-based approach shaped our global view on the composition, topology and performance of molecular networks moving from the initial gene discovery stage towards global views of network’s performance. Based on this work, novel targets for therapeutic intervention in pathologies associated with accelerated cell death and new cancer related prognostic tools have been and will be continued to be discovered as detailed below (reviewed by [7,8]).
2. DAP gene discovery

As predicted by our initial working hypothesis, the repertoire of genes that were selected by our screen was very large and included many new components of the cell death machinery which were not discovered by the C. elegans screens. This means that the molecular basis of cell death is larger than initially thought. Most of the death protective anti-sense cDNA fragments which we isolated knocked down the expression of genes which were unknown at that time and therefore we named them death associated proteins (DAPs). The first round of selection yielded five novel DAP genes [2], and the second round added six more candidates. Additionally, two known genes were rescued by this genetic screen as well, i.e., thio-redoxin [1] and the cathepsin D lysosomal protease [9], thus attributing new functions to known genes. Of note, our seminal paper on cathepsin D, was among the first studies which initiated the direction of causal roles of lysosomal proteases in programmed cells death. DAP genes were thoroughly characterized over the years at the structural and functional levels. The following reviews summarize the detailed information coming from many laboratories including ours on the different DAP genes [7,8,10–15]. Here, the major characteristics of the DAP proteins which we discovered over the years are briefly discussed in the context of our global view of the cell death network.

DAP-kinase (DAPk), a Ca2+/calmodulin (CaM) regulated, Ser/Thr kinase, is one of the most extensively studied proteins isolated by this screen. We determined how DAPk remains silent in healthy cells and becomes activated by various death signals. We identified in this respect a single inhibitory event of auto-phosphorylation, mapped into the calmodulin binding segment of the kinase, and showed that dephosphorylation of this site was necessary for the activation of the kinase concomitant with the increase in cellular calcium concentrations [16,17]. We investigated in details the various functional domains which dictate the biochemical mode of action of this kinase including the death domain at the C-term inus which mediates interaction with other proteins [18–23]. In parallel, we investigated the signaling pathways activated downstream of DAPk that lead to cell death. To this end, we focused on identifying DAP-kinase substrates and interacting proteins that form part of the multiple functional arms of this kinase (see also Section 4). The first identified DAPk substrate was the myosin II regulatory light chain, phosphorylation of which mediates the process of membrane blebbing [18,19,24]. In another cellular setting, initiated in primary fibroblasts by activated oncogenes, a link of DAPk to p19ARF was discovered [25]. Subsequent work illustrated that at least two pathways emanate from p19ARF one resulting in apoptotic cell death and the second in autophagic cell death. The first involves the nucleolar p19ARF isoform which activates p53 via Mdm2 binding while the second pathway is dependent on yet another new isoform of p19ARF which is excluded from the nucleolar compartment of the cell. This novel small isoform of p19ARF, named smARF, is produced by internal initiation of translation, localizes to the mitochondria, and disrupts mitochondrial membrane potential, ultimately leading to caspase-independent autophagic cell death [26–28].

The discovery of DAPk was followed by the identification of four additional closely related death-promoting kinases, named ZIPk, DRP-1, DRAK-1 and DRAK-2 thus establishing the existence of a new family of death kinases [29]. The homology between the members resides in the catalytic domains while they differ substantially in their extra-catalytic features. Among the common features, a unique highly ordered basic loop was identified, located at the surface of the upper lobe of the catalytic domain, named the ‘fingerprint’ of the DAPk family. This basic loop mediates heterodimerization between DAPk and ZIPk, which results in trans-phosphorylation and subsequent functional activation of ZIPk [30]. In addition, the basic loop enables the homodimerization of DRP-1 (unpublished results), which is also critical for activation [31]. Thus, members of the DAP-kinase family generate multi-protein complexes in which a direct binding through their catalytic domains is a critical step, generating feed forward amplification loops by trans phosphorylations [30].

In addition to DAPk and its family members, many efforts were invested in our laboratory on the other DAP genes. We found that DAP5 is a translation initiation factor that directs IRES-dependent translation under stress conditions when cap-dependent translation is compromised [5,32,33]. Several cellular IRESes that are DAP5 targets were identified in our laboratory including Apaf-1, c-myc, and XIAP. Analysis of the entire profile of DAP5 targets indicated that by changing the relative steady state levels of critical nodes in the network, the DAP5 protein can either stimulate or inhibit the process of cell death (reviewed by [12]). Other DAP genes include DAP1, a small basic protein that binds to the ER, and DAP4 which is a PKR activator modulating protein translation through eIF2-α phosphorylation. DAP3, turned out to be a GTP-binding protein which is localized in the mitochondrial matrix and is not released to the cytosol during cell death. We found that DAP3 impairs the mitochondrial morphology by increasing the fission process [10,34–36].

