Introduction

1.1. Cell death

Upon a lethal stimulus, a cell can die in different ways that can be classified according to their nuclear morphology as it has now become clear that a cell can not only die from apoptosis (as defined by Kerr *et al.*, 1972) or necrosis; several models have been proposed to define the process of cell death.

Necrosis is the premature death of cells and living tissues. It is an unordered and accidental form of cell death. Cellular necrosis can be induced by a number of sources, including injury, infection, cancer, infarction, poisons, and inflammation. It is a severe form of cell death resulting from the additive effect of a number of independent biochemical events that are activated by severe depletion of cellular energy stores and thus is a passive phenomenon (Kanduc *et al.*, 2002). Necrosis typically begins with cell swelling, chromatin degradation, and disruption of the plasma membrane as well as organelle membranes. Late necrosis is characterized by extensive DNA fragmentation, organelle breakdown, and cell lysis. The release of intracellular content after plasma membrane rupture is the cause of inflammation in necrosis. Autophagy is characterized by sequestration of cytoplasm or organelles in autophagic vesicles and their subsequent degradation by the cell's own lysosomal system (Schweichel and Merker, 1973).

Apoptosis, one form of PCD (programmed cell death) is frequently viewed as the result of an explosive activation of the 'caspase cascade'. In this version, the regulation is a multi dimensional network of activatory chain reactions involving activators, inhibitors, and even inhibitors of inhibitors, much like the complement system. Nonetheless, it appears clear that apoptosis is not just synonymous of caspase activation. The idea that apoptosis can be initiated and sometimes executed by caspase independent processes has been challenged by the difficulty of outlining complete pathways in which each of the molecular actors is defined. Although we are still far from a complete view of the caspase-independent death-initiating machinery, an attempt has been made to understand this relatively new mechanism of apoptosis in a primary eukaryote *Dictyostelium discoideum* in this study. To avoid ambiguity in understanding 'apoptosis' term is used for classical caspase dependent apoptosis and 'paraptosis' for caspase independent apoptosis.

1.1.1. Classification based on morphology of different cell death processes

In apoptosis, chromatin condensation takes place into compact figures, which are often globular or crescent shaped. Apoptotic morphology further includes cytoplasmic condensation, membrane blebbing, and the formation of apoptotic bodies. Apoptosis is dependent on caspase 3 and caspase-activated DNAse. Paraptosis on the other hand is characterized by chromatin condensation that is less compact but which gives more complex and lumpy shapes and is caused by apoptosis inducing factor (AIF), endonuclease G, cathepsins, or other proteases. It involves cytoplasmic vacuolation, mitochondrial swelling in the absence of caspase activation or typical nuclear changes (Sperandio and Bredesen, 2000). Any degree or combination with other apoptotic features can be found.

Despite of the numerous models proposed to categorize cell death, exclusive definitions are difficult to make and are probably artificial due to the overlap and shared signaling pathways between the different death programs. It has been shown that apoptotic and necrotic death markers can concomitantly be present in the same cell after cerebral ischemia, indicating that more than one death program may be activated at the same time (Unal-Cevik *et al.*, 2004). In addition, a cell may switch back and forth between different death pathways.

1.1.2. Molecular mechanisms of apoptosis and paraptosis

Apoptosis has been studied extensively and two separate pathways leading to caspase activation: the intrinsic and the extrinsic pathways have been characterized (Green, 2000, Johnstone *et al.*, 2002). The extrinsic apoptotic pathway is activated *via* tumor necrosis factor alpha (TNF-α) superfamily death receptors (Almasan and Ashkenazi, 2003, Baines *et al.*, 2003), while the intrinsic apoptotic pathway involves mitochondria (Fig. 1.1).

The intrinsic apoptotic pathway and paraptosis share lot of similarity despite subtle variations with respect to the executioners of the cell death processes. The intrinsic pathway is initiated in mitochondria in response to different stimuli (Costantini *et al.*, 2000, Green and Reed, 1998, Kroemer, 2003) which include high levels of Ca^{2+}, reactive oxygen species-ROS, the activation of pro-apoptotic Bcl-2 family proteins (Keeble and Gilmore (Keeble and Gilmore, 2007, Kroemer *et al.*, 2007), UV damage (Denning *et al.*, 2002) etc.

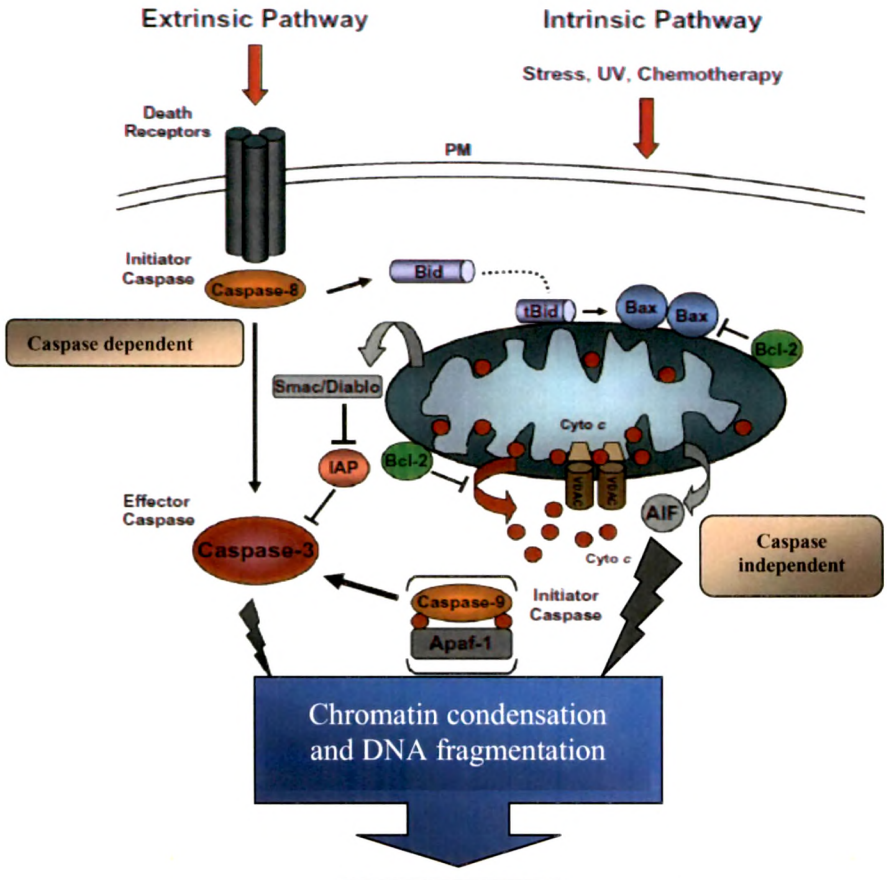

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Figure 1.1: PCD network- A simplified model of the two cell death signaling pathways converging to a common execution phase. In the extrinsic, the death receptor-mediated pathway, the death receptor at the plasma membrane (PM) is activated by specific ligands, leading to activation of initiator caspase-8 that activates the executioner caspase-3, which in turn, cleaves cellular substrates and brings about formation of apoptotic bodies. In the intrinsic, mitochondria mediated pathway (which could be caspase dependent/independent), multiple stimuli such as UV, DNA damage, oxidants, chemotherapies, Ca^{2+} overload and ceramide can trigger OMM (outer mitochondrial membrane) permeabilization, leading to the release of mitochondrial apoptogenic factors (Cytochrome c, AIF, Smac/Diablo) from the IMS (intermembrane space of mitochondria) into the cytosol. The released Cyto c and Apaf-1 form the apoptosome together with procaspase-9, triggering caspase-9 activation that cleaves effector caspase-3. Extrinsic pathway can activate the mitochondria mediated intrinsic pathway when caspase-8 cleaves Bid. Truncated Bid translocates to the mitochondria and activates the intrinsic pathway. The pro-apoptotic molecule, AIF, also released from the mitochondria, is cleaved by calpains and/or cathepsins and translocates to the nucleus, leading to chromatin condensation. Smac/Diablo that is also released from the mitochondria, interacts with Inhibitor of Apoptosis Proteins (IAPs)- inhibitors of caspases.

The extrinsic apoptotic pathway, however, can also induce activation of the intrinsic pathway, *via* caspase 8-dependent cleavage of the BH3-only Bcl-2 family protein member, Bid, with its truncated form (tBid) translocating to the mitochondria to activate the intrinsic apoptotic pathway (Berry and Boulton, 2000, Yin, 2000). Therefore, tBid links apoptotic signals initiated by death receptors to mitochondria-mediated apoptosis. Also as is discussed in section 1.1.3, lysosomes play role in triggering the intrinsic mitochondrial pathway.

1.1.3. Selective permeabilization of organelle membranes during cell death

During apoptosis and paraptosis, the strict compartmentalization of potentially lethal proteins such as cytochrome c, cathepsins, and AIF is selectively disrupted. As suggested by Bidere *et al.*, (2003) activated T cells treated for a short period with staurosporine exhibit a major change in lysosomal function. Cathepsins (~30 kDa) and FITC-dextran conjugates of up to 40 kDa but not β-hexosaminidase (250 kDa) or FITC-dextran molecules ≥70 kDa are released from lysosomes. Simultaneously, however, the cytosol-lysosome pH gradient, as indicated by labeling with an acidophilic fluorochrome1, is at least partially preserved. These studies point to a highly selective membrane permeabilization process to occur in which membrane pores selective for proteins of a limited size open, presumably in a transient fashion. Alternatively, a selective transport system could become activated during the commitment phase of apoptosis.

Mitochondria and various models proposed for its permeabilisation-a key step in PCD

Mitochondria are surrounded by a double membrane that divides it into two compartments: the inter-membrane space (IMS) and the inner compartment called the matrix. Mitochondria are increasingly being thought of as cell death check points at which signals for necrosis and apoptosis are sent for downstream processing. The mechanisms by which mitochondria are involved in the process of PCD have been intensively studied and the predominant version of PCD proceeds through the mitochondrial pathway, with OMM (outer mitochondrial membrane) permeabilization leading to the release of IMS content. IMS contains an arsenal of apoptogenic factors such as cytochrome c, AIF, Smac/DIABLO, and endonuclease G, molecules that are critical for the execution stage of PCD. In response to an apoptotic signal, these factors

are released into the cytosol, as a result of mitochondrial outer membrane permeabilization (MOMP) (Kroemer *et al.*, 2007). Release of toxic proteins form the intermembrane space of the mitochondria triggered by permeabilization of the outer mitochondrial membrane constitutes the "point of no return" in most cases of PCD (Sevrioukova and Churbanova, 2008).

Cytochrome c, Apaf-1 and ATP form apoptosome, (ATP/Apaf-1/Cyto c) complex which is fully functional in recruiting and activating procaspase-9. Activated caspase-9 then cleaves and activates downstream caspases, such as caspase-3, -6, and -7. (Riedl and Salvesen, 2007, Shi, 2002), which are responsible for destroying the cell from within (Li and Yuan, 2008, Philchenkov, 2003, Salvesen, 2002, Stegh and Peter, 2001). The AIF released from mitochondria also acts as an effector of apoptotic cell death (Cande *et al.*, 2002). Upon cleavage by calpains and/or cathepsins, AIF is translocated to the nucleus, leading to chromatin condensation (Yuste *et al.*, 2005) and DNA fragmentation (Susin *et al.*, 1999). Thus, release of cytochrome c and AIF are the key steps in apoptosis induction.

All of the mitochondrial components known to translocate to the cytoplasm following death stimulus reside in the IMS. Hence, alteration in OMM permeability is sufficient to release these proapoptotic effector molecules into cytosol. Accordingly, several competing models (Fig.1. 2) (Shoshan-Barmatz *et al.*, 2008) have been proposed for the MOMP. Some models suggest that release exclusively involves an increase in OMM permeability due to the formation of a channel large enough to allow release of proteins while others consider release is due to disruption of OMM integrity.

Osmotic matrix swelling and OMM rupture, leads to nonspecific release of inter-membrane proteins into the cytosol. A sudden increase in IMM permeability to solutes of low molecular weight, leads to MPT. Initial permeabilization of the IMM exerts osmotic forces driving water into the matrix, this leads to swelling and distension of the IMM and eventually, rupture of the OMM, allowing the efflux of IMS proteins to the cytosol (Feldmann *et al.*, 2000). It has been suggested that matrix swelling and OMM rupture results from a defect in mitochondrial ATP/ADP exchange due to VDAC closure (Tan and Colombini, 2007, Vander Heiden *et al.*, 2000, 2001) (Model A, in Fig 1.2).

Mitochondria maintain an electrochemical potential required to drive mitochondrial ATP synthesis by oxidative phosphorylation, providing the cell with ATP.

During mitochondrial respiration, five multiprotein complexes situated in the IMM mediate the electron transfer from NADH or $FADH_2$ to oxygen as final acceptor. The electron transport generates proton-motive force across the IMM. The PMF is a combination of the proton concentration gradient (pH) and the membrane electric potential ($\Delta\psi_m$). The movement of protons back into the matrix, down the concentration gradient, is coupled to the synthesis of ATP from ADP and Pi. The ANT mediates the exchange of ADP for ATP. Mitochondrial ATP production is absolutely dependent on the integrity of the IMM. However, it has become apparent that mitochondria possess a latent mechanism called MPT (Mitochondria permeability transition) that, when activated, destroys this permeability barrier and disrupts normal mitochondrial function. An important consequence of an activated MPT is uncoupling of oxidative phosphorylation (oxidation of the metabolites by O_2 still occurs with concomitant electron flux, but this flux is no longer coupled to proton pumping). Consequently, no transmembrane proton gradient can be maintained abolishing the production of ATP. Under these conditions, electrons can escape from ETC to form ROS.

A second model (Model B, in Fig. 1.2) for MMP suggests the formation of a permeability transition pore (PTP) complex, a large high conductance multiprotein complex comprising several components and spanning both the IMM and OMM (Bernardi, 1999, Halestrap *et al.*, 2000, Shoshan-Barmatz and Gincel, 2003). The PTP is formed at the contact sites between the inner and outer mitochondrial membranes. The major PTP complex components include VDAC at the OMM, ANT in the IMM, and cyclophilin D (CypD), a mitochondrial peptidyl prolyl cis-trans isomerase resident in the matrix (Bernardi, 1999, Shoshan-Barmatz and Gincel, 2003, Tsujimoto and Shimizu, 2007). PTP is a regulated pore, corresponding to a large voltage-dependent, non-selective conducting channel with a pore diameter of about 3 nm, features that are compatible with those of VDAC (Zoratti and Szabo, 1995). VDAC makes the OMM permeable to most small molecules (< 5 kDa), allowing free exchange of respiratory chain substrates. Cardiolipin makes the membrane impermeable to all but a few selected ions and metabolites. PTP opening could be caused by factors, such as changes in the energetic balance of the mitochondria, anoxia and ROS (Crompton, 1999, Kroemer and Reed, 2000, Zoratti and Szabo, 1995). Shimizu *et al* (1999) have shown that VDAC is required for apoptotic $\Delta\psi_m$ loss. VDAC communicates with other channels on the inner membrane, opening VDAC sufficiently for proapoptotic factors release might trigger the

opening of inner membrane channels resulting in $\Delta\psi_m$ loss. Direct interaction between Bax/Bak with ANT and other ANT-like proteins, such as the Pi/OH- antiporter and uncoupling proteins, may lead to $\Delta\psi_m$ loss.

This model is based on the finding that following Ca^{2+} accumulation in the matrix, a sudden increase in permeability to solutes (up to 1500 Da) is seen, representing PTP opening (Bernardi, 1999, Halestrap *et al.*, 2000, Shoshan-Barmatz and Gincel, 2003). Although the mechanism(s) responsible for PTP opening and its physiological function have not yet been entirely resolved, a variety of agents have been found to promote or inhibit PTP opening, including Ca^{2+}, inorganic phosphate, various oxidizing agents, glutamate, nucleotides, CypD ligands, gelsolin, ANT, TSPO (PBR), HK, and proteins of the Bcl-2 family. Some of these molecules, such as Bax or Bak, have also been shown to interact with VDAC directly and modify its channel activity or become part of the mega-channel that contains VDAC (Gincel and Shoshan-Barmatz, 2004, Shimizu *et al.*, 2000, Shoshan-Barmatz and Gincel, 2003, Shoshan-Barmatz *et al.*, 2006, Sugiyama *et al.*, 2002, Tsujimoto and Shimizu, 2002).

A third proposed mechanism for MMP suggests that proapoptotic proteins like Bax form homo or hetero- oligomeric channels for apoptogenic protein release (Model C and D, Fig. 1.2) (Antonsson *et al.*, 2000, 2001, Eskes *et al.*, 2000, Reed, 2006, Bidere *et al.*, 2003). Cytosolic Bax is weakly associated with mitochondria as a monomer, whereas following apoptosis induction with STS (staurosporine) or UV irradiation, Bax associates with mitochondria as a large oligomer/complex of 96 kDa and 260 kDa. Bcl-2 prevents Bax oligomerization and insertion into the mitochondrial membrane. It has also been demonstrated that tBid directly binds to Bax on mitochondrial membrane, followed by integration of Bax into the bilayer and subsequent oligomerization of Bax resulting in the formation of pores in membranes, with tBid remaining associated with the pore (Lovell *et al.*, 2008). Although it has been suggested that Bax or Bak homo-oligomerization is sufficient for OMM permeabilization, others suggest that Bax and Bak or VDAC function cooperatively to induce OMM permeabilization (Models D and E in Fig. 1.2). Similarly Bax and Bak oligomers form pores for proapoptotic factor efflux during apoptosis (Model C Fig. 1.2) (Antignani and Youle, 2006, Desagher *et al.*, 1999, Gross *et al.*, 1998, Wei *et al.*, 2000, 2001). Indeed, Bax and Bak interactions were shown to be augmented by various apoptosis stimuli, resulting in cooperative oligomerization, acting to facilitate formation of mitochondrial membrane pores and cell death in some apoptotic models

(Annis *et al.*, 2005, Antonsson *et al.*, 2000, Sundararajan *et al.*, 2001). At the same time, anti-apoptotic proteins, such as Bcl-2 and Bcl-XL, were shown to protect cells from apoptosis *via* a blockage of the Bax–Bak interaction, subsequently preventing cytochrome c release. Mitochondria incorporating Bax were found to release AIF selectively. This is in contrast to the reports suggesting that Bax and staurosporine trigger an early caspase-independent cytochrome c release and late caspase-dependent AIF release (Arnoult *et al.*, 2002), or that Bax suffices to release all mitochondrial inter-membrane proteins (and FITC–dextran molecules of up to 2000 kDa) (Kuwana *et al.*, 2002), yet in accord with previous observations indicating that AIF can be released from mitochondria in a selective, caspase-independent fashion (Susin *et al.*, 1999). Reversible and partial membrane permeabilization has, however, been observed for the inner mitochondrial membrane during the prelude of PCD (Zamzami *et al.*, 2003, Poncet *et al.*, 2003). How exactly such a selective permeabilization is obtained remains an enigma.

There are reports implying that VDAC can induce membrane permeability independently and in the presence of Bax (Shimizu and Tsujimoto, 2000, Banerjee and Ghosh, 2004) (Model H and E, Fig. 1.2). Furthermore, mitochondria isolated from VDAC-deficient yeast did not release Cytochrome c in the presence of Bax, whereas mitochondria from VDAC-deficient yeast expressing the human VDAC1 gene released Cytochrome c in presence of Bax (Shimizu *et al.*, 1999). In addition, intra-cellular microinjection of neutralizing anti-VDAC antibodies prevents Bax-induced Cytochrome release (Shimizu *et al.*, 2001). Independent evidence supporting the participation of a Bax–VDAC interaction in PCD was provided by the fact that HK-I (hexokinase) and HK-II can prevent apoptosis by inhibiting interaction of Bax with VDAC, upon binding to VDAC. Another version of this model suggests that oligomeric VDAC is the prime Cytochrome c release channel and that its pore is regulated by Bax (Debatin *et al.*, 2002). More recently, it was demonstrated that siRNA-mediated down-expression of VDAC1 strongly suppressed cisplatin-induced activation of Bax (Tajeddine *et al.*, 2008).

Yet another model in which Cyto c release takes place through the formation of larger pores by oligomerization of VDAC has been proposed (Shoshan-Barmatz *et al.*, 2006, 2008, Zalk *et al.*, 2005) (Model H, Fig. 1.2). In addition, nuclear magnetic resonance (NMR) studies imply that Bcl-XL mediates formation of heterotrimers including two VDAC1 molecules (Bayrhuber *et al.*, 2008, Hiller *et al.*, 2008). Apoptosis mediated enhancement of VDAC oligomerization was obtained regardless of the cell type

or apoptosis inducer used, including staurosporine (STS), curcumin, As2O3, etoposide, cisplatin, selenite, TNF-α, H2O2 or UV, all affecting mitochondria yet acting through different mechanisms. Conversely, the apoptosis inhibitor, 4,40-diisothiocyanostilbene-2,20-disulfonic acid (DIDS), prevented STS-induced VDAC oligomerization and apoptosis (Keinan *et al.*, 2010, Shoshan-Barmatz *et al.*, 2008).

Though the signaling mechanism responsible for induction of VDAC oligomerization *in vivo* is not yet known, the dynamic equilibrium between monomers and oligomers can be influenced by Ca^{2+}, ROS, low ATP levels and associated proteins, such as HK-I, or proteins from the Bcl-2 family, all known to interact with VDAC (Shoshan-Barmatz *et al.*, 2004, 2008).

An additional proposed mechanism for the release of proapoptotic proteins (Model G, in Fig. 1.2) suggests the involvement of the lipid, ceramide, acting through the formation of a lipidic channel in the OMM (Siskind *et al.*, 2006, Stiban *et al.*, 2008, Scharstuhl *et al.*, 2009). Ceramides have been proposed to function as important second messengers in apoptosis signaling pathways and are shown to be produced during apoptosis, where they directly target mitochondria. Ceramides are shown to promote Cytochrome c release from isolated rat liver mitochondria (Ghafourifar *et al.*, 1999) and AIF release from mitochondria of human foreskin-derived fibroblasts (Scharstuhl *et al.*, 2009). Three mechanisms have been proposed for the action of ceramides in apoptosis: a) they can form large stable channels in OMM (Siskind and Colombini, 2000, Siskind *et al.*, 2006, Stiban *et al.*, 2008), b) affect membrane organization (Cremesti *et al.*, 2001), c) ceramide and cholesterol in the membrane microenvironments favor Bax translocation to mitochondria *via* Bax activation, ultimately fostering propagation of the apoptotic cascade (Martinez-Abundis *et al.*, 2009).

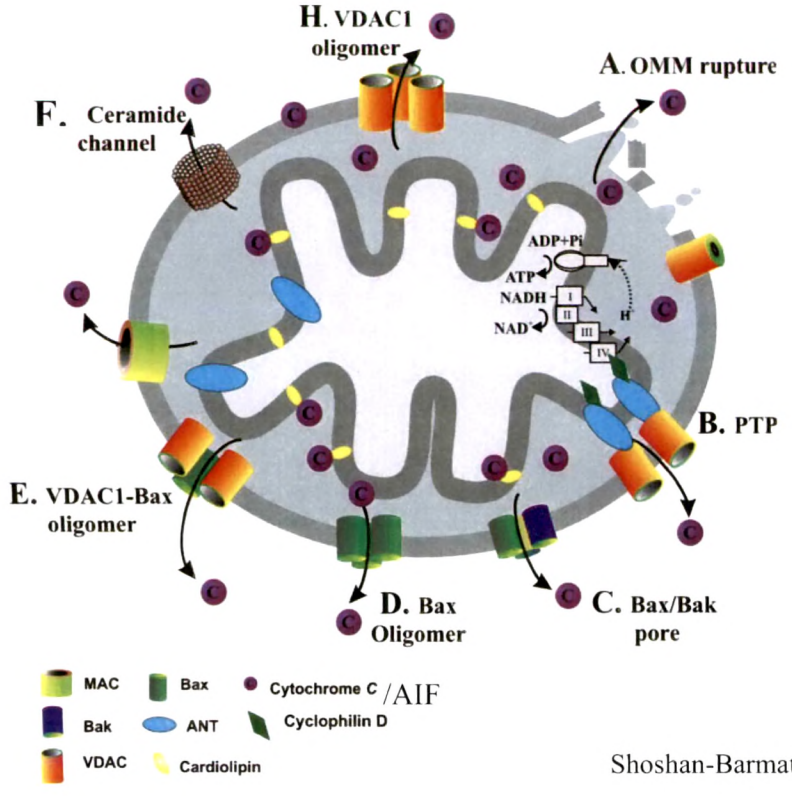

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Figure 1.2: Schematic representation of proposed models for the release of apoptogenic proteins from the mitochondrial inter-membrane space. Different models explaining how the OMM permeability changes during apoptosis induction, allowing the release of apoptogenic factors, such as Cyto c and AIF: A: OMM rupture caused by VDAC closure. B: PTP composed of VDAC1 at the OMM, ANT at the IMM and CypD in the matrix, allows apoptogenic proteins release. C: A pore formed by oligomerized forms of Bax and Bak after their activation by tBid, causing membrane permeabilization. D: Oligomeric Bax forming an apoptogenic release channel. E: (Bax/Bak) and VDAC1-based hetero-oligomer mediates the release. G: A lipid channel formed by ceramide – a self-assembled ceramide channel is proposed to act as the apoptotic protein release pathway. H, Oligomeric VDAC1 as a channel for the release of apoptotic proteins. In this manner, VDAC1 oligomerization functions in mitochondria mediated apoptosis.

Multiple pathways and mechanisms can coexist within a single model of cell death (Galluzzi and Kroemer, 2007, Gogvadze *et al.*, 2006) for such release of proapototic proteins from mitochondria which is of much significance in paraptosis.

1.1.4. Links between Lysosomes and Mitochondria

During STS stress a linear sequence of events in which cathepsin D translocation is required for the Bax-mediated release of AIF, which in turn causes chromatin

condensation and commitment to paraptosis. Similarly, it has been found that lysosomotropic toxins fail to kill Bax and/or Bak null cells and also in cells overexpressing Bcl-2 (or the strictly mitochondrion-specific vMIA protein from Cytomegalovirus) (Boya *et al.*, 2003 a,b). In such a system, the absence of Bax and/or Bak or the presence of Bcl-2 or vMIA fails to affect the redistribution of cathepsins B and D, yet does block the release of mitochondrial death effectors (Boya *et al.*, 2003 a,b). These data point to an obligatory participation of mitochondria in the transmission of lethal signal initially perceived at the lysosomal level.

In preapoptotic-activated T cells, apparently the enzymatic activity of cathepsin D is required for Bax to become inserted into the mitochondrial membrane (Bidere *et al.*, 2003). No signs of cathepsin D mediated cleavage of either Bid or Bax were readily detectable in staurosporine treated activated T cells (Bidere *et al.*, 2003). Thus, the exact mechanism of cathepsin D-mediated activation of Bax in this model remains unclear. In several other LMP-mediated cell death models, individual cathepsins' inhibition (or their knockout) is not sufficient to block the activation of Bax (Boya *et al.*, 2003 a,b). Thus, several cathepsins (and perhaps even other lysosomal hydrolases) may be able to constitute the link between LMP and MMP. Indeed, cysteine cathepsins B, H, L, S, and K have recently been shown to cleave and activate the Bax-activating Bcl-2 family member, Bid, *in vitro*, and the TNF-induced MMP in hepatocytes depends on the activity of cathepsin B rather than cathepsin D (Guicciardi *et al.*, 2000). In addition to Bid and Bax, other mediators like phospholipase A2 activation (Zhao *et al.*, 2001), arachidonic acid, (Foghsgaard *et al.*, 2002) and a potential MMP inducer (Scorrano *et al.*, 200, Scorrano *et al.*, 2003) may modify the link between LMP and MMP. Inspite of the exhaustive literature available in the context the exact molecular cascade leading from LMP to MMP still remains a conundrum.