3. Clinical implications in cancer

Obviously, one of the challenges in the study of the DAP genes was to find out whether one or more of these pro-cell death genes is a potential tumor suppressor subjected to loss or inactivation in cancer. To this end, several independent directions were undertaken in our laboratory over the past years, including experimental systems which assess the effects of loss of DAPk on tumorigenesis, as well as initial screens of human tumors (reviewed in [7,14,37]). The
experimental approaches that initiated this research direction were performed in two different systems at different time periods in the laboratory. One was in primary cell cultures and the second on mouse model systems. They established the notion that DAPK functions as a tumor suppressor in at least two different stages of cancer development. That is, it operates in an apoptotic checkpoint functioning early during oncogenic cell transformation [25], and in a second one that occurs later at the metastatic stage [38]. The anti-oncogenic effects of DAPK were documented in cultures of mouse embryonic primary fibroblasts (wt and DAPK−/−) transformed by various oncogenes [25] and the anti-metastatic properties were established in mouse model systems of lung metastasis [38]. Early examination of human tumor cell lines showed that DAPK expression is lost at high frequency by promoter silencing through DNA methylation [39,40]. Altogether this seminal work prompted many groups to assess the status of DAPK in human tumors. A comprehensive screen of human tumor specimens was conducted in many cancer centers and correlations were made with respect to the staging and aggressiveness of the disease in B cell lymphomas, as well as many types of solid carcinomas and sarcomas. As a result, in the past 4–5 years alone, over 150 articles have been published on the loss of DAPK expression in human cancer as we summarized in details in a few reviews [7,13,14]. These clinical data indicate that DAPK promoter methylation/gene mutations serve as prognostic tools in cancer development and that attempts should be developed at restoring gene expression/protein function, where possible, for cancer therapy.

4. Implications in pathological cell death: designing novel death protective drugs based on inhibitors of DAP-kinase

The other side of the coin concerning DAPK’s structure/function analysis relates to its hyper-activation or gain of function abnormalities, which might lead to excessive cell death in some human pathologies associated with cell loss. Of special interest is the DAPK involvement in neuronal cell death. Mature neurons in adult organisms undergo cell death in response to a variety of stress conditions, including lack of neurotrophic factors, anoxia, excitotoxicity, traumatic injury and neurodegenerative disorders. Obviously, identifying the relevant genes carrying the causal killing effects provides new targets for drug design. Recent information about the activation of DAPK in hippocampal neurons by various stress signals and the determination of its participation in multiple death processes, raised the possibility of the kinase’s involvement in pathologies of the nervous system [7,23,41]. Furthermore, the advanced understanding of the protein’s structure and mode of action that has been achieved to date enabled the design of specific inhibitors of DAPK which may serve as candidates for a potential clinical use. We characterized the C-terminal end of DAP-kinase as being inhibitory to the death promoting function of DAPK [21]. To this end, a peptide corresponding to the 17-amino acid tail region of DAPK was synthesized and covalently linked at its N-terminus to tetramethylrhodamine isothiocyanate, in order to increase the hydrophobicity of the peptide, and to enable its detection upon uptake into cells [41]. Significantly, administration of micromolar concentrations of the labeled peptide to the culture media partially protected primary hippocampal neurons from cell death induced by C6-ceramide or by expression of sphingomyelinase. In fact, the reduced sensitivity of hippocampal neurons to various death signals perfectly resembled the behavior of DAPK deficient neurons [41]. This encouraging data suggests that the tail peptide can act as a starting point for optimization of a peptide-based therapy, or of mimicking molecules, to generate specific inhibitors of DAPK that can be useful for in vivo delivery.

5. Network characteristics of programmed cell death that emerged from DAP gene discovery

Programmed cell death displays several cellular phenotypes affecting various intracellular organelles, membranes, and nuclei. For example, the well characterized chromatin condensation, nuclear and cytoplasmic fragmentations, membrane blebbing, and mitochondrial membrane permeabilization—are part of what is classified as type I apoptotic cell death. The autophagosome and autolysosome formation, and mitochondrial fragmentation followed by engulfment by autophagosomes are characteristics of type II autophagic cell death. Along these lines, the novel DAP proteins were assigned to one or more of these sub-cellular events after being characterized at the molecular level. For example, DAP3 was assigned to the process of mitochondrial fragmentation [36]. DAPK was assigned to several phenotypic outcomes. The latter include membrane blebbing [24], formation of autophagosomes [42], and a link to cytoplasmic/nuclear fragmentations that occurs in the previously mentioned primary embryonic fibroblasts in which the nucleolar p19ARF and p53 are activated [25]. We found that these different phenotypic outcomes result from multiple signaling pathways initiated by DAPK, each involving different sets of substrates and interacting proteins. The multiple ‘functional arms’ defines DAPK as a molecular switch capable of driving the phenotypic outcome into different directions (reviewed by [8]). Thus, our study provides a well studied example where a single pro-death gene can be wired to various sub-cellular changes. Recently by performing high throughput silencing perturbations we characterized the conditions in which this molecular switch can take place (Zalcikvar and Kimchi, unpublished data). This causes an unpredicted paradigm of ‘system plasticity’ which contributes to the robustness of the system—i.e., the capability to shift from one type of cell death to another, e.g., from type I apoptotic cell death to type II autophagic cell death.
The differential contribution of each DAP gene to the final system performance was already observed at the early stages of our TKO selection procedure where the knock down of DAP1, DAPk, DAP3 or cathepsin D reduced the system performance by a factor which was at least 100 higher than DAP4 or DAP5 knock down [5].