1.1.5. Calpain: an addition to the cell death complexity

Calpains are a family of calcium activated cysteine proteases that are widely distributed in cells and sub-organelles along with their endogenous inhibitor, calpastatin (Goll *et al.*, 2003). The calpain family is made up of the ubiquitous calpains such as α-calpain (calpain 1), m-calpain (calpain 2), calpain 10 and the tissue-specific calpains, calpain 3 (muscle-specific p94), calpain 8 (stomach nCl-2), calpain 9 (digestive tubule nCl-4), lens Lp82 and Lp85, retinal Rt88 and corneal Cn94 (Ozaki *et al.*, 2007). The α-

and m-calpains coexist in most cells as a low micromolar order; Ca^{2+} requiring form (α-calpain) and a high millimolar order Ca2+-requiring form (m-calpain) (Goll *et al.*, 2003).

Calpain may also play an important role in apoptotic cell death through direct and indirect interactions with members of the caspase family of proteases. Apoptosis protease-activating factor-1 has been demonstrated to be cleaved by calpain (Bevers and Neumar, 2008) resulting in the reduced activity of caspase-3 like protease. The effect of calpain on caspase-9 also results in a loss of ability to activate caspase-3. Conversely, calpain cleavage converts procaspase-7 to the active form, which is independent of the activity of other caspases (Bevers and Neumar, 2008). Cross-talk between the calpain and caspase systems also occurs *via* calpastatin. Caspases have been shown to cleave calpastatin and reduce its inhibitory activity, thereby setting up a complex interaction between the calpain and calpastatin systems (Bevers and Neumar, 2008).

An increase in $[Ca^{2+}]i$ (intracellular Ca^{2+} concentration) leads to mitochondrial Ca^{2+} overload, which activates mitochondrial calpains (Norberg *et al.*, 2008). Ca^{2+} activation of calpain is important for physiological functions, but excessive Ca^{2+} can produce abnormal proteolytic activity, MPT and death (Liu *et al.*, 2003). Calpain 10 over expression has been shown to induce mitochondrial fragmentation and swelling, which is consistent with MPT (Pariat *et al.*, 1997) and this altered mitochondrial morphology was blocked by MPT inhibitors. Kar *et al* (2009) have demonstrated that mitochondrial major Ca^{2+} extruding pathway $Na+/Ca^{2+}$ exchanger is cleaved by the mitochondrial α-calpain in an increased $[Ca^{2+}]m$ and that could play important roles in smooth muscle cell demise (Kar *et al.*, 2009, Norberg *et al.*, 2008) In addition to promoting formation of the mitochondrial permeability transition pore, calpain plays a more direct role in the release of AIF. Calpain has recently been shown to cleave AIF and to induce its release from mitochondria (Cao *et al.*, 2007, Polster *et al.*, 2005, Sanges *et al.*, 2006). Release of AIF from the mitochondria has also been observed in concert with activation of caspase-12, another calpain substrate (Nakagawa and Yuan, 2000). Therefore, although calpain appears to down regulate caspase mediated cell death, it may simultaneously work to promote caspase- independent programmed cell death *via* an AIF-mediated mechanism. However, the role of calpain in the release of AIF from mitochondria is found to be tissue specific (Kar *et al.*, 2010).

1.2. Main proteins in Paraptosis

Paraptosis is mainly mediated by three proteins: Poly(ADP-ribose) polymerase (PARP) and Apoptosis inducing factor (AIF).

1.2.1. Poly ADP-Ribose Polymerase (PARP)

One of the most drastic post-translational modifications of proteins in multicellular organisms including the unicellular eukaryote *Dictyostelium discoideum* is poly (ADP-ribosyl) ation (PARylation). This modification is not seen in prokaryotes and yeast. The DNA-repair and protein-modifying enzyme PARP-1 is responsible for this modification. It is also called poly(ADP-ribose) synthetase and poly(ADP-ribose) transferase and is an abundant nuclear protein that is involved in the DNA-base-excision-repair system. It is the first identified substrate of caspases. It belongs to a large family of proteins that includes PARP-2, PARP-3, vault PARP and tankyrases. On average, approximately one molecule of PARP-1 is present per 1000 bp of DNA.

Structure of PARP

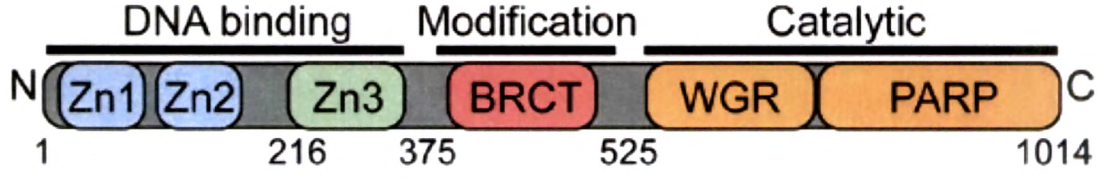

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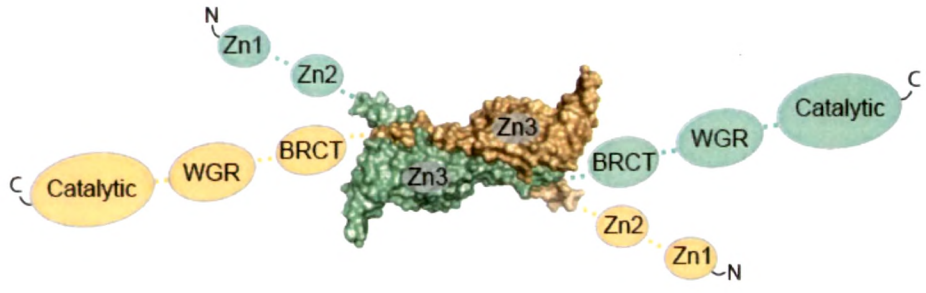
Figure 1.3: A schematic representation of the modular organization of human PARP

PARP-1 is a 116 kDa protein. It contains three functionally distinct domains: an amino terminal DNA binding domain (DBD), modification domain (AD) and a carboxyl terminal PARP homology domain that includes the catalytic domain (CAT) (Fig. 1.3) responsible for PAR formation (Mazen *et al.*, 1989, de Murcia *et al.*, 1994, Ruf *et al.*, 1996, Smith, 2001). AD is rich in glutamic acid residues consistent with the fact that Poly-(ADP ribosy) lation occurs on such a residue (Schreiber *et al.*, 2006). Recent studies identified a weak leucine-zipper motif in the amino-terminal region of the AD, which suggests that this motif might be involved in homo and/or hetero-dimerization

(D'Amours *et al.,* 1999). This domain also comprises of BRCT (breast cancer 1 protein {BRCA 1} C-terminus) motif that is present in many DNA damage and cell cycle check point proteins. DBD known to contain two structurally and functionally unique zinc fingers (Zn1: aa, amino acid, 11–89, Zn2: aa 115–199) (D'Amours *et al.,* 1999, Hassa *et al.,* 2006). Recently, a third and so far unrecognized zinc binding motif is discovered (Zn3: aa 216–375) (Langelier *et al.,* 2008, Tao *et al.,* 2008). The DNA-binding domain of PARP-1 binds to single or double strand breaks with high affinity *via* zinc fingers. The Zn1 is essential for PARP activation by DNA strand breaks, where as Zn2 is essential for PARP activation by DNA single strand breaks but not double strand breaks (Kun *et al.,* 2002). Langlier *et al* (2010) define two roles for the Zn3 domain of PARP-1. One role is to contribute to the chromatin compaction activity of PARP-1 either *via* homodimerization of chromatin-bound PARP-1 molecules, or optimal spatial positioning of the Zn1-Zn2 domains and the CAT domain for maximal chromatin compaction (Fig. 1.4). In the second role, the zinc-ribbon fold of the Zn3 domain is a vital component of DNA-dependent PAR synthesis activity. It is proposed that the zinc-ribbon fold mediates an interdomain contact that is essential to form the DNA-activated conformation of PARP-1. Thus the DNA binding domain is also involved in protein-protein interactions. The DBD also contains a bipartite nuclear localization signal (NLS) that targets PARP-1 to the nucleus (Schreiber *et al.,* 2006). The CAT domain has been suggested to catalyze at least three different enzymatic reactions: the attachment of the first ADP-ribose moiety onto an acceptor amino acid (initiation reaction), the addition of further ADP-ribose units onto already existing ones (elongation reaction) and the generation of branching points (branching reaction) (Ruf *et al.,* 1996). The active site is formed by a phylogenetically well-conserved sequence of ~50 residues (aa 859–908 of hPARP1). This PARP signature sequence, a 50 amino acid sequence shows 100% homology between vertebrates and contains the NAD^+ acceptor sites and critical residues involved in the initiation, elongation and branching of PAR. WGR domain of PARP is also a highly conserved domain that is involved in the activation.

The crystal structure of the C-terminal catalytic fragment revealed a striking homology with bacterial toxins that act as a mono(ADP ribosyl) transferase (Burkle, 2005) also. PARP in *D. discoideum* (Dd PARP) has been shown to have features of both PARP-1 and PARP-2. Dd PARP is shown to have > 50% sequence homology with human PARP-2. There are nine PARPs reported in *D. discoideum* (Otto *et al.,* 2005).



Langlier *et al.*, 2007

Figure 1.4: Self-association of the third Zn-binding domain of PARP-1 provides a structural scaffold for assembling the three-dimensional arrangement of PARP-1 domains. The two molecules of the PARP-1216–366 homodimer are shown as molecular surfaces (green and orange). The precise anchoring of the two molecules could serve as a molecular cross brace that organizes the three-dimensional arrangement of attached PARP-1 domains.

Mode of action of PARP

PARP functions as a DNA damage sensor and signaling molecule binding to both single- and double-stranded DNA breaks. Upon binding to damaged DNA mainly through the second zinc-finger, it forms homodimers and catalyzes the cleavage of NAD into nicotinamide and ADP-ribose and then uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins (de Murcia *et al.*, 1994, de Murcia and Menissier de Murcia, 1994, Lindahl *et al.*, 1995, Burkle, 2001, Smith, 2001). The covalent transfer of ADP-ribose unit onto glutamic acid, aspartic acid or lysine residues of target proteins is followed by successive transfer reactions onto the protein-mono (ADP-ribosyl) adduct and subsequently onto the emerging chain of several covalently linked ADP- ribosyl residues (Fig. 1.5). PARP-1 transfers 50-200 residues of PAR to itself and to acceptor proteins such as histones, DNA polymerases, topoisomerases, DNA ligase-2, high-mobility-group proteins and transcription factors. Poly (ADP-ribose) can be regarded as the cell's most elaborate metabolite of NAD^+ (de Murcia *et al.*, 1994, Smith, 2001, Burkle, 2004). The high negative charge, in addition to the bulky size of the polymer, imparted to the protein by poly (ADP-ribosyl) ation dramatically alters the function of the target protein. The synthesis and turnover of ADP-ribose polymers is a dynamic cellular response to DNA

damage. Also the extent of PARP activation is directly proportional to the extent of DNA damage.

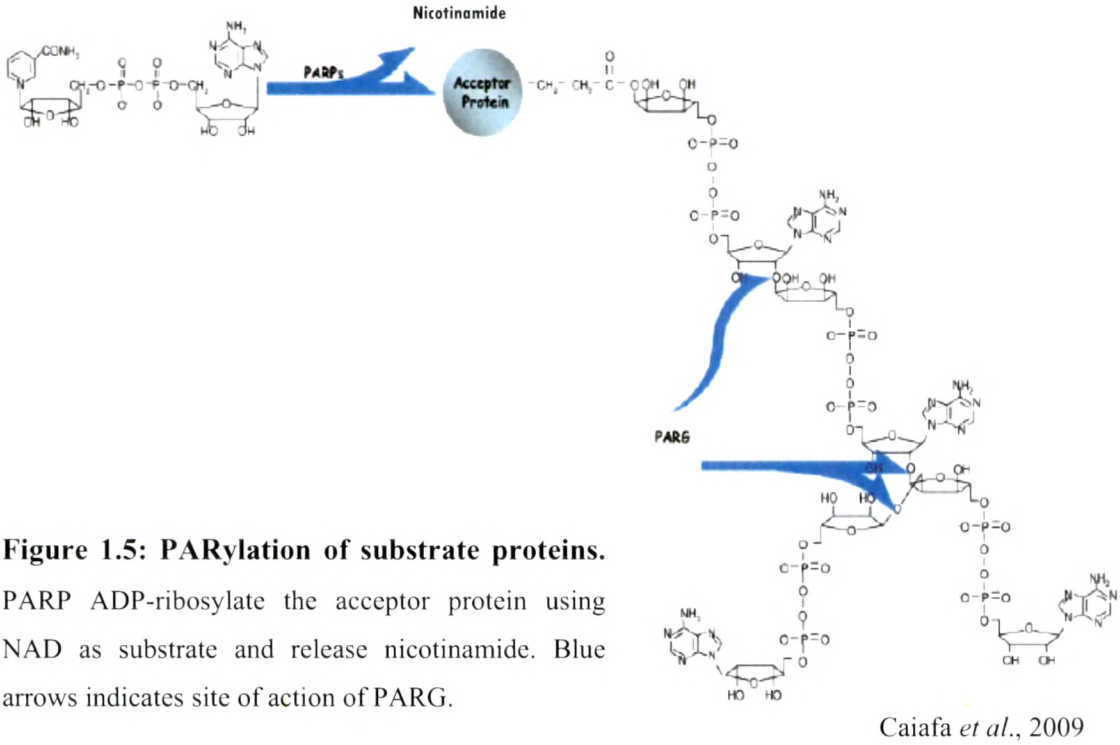
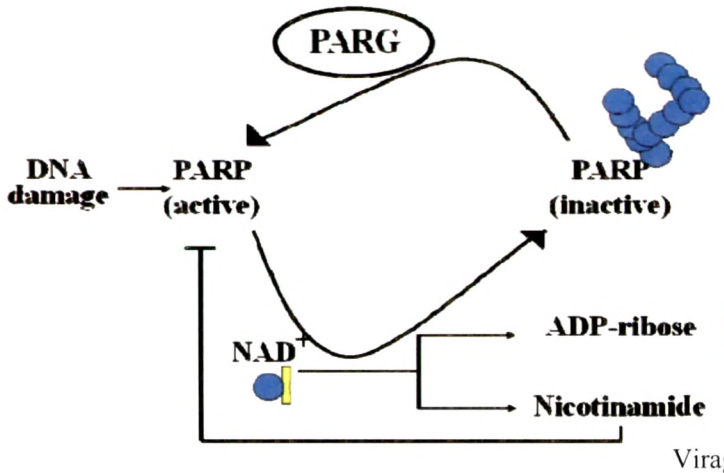


Figure 1.5: PARylation of substrate proteins. PARP ADP-ribosylate the acceptor protein using NAD as substrate and release nicotinamide. Blue arrows indicates site of action of PARG.

Caiafa *et al.*, 2009

Regulation of PARP activity and PAR metabolism

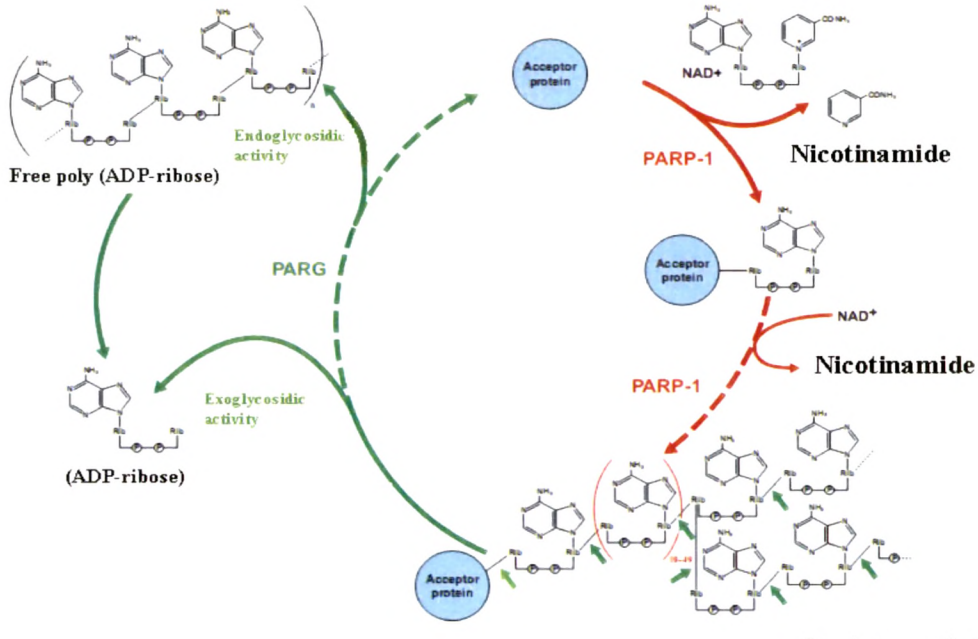
The regulation of PARP activity is established through different mechanisms (Fig. 1.6). The best characterized mechanism is the down-regulation of enzyme activity through auto-poly-ADP-ribosylation (Kawaichi *et al.*, 1981). Furthermore, nicotinamide, the smaller cleavage product of NAD, also exerts inhibitory effect on PARP, allowing negative feedback regulation. The purines hypoxanthine, inosine, and adenosine also are identified as another class of endogenous PARP inhibitors (Virag and Szabo, 2002). The regulation of PARP activity by purines is possibly relevant under pathophysiological conditions in which intracellular levels of these metabolites reach levels that are high enough to efficiently inhibit PARP. Phosphorylation of PARP by protein kinase C also results in its inhibition (Tanaka *et al.*, 1987, Bauer *et al.*, 1992). The abundance of PARP may also change under certain conditions, suggesting a transcriptional or posttranscriptional regulation (Tramontano *et al.*, 2000, Doucet-Chabeaud *et al.*, 2001).



Virag and Szabo, 2002

Figure 1.6: Regulation of PARP activity. PARG and nicotinamide play significant role in the regulation of PARP activity.

PARylation is a dynamic process owing to the activities of PARP and Poly{ADP-ribose)}glycohydrolase (PARG). A family of PARP enzymes catalyze the addition of poly ADP-ribose (PAR) adducts to the proteins whereas PARG is responsible for the short half life of PAR during PARP activation, as it cleaves the PAR polymer (D'Amours *et al.*, 1999, Alvarez-Gonzalez & Althaus, 1989, Chiarugi, 2002). In



Rouleau *et al.*, 2004

Figure 1.7: PAR metabolism. PARP catalyses PARylation using NAD^+ as the substrate, transferring the ADP-ribose moiety to the acceptor protein and releasing nicotinamide. PARG via its endo and exo glycosidase activity constitutes the major catabolic enzyme for PAR metabolism.

addition to PARG, ADP- ribosyl lyase is also involved in the catabolism of PAR. The linear and branched portions are cleaved by PARG (Fig. 1.7) while lyase removes the proximal ADP-ribose monomers (Davidovic *et al.*, 2001). PARG like PARP has been addressed as a target for various therapeutic diseases.

Functions of PARP

Owing to the wide array of proteins that serve as a target for its activity (Table 1.1) PARP has been associated with virtually all the physiological processes of the cell. The biological role of poly(ADP-ribose) is complex and involves nine main functions as follows:

First, PARP-1 has been implicated in chromatin remodeling, DNA repair and maintenance of genomic integrity (de Murcia and Menissier de Murcia, 1994, deMurcia *et al.*, 1994,1997, Schreiber *et al.*, 1995, Chatterjee *et al.*, 1999, Shall and de Murcia, 2000). Its involvement in chromatin remodeling is owing to histone being its second major substrate. This "guardian angel" function is indicated by delayed DNA base-excision repair and by a high frequency of sister chromatid exchange in PARP-1-deficient cells exposed to ionizing radiation or treated with alkylating agents (deMurcia *et al.*, 1997).

Second, PARP-1 also regulates the expression of various proteins at the transcriptional level. Of special importance is the regulation by PARP-1 of the production of inflammatory mediator such as the inducible nitric-oxide synthase (iNOS) (Hauschildt *et al.*, 1992, Le Page *et al.*, 1998, Szabo *et al.*, 1998, Oliver *et al.*, 1999), intercellular adhesion molecule 1 (ICAM-1) (Zingarelli *et al.*, 1998, Szabo *et al.*, 2001), and major histocompatibility complex class II (Otsuka *et al.*, 1991). NF-kB is a key transcription factor in the regulation of this set of proteins, and PARP has been shown to act as a coactivator in the NF-kB mediated transcription (Oliver *et al.*, 1999).

Third, PARP-1 regulates replication and differentiation. PARP-1 is part of the multiprotein replication complex (MRC) (Simbulan-Rosenthal *et al.*, 1996), indicated by co-purification of PARP-1 with key components of MRC. Another mechanism by which PARP may regulate nuclear processes is poly(ADP-ribosylation) of histones facilitating the disassembly of histone complexes on DNA during replication (Boulikas, 1990).

Fourth, poly(ADP-ribosylation) has been implicated in the regulation of telomerase activity. PARP-1 has also been implicated in the maintenance of telomere length (Virag and Szabo, 2002).

Fifth, PARP-1 activation has been proposed to represent a cell elimination pathway (Berger *et al.*, 1983, Berger and Berger, 1986, Schraufstatter *et al.*, 1986, Sims and Benjamin, 1987, Kleczkowska and Althaus, 1996) through which severely damaged cells are removed from tissues. Mild genotoxic noxa cause PARP activation that facilitates DNA repair and cell survival. Severe DNA damage, however, causes overactivation of PARP resulting in the depletion of NAD and ATP and consequently in necrotic cell death (Fig. 1.9). PARP activation has also been implicated in triggering programmed cell death that is discussed in detail later.

Sixth, poly(ADP-ribose) polymer has been identified recently as an emergency source of energy used by the base-excision machinery to synthesize ATP (Maruta *et al.*, 1997, Oei and Ziegler, 2000).

Seventh, similarly to ubiquitination, poly(ADP-ribose) may also serve as a signal for protein degradation in oxidatively injured cells (Ciftci *et al.*, 2001, Ullrich andGrune, 2001, Ullrich *et al.*, 2001).

Eighth, in addition to PARP-catalyzed covalent poly-(ADP-ribosylation), poly(ADP-ribose) polymers can non-covalently bind to specific (ADP-ribose) binding motifs in proteins, such as histones, XRCC1, p53, and DNA polymerase , and thereby modify their function (Althaus *et al.*, 1993, Pleschke *et al.*, 2000).

In the ninth and final function, poly(ADP-ribosyla-tion) may also be involved in the regulation of cytoskeletal organization. A recent study reported morphological alterations in Drosophila overexpressing PARP-1(Uchida *et al.*, 2001).

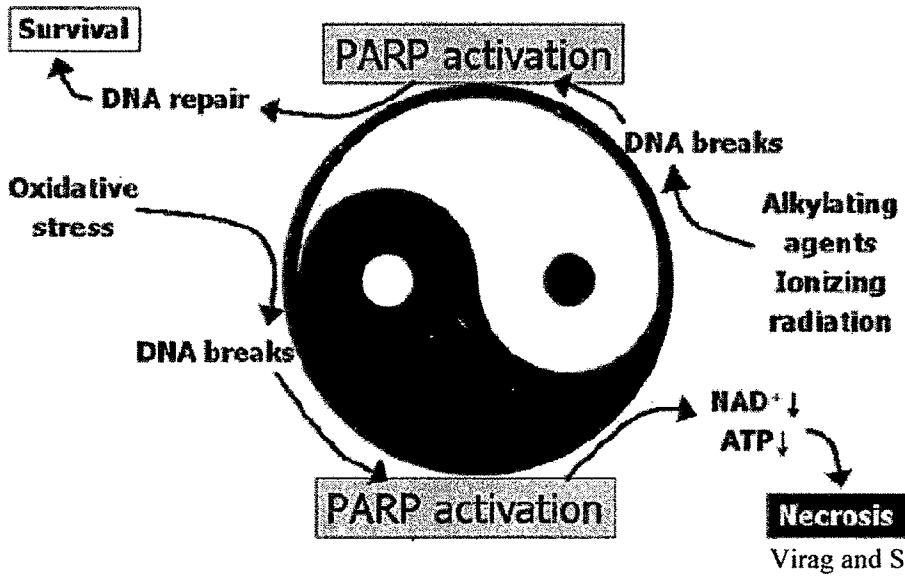
Table 1.1: Examples of target proteins for PARylation.

Potential function	Acceptors
Modulation of chromatin structure, repair	Histone H1, H2a, H2b, H3, H4, Topoisomerase I and II, HMG proteins, PARP.
DNA synthesis, repair	DNA ligase I and II (*in vitro*), DNA polymerase α,β (*in vitro*), Terminal transferase, PCNA.
Transcription	RNA polymerase I and II, hnRNP, Fos, p53, $NF\text{-}_{K}B$
Base excision repair	XRCCi, DNA ligase III, DNA polymerase beta
Cell cycle	p53, cFOS, PCNA

(Table adapted from D' amours, *et al*, 1999)

PARP and cell death:

PARP plays a crucial role in DNA damage induced cell death. Its role is seen as the cellular survival factor and also as a perpetrator of cell death (Fig. 1.8).



Virag and Szabo, 2002

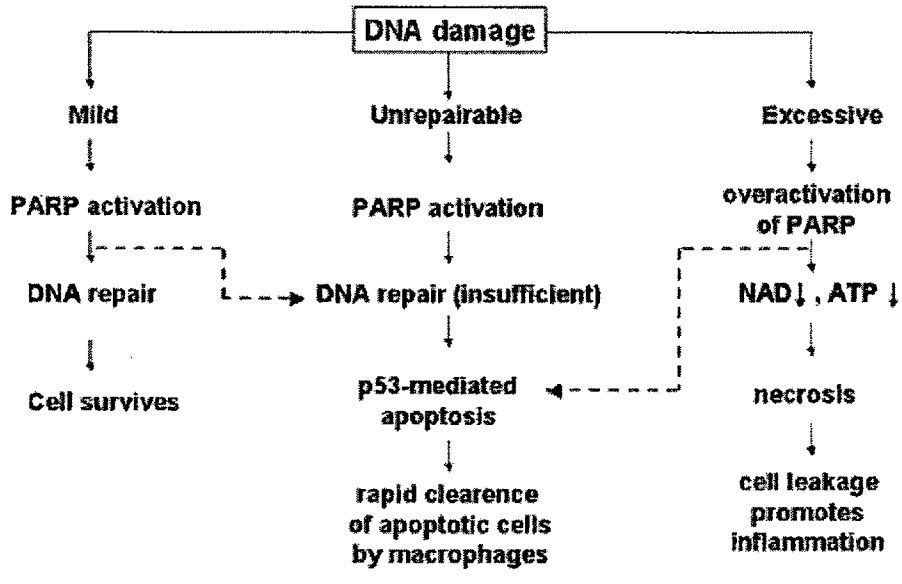
Figure 1.8: The ying-yang model for PARP activation after DNA damage.