Finally it should be stressed again that although the DAP genes were each rate limiting in global network performance (the property which served as the basis for their selection), they differed substantially in their biochemical properties and intracellular localization. Thus, each of the well characterized DAP genes is currently defined as part of a different functional module within the death signaling network and their ultimate connectivity occurs at the level of inter-module interaction. (A module is defined here on a functional basis—i.e., as a set of close molecular interactions which display high degree of connectivity). The translational module in which DAP5 participates is the best studied example in this respect. The DAP5 translation factor promotes during cell death the cap-independent translation of several proteins, including Apaf-1 [33]. Thus, by changing the relative steady state levels of some critical nodes in defined modules (which obviously display their own intramodular connectivity), the impact of the translation module is further manifested. For example, the activation of Apaf-1 within its module depends on binding to cytochrome C which eventually leads to caspase activation. Yet, in parallel, the cap-independent increase in Apaf-1 translation by DAP5 continues to feed into the system this critical protein reflecting the impact that such inter-modular interactions impose on final system performance. We also found that the inter-module connectivity can occur at the reciprocal direction as well, since DAP5 protein is activated by caspase cleavage thus creating a feed back positive loop between modules [32]. Thus, DAP5 lies at a critical junction of inter-module connectivity within the cell death network.

6. Conclusions and perspectives

In conclusion, our genome wide genetic screens added a collection of new genes and proteins to the field of programmed cell death resulting in the establishment of novel mechanisms and unexpected concepts. The initial set of anti-sense cDNA fragments, which corresponded to unknown genes when they were functionally selected, led to the characterization of the DAP proteins at the structural, biochemical, and cellular levels. This was followed by wiring the DAP proteins to their upstream and downstream modulators/effectors to construct new pathways within the various functional modules and their assignment to the phenotypic outcome. Next, we analyzed the contribution of inter-module connectivity to the final network performance which represents another level of complexity to be considered when analyzing the composition, topology and performance of molecular networks. Altogether, our findings highlight the unusually high robustness of the cell death machinery and its ability to switch into alternative routes when necessary.

The biomedical implications of our study emerge from both the gene discovery itself and the novel concepts that were extracted from our study on the network’s connectivity. The identification of DAP-kinase as a tumor suppressor gene, the expression of which is lost in a variety of human tumors, became a strong prognostic tool commonly used in many cancer centers to assess the aggressiveness of the cancer disease. A PCR-based assay was developed in this respect that measures the specific methylation of the DAPk promoter. The role of DAPk in the death of neurons established additional milestones in studying its clinical relevance as a target for interruption in various types of pathological cell death. Inhibitors of DAP-kinase were designed in our laboratory, as well as in other laboratories, to be used in pathological conditions where accelerated cell death takes place, especially in the nerve system. Our broader understanding of the cell death network brought us to the concept that a combinatorial approach of several drugs, hitting different nodes (proteins) in the various network’s modules, should be designed in attempting to negate cell death in ischemia, stroke or neurodegenerative diseases. The targets should be chosen in a smart way which is based on our progressive understanding of network’s connectivity and special emphasis should be put on preventing switches from one module to another. Conversely, efficient killing of cancer cells by chemo-therapeutic drugs should be also conducted by educated simulations which take in account the routes in the network which are still functioning in the tumor cells and by choosing the right drug combination capable of turning on these pathways and getting the maximal efficiency of cell killing.

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Adi Kimchi is a full professor at the Weizmann Institute in the Department of Molecular Genetics which she chaired over the last six years. Her group has developed high throughput genetic screens in mammalian cell cultures, which were successfully used for the isolation of pro-death genes. This led to the discovery of a group of novel death-promoting genes (the DAP genes) and through their study to the establishment of mechanistic views on autophagic cell death and...
on switches between different forms of cell death. Kimchi is a member of EMBO, and was a member of the Council for Higher Education in Israel between 1996 and 2003. Kimchi was the Director of the Leo and Julia Forchheimer Center for Molecular Genetics between 2001 and 2007. She currently serves in many institutional, national and international scientific committees. Among her list of awards is the Milstein Award for Excellency in Cytokine Research (1999), the Landau Award for Excellency in Biology and Biotechnology (1999), the Seroussi Award for Cancer Research (2002), and the Lombroso Prize for Cancer Research (2006).