According to this concept, cells exposed to DNA-damaging agents can enter three pathways determined by the intensity of stimulus (Fig. 1.9). PARP activated by mild genotoxic stimuli facilitates DNA repair by signaling cell-cycle arrest and by interacting with DNA repair enzymes such as XRCC1 and DNA-dependent protein kinase. As a result, DNA damage is repaired, and cells survive without the risk of passing on mutated genes. More severe DNA damage induces apoptotic cell death during which caspases (3 and 7), the main executor enzymes of the apoptotic process, inactivate PARP by cleaving it into two fragments (p89 and p24). The cleavage of PARP-1 separates its DBD from its catalytic domain, which inactivates the enzyme, and is one of the hallmark features of apoptosis. This pathway allows cells with irreparable DNA damage to become eliminated in a safe way. The normal development pattern that is seen in PARP-1 deficient mice is indicative that PARP-1 does not have an active role in apoptosis. Cleavage of PARP is believed to aim at preventing the activation of PARP by the ensuing DNA fragmentation and thereby preventing cells from the pathological squeal and maintaining cellular energy for certain ATP dependent steps of apoptosis (Herceg and Wang, 1999, Aikin *et al.*,

2004). The third route is induced by extensive DNA breakage that is usually triggered by a massive degree of oxidative or nitrosative stress (hydroxyl radical, peroxynitrite, nitroxyl anion). The over activation of PARP depletes the cellular stores of its substrate NAD^+ and consequently ATP. The severely compromised cellular energetic state inhibits the apoptotic cell death process to proceed, because many steps of apoptosis are known to depend on ATP (Kass *et al.*, 1996, Richter *et al.*, 1996, Stefanelli *et al.*, 1997, Ferrari *et al.*, 1997, Feldenberg *et al.*, 1999, Chalmers-Redman *et al.*, 1999, Leist *et al.*, 1999).

Thus PAR metabolism plays a role in a wide range of biological processes including DNA repair and maintenance of genomic stability (D'Amours *et al.*, 1999, Alvarez-Gonzalez & Althaus, 1989, Chiarugi, 2002) during basal level of DNA damage and hence PARP activity. However, during conditions of extensive DNA damage, PARP gets over activated and PARylation paradoxically contributes to mitochondrial failure and cell death. PARP is found to play an important role in all three kinds of cell death i.e., necrosis, caspase dependent (Tewari *et al.*, 1995, Ciprani *et al.*, 2005) and caspase independent programmed cell death.

Molecular mechanism of PARP over activation that is localized to the nucleus leads to programmed cell death is still under investigation. Some studies indicate a direct action on mitochondrial function. A report suggests that PARP localizes to mitochondria and, thus, could directly mediate these effects (Du *et al.*, 2003). However, other investigations using carefully controlled sub cellular fractions have not observed mitochondrial localization of PARP-1. A more common hypothesis of PARP mediated cell injury involves the observation that over activation of PARP and (ADP-ribosyl)ation leads to massive utilization of NAD^+ and a rapid loss of cellular NAD^+ and ATP (Ha *et al.*, 2000). This observation has led to the 'suicide hypothesis', in which rapid catabolism of NAD^+ by PARP activation affects cellular energy metabolism and, ultimately, lead to cell death. This may be true with necrotic cell death however, direct evidence that energy depletion has a role in PARP-dependent cell death is lacking in caspase independent apoptosis or paraptosis.



Virag and Szabo, 2002

Figure 1.9: Intensity of DNA damaging stimuli determines the fate of cell. PARP senses the state of DNA and directs the cell towards either survival or death.

Mitochondrial changes downstream to PARP activation and altered cellular energy status are accompanied with the release of apoptosis inducing factor for mitochondrial intermembrane space to cytosol. However, the signal employed for this nuclear and mitochondrial crosstalk is not yet identified. Although studies indicate that NAD^+ depletion, modified acceptor proteins and PAR polymer as the potent candidates that could serve as the signals for the induction of release of AIF from mitochondria, however the results are conflicting (Goto *et al.*, 2002, Hong *et al.*, 2004).

1.2.2. Apoptosis Inducing Factor (AIF)

Structural similarity to flavoproteins indicates that AIF acts as an oxidoreductase in mitochondrial electron transport (Lipton and Wetzel, 2002). The oxidoreductase domain confers it a local redox function that is essential for optimal oxidative phosphorylation and for an efficient antioxidant defense. The importance of NADH oxidase activity of AIF in the maintenance of oxidative phosphorylation and antioxidant defense has been recently unraveled. It reduces O2 to $O2^{\cdot-}$ producing superoxide radicals in *in vitro* conditions. However, considering the rate of electron transfer it is difficult to conceive both the natural electron acceptor of AIF and the physiological relevance of the redox behaviour. Cerebellar granule cells from Harlequin (Hq) mice in which AIF expression is reduced markedly are susceptible to oxidative stress. Retroviral transfection

of AIF eliminates this difference between wild type (WT) and Hq mice. However *aif* gene silencing has adverse effect on the functioning of ETC (Vahsen *et al.*, 2004, Joza *et al.*, 2005, Brown *et al.*, 2006, Hangen *et al.*, 2010) (discussed in detail in chapter 7). The mitochondrial localization, sensitivity to oxidative stress in Hq mice and NADH oxidase activity of AIF indicates that AIF might be involved in scavenging reactive oxygen species under normal physiological circumstances, however, it is also evident that AIF is an important factor for cell death.

AIF and cell death

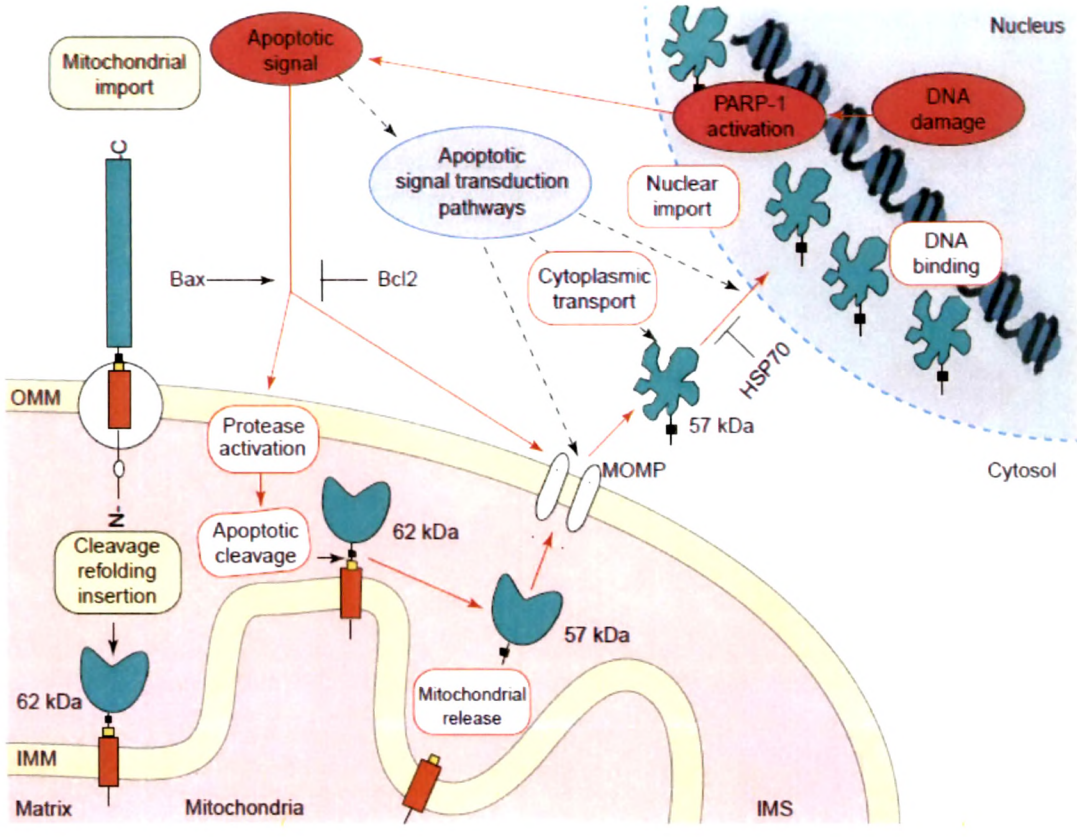
In many models of caspase independent cell death (paraptosis) AIF translocates to the nucleus, where it induces chromatin condensation and DNA fragmentation. The nuclear localization of AIF can be inhibited by blocking upstream signals of paraptosis. The contribution of AIF to cell death depends on the cell type and cell death stimuli and is clearly seen when caspases are inhibited or not activated. It is unknown to what extent and through which mechanisms AIF contributes to the induction of cell death.

Parallels can be drawn between AIF and cytochrome c: both are important for cell viability when they are located in mitochondria, but when either is released from the mitochondria, they activate death programs (Hong *et al.*, 2004). AIF induces caspase-independent cell death primarily. Following AIF translocation, classical apoptotic features, such as phosphatidylserine exposure, partial chromatin condensation and nuclear condensation, occur in the absence of caspase activation. This apoptogenic activity of AIF is independent of its oxidoreductase domain (Loeffler *et al.*, 2001, Miramar *et al.*, 2001).

Thus AIF could function both as a free radical scavenger as well as a promoter of cell death according to the location of the protein.

Release of AIF

Earlier AIF was believed to be a soluble protein in the intermembrane space of mitochondria (Susin *et al.*, 1999, Daugas *et al.*, 2000) which could be released due to MOMP would be sufficient to release AIF from mitochondria. Later, few reports (Arnoult *et al.*, 2003, Uren *et al.*, 2005) showed that AIF is tethered to the inner membrane of mitochondria through a transmembrane segment (TMS) comprising of residues 67–83 (Otera *et al.*, 2005). The Fig.1.10 shows the import of unprocessed full length AIF into the mitochondrion of a healthy cell.



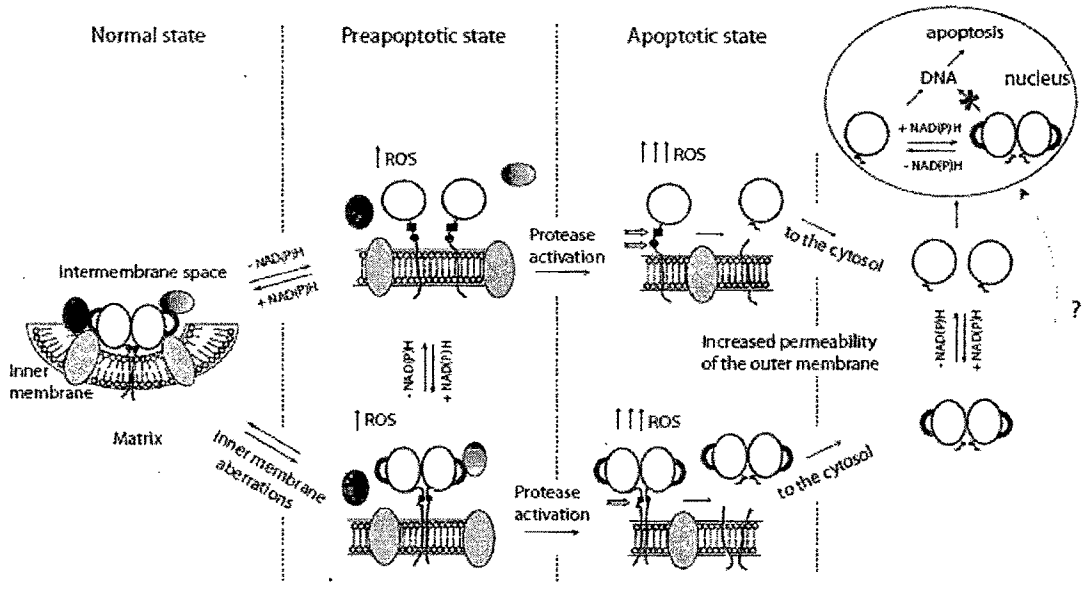
Modjtahedi *et al.*, 2006

Figure 1.10: Subcellular localization of AIF and its release during paraptosis.

After the cleavage of its N-terminal MLS by a mitochondrial peptidase, AIF is inserted in the inner mitochondrial membrane (IMM), via its N-terminal transmembrane region (orange). The rest of the protein faces the intermembrane space (IMS), where it participates in the regulation of the activity of the respiratory chain. The apoptotic release of AIF from mitochondria requires the inducible activity of an unknown protease and MOMP. This explains why, in some instances, factors such as cytochrome c are released from mitochondria but AIF is retained (Uren *et al.*, 2005). Conversely, AIF could be released before cytochrome c (Uren *et al.*, 2005, Zhu *et al.*, 2003), for example, when cytochrome c is retained at the inner mitochondrial membrane through electrostatic interactions with cardiolipin, a lipid that is confined to the inner mitochondrial membrane.

Some evidences link the release of AIF with the metabolic state of the cell. These reports show that AIF exists as a dimer bound to NAD(P)H and participates in redox cycling, cristae formation, and assembly/functioning of the respiratory chain directly or

indirectly *via* protein-protein interactions. This dimer serves as a FADH2-NAD(P) charge-transfer complex which upon reduction of FAD undergoes a conformational change in such a way that AIF now becomes susceptible to calpain as well as its interaction with DNA is favored. Both these events are critical for paraptosis.



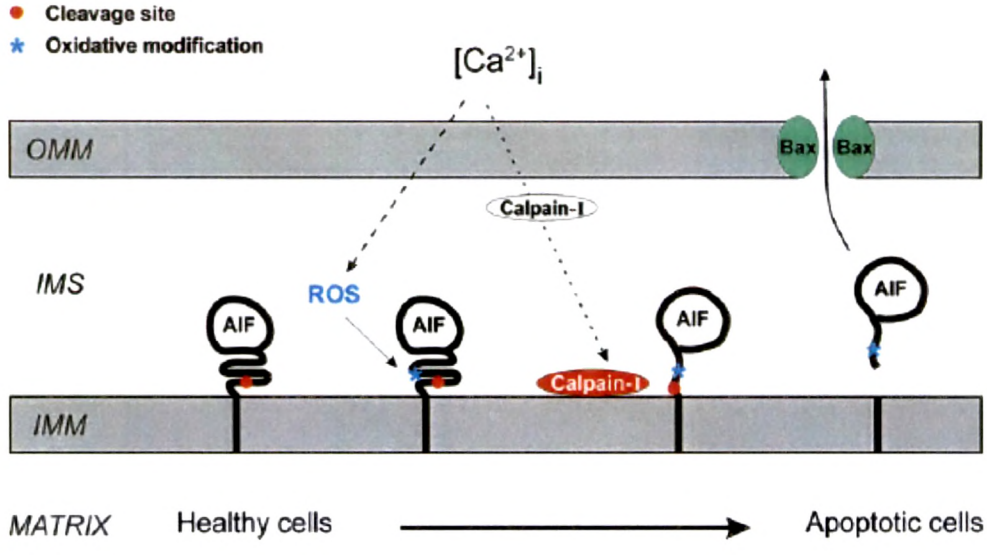
Churbanova and Sevrioukova, 2008

Figure 1.11: Possible mechanism for mitochondrial AIF acting as a redox sensor. Normal and apoptogenic functions of AIF are controlled by NAD(P)H levels and hence metabolic state of the cell.

Churbanova and Sevrioukova (2008) have proposed a possible mechanism as outlined in Fig. 1.11 that links pyridine nucleotide dependent metabolic pathways with cell death *via* AIF. The figure shows how either of the two factors namely depletion of pyridine nucleotide or MOMP and protease activation could result in AIF proteolysis and release of AIF from the IMS (point of no return).

In STS treated cells, cellular and subsequently mitochondrial Ca^{2+} level rises. The Ca^{2+} elevation leads to activation of mitochondrial calpain located in the intermembrane space and ROS production (Norberg *et al.*, 2008). This leads to oxidative modification (carbonylation) of AIF, which presumably is associated with increased accessibility of the calpain cleavage site to proteolytic activity (Fig.1.12) (Norberg *et al.*, 2010). The soluble 57 kDa AIF is then released through Bax/Bak pores in the OMM. Co-immunoprecipitation experiments by Lui and Kong (2007) show that HSP 70 directly

interacts with AIF and thereby prevents its translocation to nucleus. The cytoplasmic transport of AIF is inhibited by the anti-apoptotic Hsp70 protein (Ravagnan *et al.*, 2001) while Bcl-2 family of proteins regulates MOMP and hence release of AIF. Upon translocation to the nucleus, AIF binds to the chromatin and induces its condensation.



Norberg *et al.*, 2010

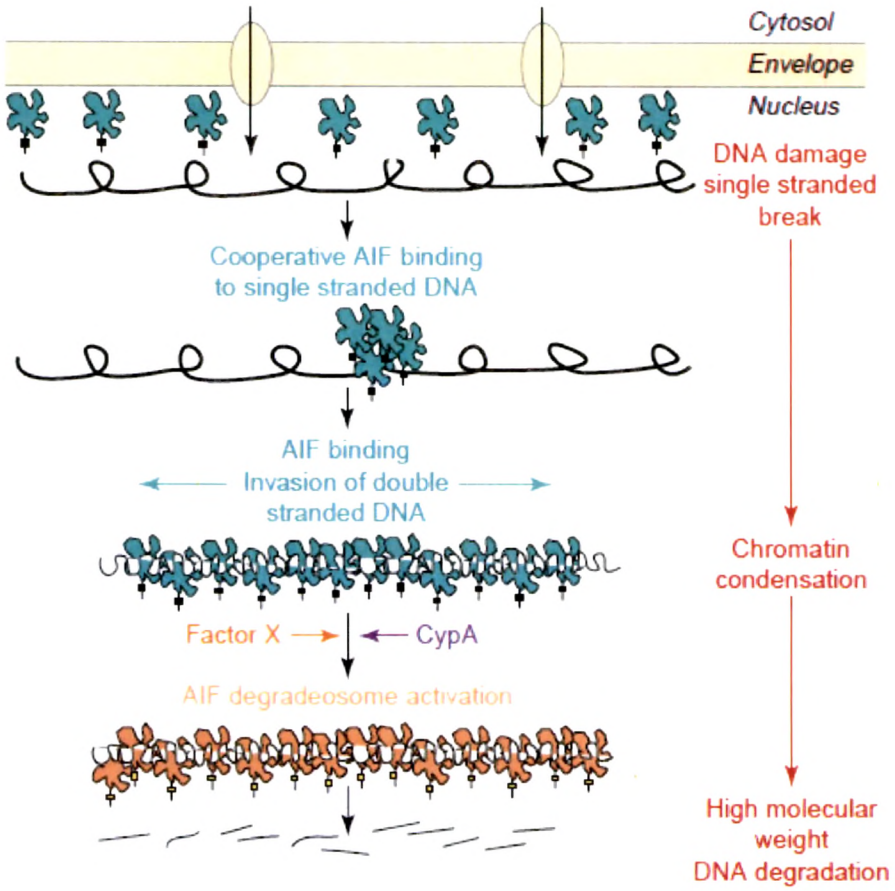
Figure 1.12: AIF processing is sequentially regulated by Ca^{2+} and ROS.

Pharmacological agents that hinder the translocation of AIF or inhibit the promotion of DNA fragmentation induced by AIF might have tremendous therapeutic potential.

Binding to DNA

The crystal structure of AIF shows that the surface of AIF protein contains positively charged amino acids, which enable it to engage in electrostatic interactions with DNA in sequence independent manner after its nuclear import. AIF invades double-stranded DNA, after initially binding to single-stranded DNA, and causes DNA condensation (symbolized by shortening of the black line representing DNA in Fig.1.14). Systematic studies performed in *C.elegans* suggest that AIF builds up a so called degradosome which includes a *C.elegans* specific cyclophilin (CYP-13) and unknown factors (factor X). CYPA is essential for the pro-apoptotic activity of AIF in Jurkat cells and *S. cerevisiae* (Cande *et al.*, 2004, Wissing *et al.*, 2004). Also Zhu *et al* (2007) suggest a model in which two proteins i.e., AIF and CYPA, which normally reside in separate compartments acquire novel properties when moving together to the nucleus. The degradosome or the

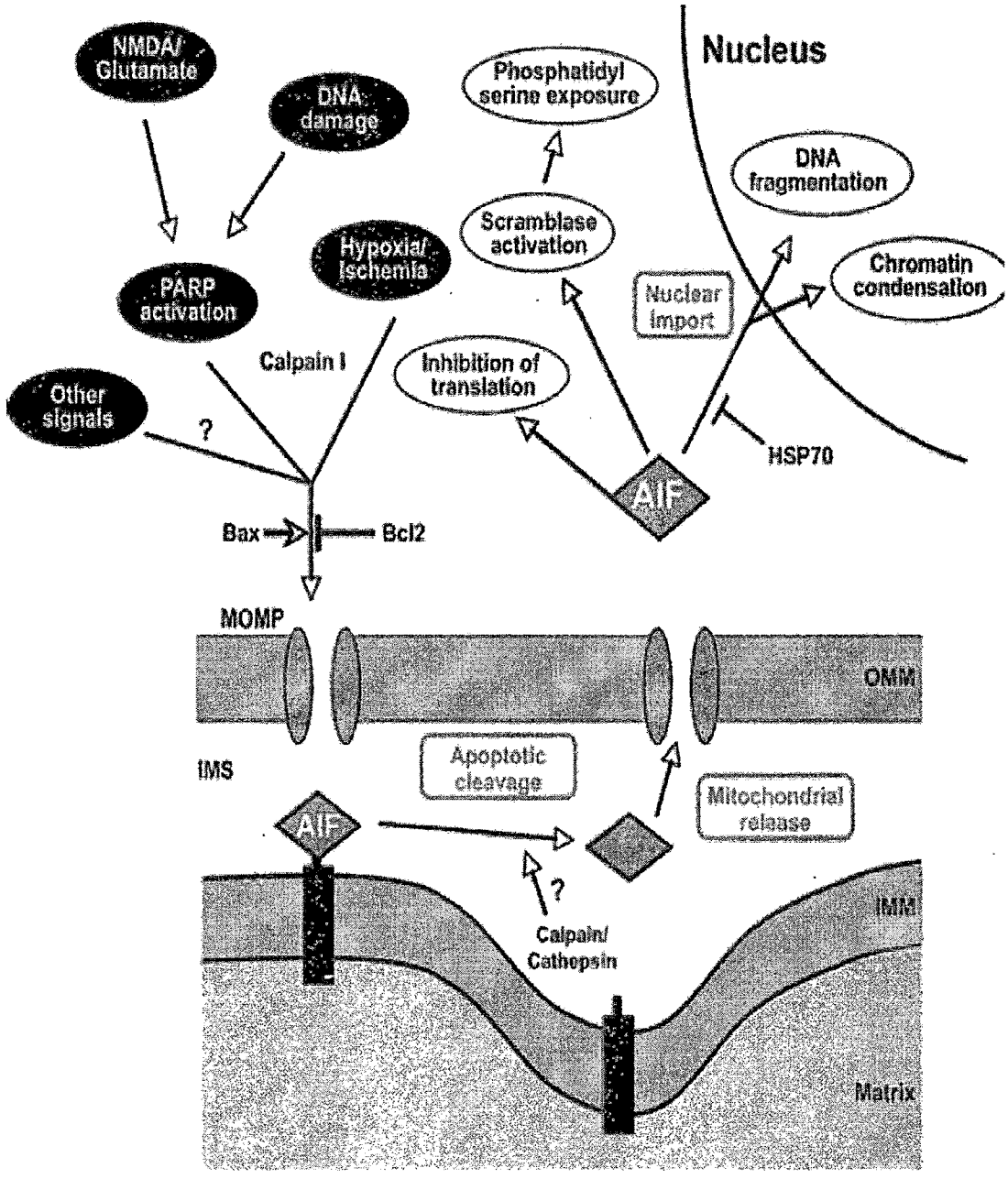
DNA degrading poly-protein complex causes large scale DNA fragmentation, yielding ~50 kbp DNA fragments (Fig. 1.13). The AIF-DNA interaction displays cooperativity. Also DNA binding and condensation by AIF requires the presence of Mg^{2+} and are stimulated by the addition of $NADP^+$. This strong influence by $NADP^+$ depicts the juncture at which apoptotic catabolism and redox metabolism meet.



Modjtahedi *et al.*, 2006

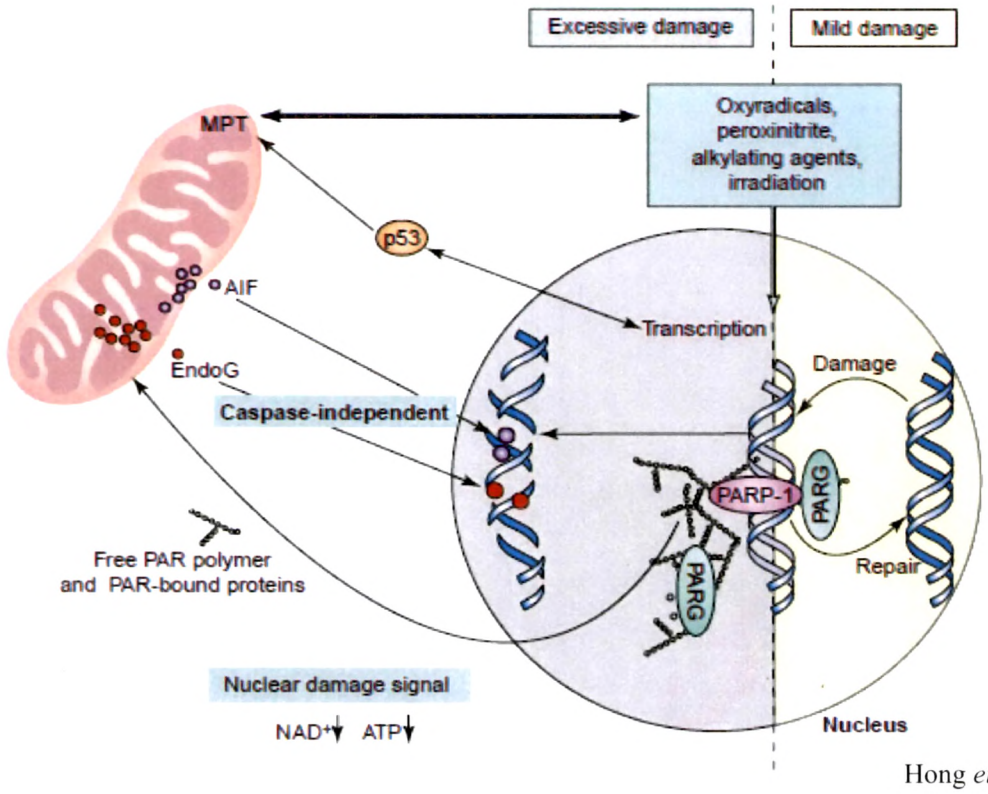
Figure 1.13: Speculative model of large scale DNA fragmentation caused by AIF.

In *C. elegans*, AIF leads to externalization of phosphatidyl serine at the plasma membrane, a potent "eat me" signal, *via* activation of a lipid translocase scramblase 1 (SCRM-1). AIF may also inhibit the translation protein eIF3G to block protein synthesis (Fig. 1.14) (Joza *et al.*, 2009). Thus AIF like many other proteins is a multifunctional protein.



Joza *et al.*, 2009

Figure 1.14: Overview of the AIF story within a cell before and after release from mitochondria.



Hong *et al.*, 2004

Figure 1.15: Caspase-independent cell death in poly(ADP-ribose) polymerase PARP mediated cell death.

As shown in the figure (Fig. 1.15) DNA-alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine, hydrogen peroxide (H_2O_2) and NMDA might activate the mitochondrial permeability transition (MPT), which leads to the release of AIF. Mild DNA damage activates repair machinery through PARP activation. However, massive DNA damage will induce PARP-1 over activation which in turn induces nuclear cell-death signaling (decreased NAD^+ and ATP) causing apoptosis inducing factor (AIF) to translocate from mitochondria to the nucleus. It is possible that AIF and EndoG act together to mediate caspase-independent cell death (Wang *et al.*, 2002).

PARP activators induce the translocation of AIF from mitochondria to the nucleus, nuclear condensation, annexin-V staining, dissipation of the mitochondrial membrane potential and cell death. Simultaneosly, Poly (ADP-ribose) glycohydrolase (PARG) degrades poly(ADP-ribose) (PAR) polymers, generating free PAR polymer and ADP-ribose. Each of these events is caspase independent as broad-spectrum caspase inhibitors do not block them (Yu *et al.*, 2002). However, each process is prevented by the presence of PARP inhibitors and in PARP knockouts. Neutralizing antibodies to AIF

block PARP-dependent cell death (Yu *et al.*, 2006). Taken together, these results indicate that AIF is an essential downstream effector of the cell death program initiated by PARP.

A *Dictyostelium* homolog of mammalian AIF is localized into mitochondria and is translocated from the mitochondria to the cytoplasm and the nucleus after the onset of cell death. Cytoplasmic extracts from dying *Dictyostelium* cells trigger the breakdown of isolated HeLa and *Dictyostelium* nuclei in a cell-free system, and this process is inhibited by a polyclonal antibody specific for *D. discoideum* apoptosis-inducing factor (*Dd*AIF), suggesting that *Dd*AIF is involved in DNA degradation during *Dictyostelium* cell death (Arnoult *et al.*, 2001). Thus AIF protein is evolutionarily conserved in the cell death pathway of a unicellular eukaryote, *Dictyostelium*.

1.3. *Dictyostelium discoideum*

D. discoideum often referred to as "slime mold" or "social amoeba", is one of the simplest studied eukaryotes that possesses true multicellularity (Raper, 1984). The cellular slime molds were formerly considered to be 'lower fungi.' Although they superficially resemble fungi in certain respects they are included in the kingdom Protista. Individual cells resemble small amoebae and move and feed in an amoeboid manner, hence they are called 'myxamoebae' (to distinguish them from true amoebae). *D. discoideum* can be found in soil and moist litter leaves. The primary diet of *D. discoideum* consists of bacteria such as *Klebsiella, E.coli* etc. that are found in soil. These bacteria secrete folic acid which attracts amoebae. Under good nutritive conditions amoebae remain unicellular and grow as primitive animal like cells either on bacteria (wild type) or in a semi defined growth medium used for the axenic laboratory strains. In addition to possessing complex individual cellular functions like a unicellular eukaryote, *D. discoideum* cells during conditions of amino acid starvation face the challenge of multicellular development and undergo a relatively simple differentiation process by the cAMP mediated pathway. Despite this relative simplicity, the regulatory signaling pathways are as complex as those seen in metazoan development. Cell cycle phase is an important determinant for differentiation of *D. discoideum,* as cells must reach a specific stage to enter into developmental phase and specific cell cycle regulators are involved in arresting growth phase genes and inducing the developmental genes.

D. discoideum development

D. discoideum has an intriguing way of becoming multicellular, under nutrient depleted conditions. This represents a developmental transition which is absent in most other multicellular lineages. The process involves the aggregation of individual cells followed by well orchestrated movements to spatially organize the cell types. Starvation initiates the creation of biochemical machinery which includes glycoproteins and adenylyl cyclases (Gilbert, 2006). The glycoproteins allow for cell-cell adhesion and adenylyl cyclases synthesize cyclic AMP. Cyclic AMP is secreted by the amoebae to attract neighboring cells to a central location. As they move towards the signal, they bump into each other and stick together using glycoprotein adhesion molecules.

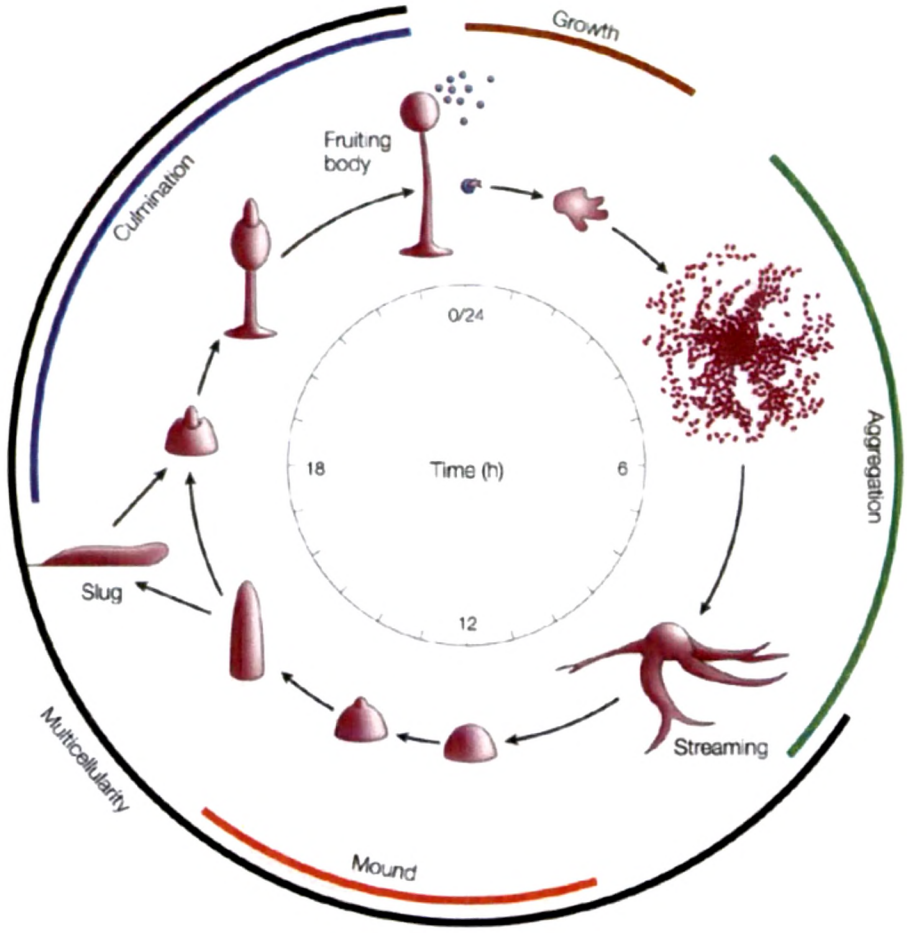
Starving cells stream together by chemotaxis towards autocrine signals and form aggregates that contain 10^5 cells, to form a multicellular mass, the mound (tight aggregate). The driving force behind this process is chemotaxis towards a pulsatile extracellular cAMP source (Roos *et al.*, 1975). When cells form an aggregate, cAMP concentration is thought to rise to the micromolar range (Abe *et al.*, 1983). During aggregation, oscillatory waves of cAMP are generated from the center of the aggregating territory and are propagated towards neighboring cells. Initially, amoebae move as individual cells towards the signal, however, as they reach near the source and cell density increases, cells coalesce into multicellular streams (Fig. 1.16).

A transcriptional cascade is activated, leading to the emergence of different cell types that self organize within the aggregate (Firtel, 1995, Kimmel and Firtel, 1991, Loomis, 1996). After about 6-8 hours of starvation, a flat loose aggregate is formed with indistinct borders. A sheath of mucopolysaccharide and cellulose is laid on a group of cells to form a tight aggregate or mound (Wilkins and Williams, 1995). During development, 20% cells differentiate into prestalk and the remaining 80% differentiate to form prespore cell types, in specified ways, and form a slug. Relative proportion of prestalk cells within slugs varies between 10-30% depending on slug size and shape (Rafols *et al.*, 2001). Differentiation inducing factor (DIF) induces stalk cell differentiation in *D. discoideum* and acts as the morphogen in the generation of the prestalk/prespore pattern during development (Masento *et al.*, 1988). The slug undergoes transient or prolonged migration depending on the environmental conditions. Recent work has focused that spatial gradients of DIF does not act as the primary signal for cell type choice (Thompson and Kay, 2000). Rather, the choice of cell type appears to rest on a

basis that is quite the opposite of morphogen dependent spontaneous patterning, i.e. the existence of functional differences, in the form of preexisting heterogeneities, between the members of an apparently homogeneous cell mass. Fate of the cells is predetermined during unicellular stage only. Pre aggregation amoebae can differ in many ways, which include nutritional status, cell size, cell cycle phase at starvation, cellular calcium content (Nanjundiah, 1997) and sensitivity to DIF. The cell cycle phase at starvation has also been implicated in determining the fate i.e. amoebae in S and early G2 phases at starvation exhibit a prestalk tendency (Weijer *et al.,* 1984, Mcdonald and Durston, 1984, Gomer and Firtel, 1987). Thus Calcium concentration and the cell cycle phase at the time of starvation decide the cell fate. High Ca^{2+} levels during S-phase is not required for cell cycle progression but for cell type choice mechanism at the onset of starvation, and these cells tend to follow the prestalk pathway while cells with low Ca^{2+} levels tend to form prespore (Azhar *et al.,* 1997, Saran, 1999). Cell fate in *D. discoideum* is thus decided based on intercellular heterogeneity as the primary factor behind cell fate choice.

After a variable period of migration, the slug settles at one place and cells near the tip form a sheath within which the cells expand and vacuolize to form the stalk, and then extends vertically. The posterior end spreads out with the anterior end raised in the air, forming what is called the "Mexican hat," and the culmination stage begins. The prestalk cells and prespore cells switch positions in the culmination stage in order to form the mature fruiting body. The anterior end of the Mexican hat forms a cellulose tube, which allows the more posterior cells to move outside of the tube to the top, and the prestalk cells move down. This rearrangement forms the stalk of the fruiting body made up of the cells from the anterior end of the slug, and the cells from the posterior end of the slug are on the top and now form the spores of the fruiting body. Thus spore is supported by a skeleton of dead cells that are arranged as a stalk and a basal disc, which anchors the stalk to the substratum.

When the spores are dispersed, under favorable conditions, they germinate by splitting the spore case longitudinally and escaping as small but normal amoebae. This complex series of stages give a two fold selective advantage to the organism i.e. to permit the dispersal of cells from an area in which they are starving and to provide a dormant stage to resist unfavorable conditions.



Fey *et al.*, 2007

Figure 1.16: Life cycle of *D. discoideum*.

1.3.1. Molecular aspects of *D. discoideum* development

The process of aggregation bridges the feeding unicellular form of *D. discoideum* to a starving multicellular form. Many biosynthetic genes expressed at growth stage are downregulated and genes involved in development are upregulated (Mir *et al.*, 2007). Amino acid starvation represses the development of these amoebae (Marin, 1976). Recent investigations have revealed several components involved in regulating the initiation of development (Souza *et al.*, 1999, Kon *et al.*, 2000, Zeng *et al.*, 2000), however little information exists on how the cells exactly sense starvation and in particular amino acid deprivation. Studies have now implicated the Target of Rapamycin (TOR) pathway in the process of sensing these two nutrients (Lee *et al.*, 2005).

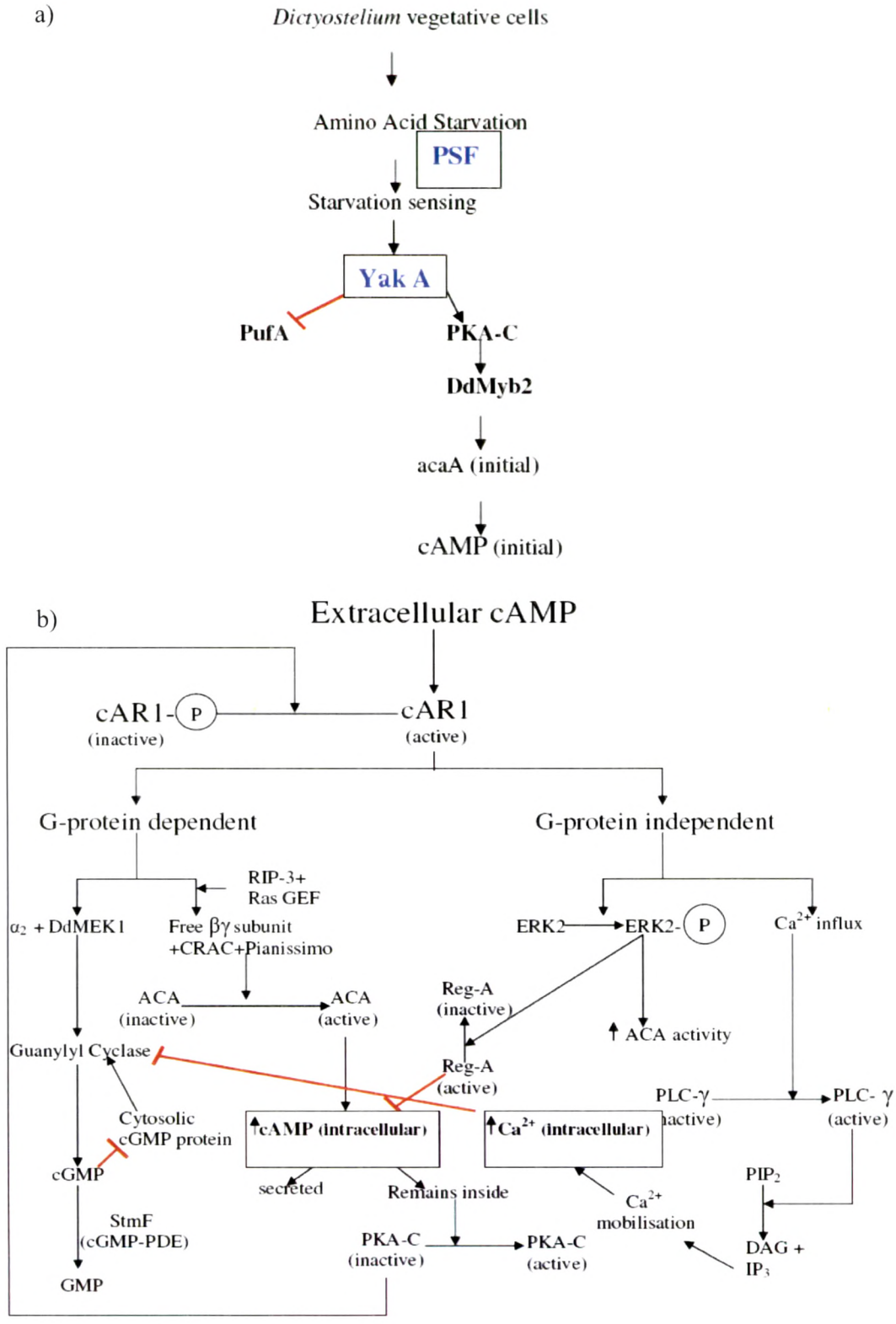
Cell cycle arrest

Upon amino acid depletion, *D. discoideum* cells undergo cell cycle arrest. YakA, a serine/threonine protein kinase governs this transition by regulating the cell cycle, repressing growth phase genes and inducing developmental genes. *yakA* is induced by starvation and its expression is controlled by an extracellular prestarvation factor (PSF) that accumulates during vegetative growth (Fig. 1.17a).

YakA mediates the initiation of development by repressing *pufA* expression. PufA, a translational regulator inhibits catalytic subunit of cAMP-dependent protein kinase A (PKA-C) translation by binding to a region at the 3'end of the *PKA-C* mRNA (Souza *et al.*, 1999). Thus, YakA acts as a regulator switch between vegetative and developmental gene expression by relieving the negative control on PKA-C expression, which in turn activates transcription factor DdMyb2 and all further downstream events which include activation of adenylyl cyclase leading to production of the differentiation inducing signal, cAMP. The adenylyl cyclase gene *aca*A is one of the first genes expressed upon starvation. ACA produces extracellular cAMP that induces chemotaxis and aggregation in neighboring cells. Intercellular signaling by secreted cAMP then induces the expression of another set of genes for further stages of development. Therefore the components that mediate the induction of adenylyl cyclase have the central role in the growth/development transition in *D. discoideum.*

Cell density sensing

Starvation and a threshold of cell density are the two known prerequisites for the transition from growth to development. Two secreted proteins, prestarvation factor (PSF) and conditioned medium factor (CMF) are involved in sensing cell density (Burdine and Clarke, 1995). PSF, the autocrine factor is a 68 kDa glycoprotein that is secreted when cells are growing and accumulates as an indicator of the ratio of the cell density relative to the food supply. PSF helps cells determine density with respect to each other as well as the bacterial population. When the bacterial population drops, the PSF inhibition is relieved, and PSF induces genes that trigger the developmental process. Once the nutrients are depleted, PSF production declines and a second cell density sensing pathway mediated by CMF is activated. CMF, a 80 kDa glycoprotein essential for early development (Yuen *et al.*, 1991, Yuen *et al.*, 1995, Jain *et al.*, 1992) is sequestered in vegetative cells but is secreted upon starvation.



Mir *et al.*, 2007

Figure 1.17: A schematic description: a) The pathway controlling the onset of *D. discoideum* development. b) The pathway induced by cAMP during *D. discoideum* development.

A critical concentration of extracellular CMF is required for subsequent development, as it is involved in regulating aggregation, cAMP pulsing and early developmental events (Gomer *et al.,* 1991, Yuen *et al.,* 1995, Jain and Gomer, 1994).

Cell size regulation

Countin proteins are thought to control the size of the multicellular structure in *D. discoideum* since the *countin* ¯ strain forms a huge fruiting body and *countin* 2 ¯ strain forms a small fruiting body. Countin is required for the proper function of a cell counting mechanism that regulates organism size in *D. discoideum* (Brown and Firtel, 2000). A field of starving amoebae first breaks up into territories. In each territory, the cells form a spider like pattern of streams of cells. As part of a negative feedback loop, counting factor (CF), a secreted protein complex whose concentration increases with the size of the stream, prevents over sized fruiting bodies being formed by increasing cell motility and decreasing cell-cell adhesion, which causes the breakup of excessively large streams (Brock and Gomer, 1999, Brock *et al.*, 2002, 2003, 2006). Thus complex counting factor regulates group size in *D. discoideum.*

Components of the cAMP relay system

The cells achieve competence to relay cAMP signals within a period of 6 hours. cAMP secreted by a starving cell in a pulsatile manner acts as the first messenger and activates several pathways in the cell itself as well as in surrounding cells through cAR mediated signal transduction (Sun and Devreotes, 1991). Initially at lower concentration of cAMP, the receptors undergo excitation leading to a cascade of processes, but as cAMP concentration rises extracellularly, the receptors become desensitized due to the modification/ sequestration/ internalization/ degradation of receptors/ uncoupling of receptor and target proteins etc. The reversal of desensitization is facilitated by removal of cAMP by extracellular phosphodiesterase (ePDE) (Fig. 1.17b).

cAMP receptors

D. discoideum also provides a powerful system for studying the role of receptors in controlling the dynamics of cell-cell signaling during multicellular development. The starvation induced chemotactic aggregation of individual cells is controlled by propagating waves of the chemoattractant cyclic AMP. During development the cAMP signal is detected and transduced by a family of at least four cAMP receptors (cAR1-cAR4), which differ in their expression levels and pattern. The cAMP receptor types

expressed sequentially during development have decreasing affinities for cAMP possibly to enable the organism to cope with an increase in extracellular cAMP concentration during the formation of the multicellular structures (Winckler *et al.*, 2004). The high affinity receptor cAR1 is the first to be expressed during early aggregation; it is the primary receptor responsible for aggregation since cells lacking cAR1 fail to aggregate. cAR1 continues to be expressed later during development in all the cells. During later aggregation a small number of cAR3 receptors are expressed. In the slug, the expression of cAR3 becomes confined to the prespore cells (Saxe *et al.*, 1993). cAR2 is first expressed at the mound stage where it is restricted to the cells that form prestalk zone. cAR4 is expressed in prestalk specific manner in slug stage.

cAMP upon binding to cAR1 transduces the signal to a trimeric G protein complex ($\alpha_2\beta\gamma$). Dissociated $\beta\gamma$ complex activates phosphatidylinositol-3 kinase (PI3K). PI3K converts phosphatidylinositol (4,5) bisphosphate (PIP2) in the plasma membrane into phosphatidylinositol (3,4,5) triphosphate (PIP3). PIP3 binds to the pleckstrin homology domain of cytosolic regulator of adenylyl cyclase (CRAC), which translocates from cytosol to membrane where it can activate adenylyl cyclase A (ACA) (Fig. 1.17b). Positive feedback causes an increase in cAMP secretion, till cARs get adapted and do not activate ACA further. Receptor phosphorylation could be the reason for receptor adaptation. The resulting drop in cAMP production combined with an active degradation mediated by phophodiesterase A (PdsA) terminates the loop. Many proteins like PdsA inhibitor (PDI), mitogen activated protein kinase (MAPK), Ras etc. play important role in this oscillatory network.

Adenylyl cyclases

Genes encoding three distinct adenylyl cyclases have been characterized and shown to be expressed at different stages of development in *D. discoideum*. G-protein coupled adenylyl cyclase A (ACA) produces extracellular cAMP. The osmosensory adenylyl cyclase, *acg* is expressed only during germination of spores (Pitt *et al.*, 1992, Van *et al.*, 1996). The other adenylyl cyclase gene, *acr*A encodes a protein (ACR) with domains related to histidine kinases and response regulators, while the C terminal portion contains the adenylyl cyclase catalytic domain. ACR is present at low levels in vegetative cells but accumulates dramatically following aggregation. The temporal expression of these genes may partially account for their stage specificity e.g. ACA is activated when

the G-protein coupled surface receptor cAR1 binds extracellular cAMP (Fig. 1.17b) however, during culmination, ACR activity is tenfold higher than ACA activity.

Protein kinase A (PKA)

cAMP-dependent protein kinase (PKA) levels increase several fold during differentiation in *D. discoideum*. PKA is a heterodimer containing the single catalytic subunit. PKA-C associated with a single regulatory subunit PKA-R (Mann *et al.*, 1992, Simon *et al.*, 1992). PKA is required for the regulation of early developmental genes, such as *aca* and *car*1, and plays a key role in cell-type differentiation. Cells lacking PKA-R are constitutively active for PKA, and early developmental genes are induced prematurely (Zhang *et al.*, 2003). The MAPK Erk2 is also required for ACA activation and is activated by cAMP but the mechanism is unknown (Schenk *et al.*, 2001, Segall *et al.*, 1995).

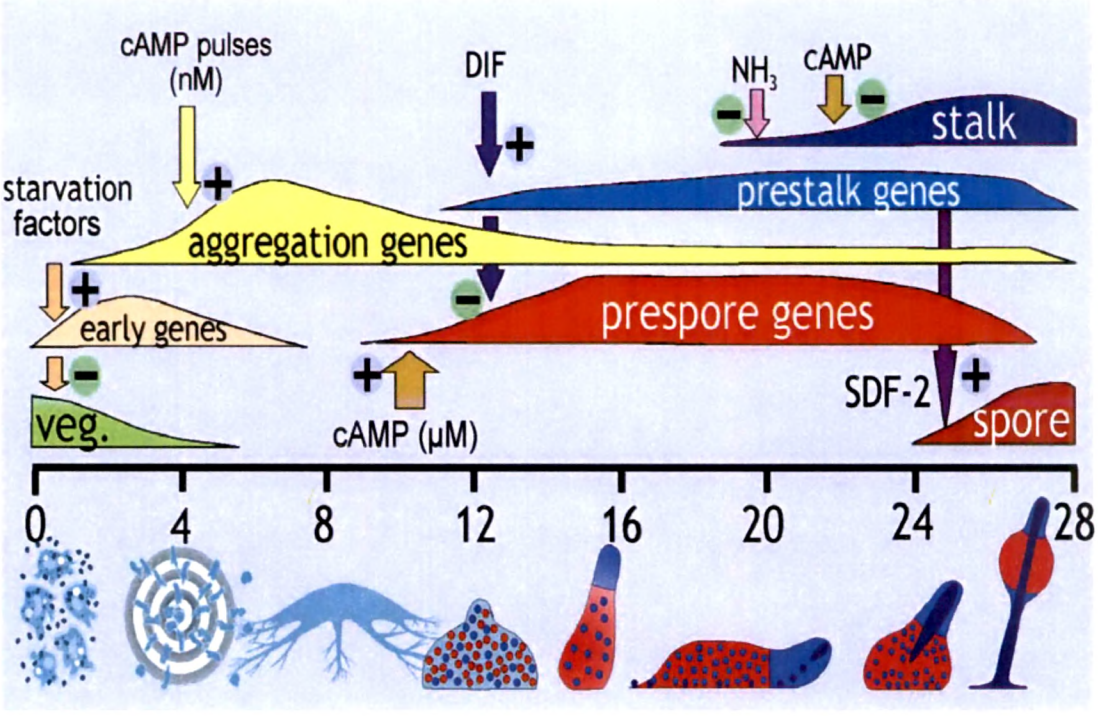
The cAMP signaling apparatus also includes polarization acquisition, actin polymerization, and cortical myosin modules, which describe the ability of a cell to create well defined anterior and posterior regions. This signaling is mediated by several kinases, one such is MEK located in cytosol and translocates to the plasma membrane. Cells lacking MEK1 develop and produce cAMP normally, but respond to chemoattractant with attenuated movements (Ma *et al.*, 1997).

Gene regulation by cAMP pulses

Oscillations in extracellular cAMP levels are essential for developmental initiation of *D. discoideum* cells. Strains that fail to produce cAMP pulses do not aggregate, but can be induced to do so by exogenous addition of cAMP pulses. Interestingly these strains can also be rescued by expression of the *pka*C (Wang and Kuspa, 1997). Many of the genes are induced by cAMP oscillations, like *cAR1*, *Myb2* which are implicated in *aca*A induction (Otsuka and Van Haastert, 1998) and also *dia2* (Hirata *et al.*, 2008), *countin*, *cs*A (contact site A) etc. cAMP also induces a set of five genes which code for Ca^{+2} binding proteins (*cbp*). cAMP oscillations and induction of developmental genes result in cell polarization, pseudopod formation, chemotaxis, cell -cell adhesion and further downstream events.

A developmental switch occurs in response to extracellular cAMP concentration within the aggregate leading to the downregulation of the aggregation stage pathways and the activation of post aggregative gene expression (Kimmel and Firtel, 1991, Williams,

1991). This is followed by the induction of cell type differentiation genes including the transcription factor GBF (G-box binding factor) and the cell surface signaling molecule LagC (Dynes *et al.*, 1994, Reymond *et al.*, 1986). The cAR1 activation of GBF-mediated pathways requires µM cAMP concentration and conditions in which the receptor is fully saturated. Figure 1.18 gives overall idea regarding the developmental gene expression.



http://www.lifesci.dundee.ac.uk/groups/pauline_schaap/generegulation.htm

Figure 1.18: Overview of gene regulation by extracellular signals during *D. discoideum* development. PSF and CMF (Clarke *et al.*, 1992) trigger the switch to multicellular development. These factors induce full expression of the early genes and induce a low rate of expression of aggregation genes, such as the cAMP receptor cAR1 and adenylyl cyclase A, which allow the cells to secrete and detect the chemotactic signal cAMP. The cells start to secrete cAMP pulses, which in turn strongly accelerate the expression of the aggregative genes (Darmon *et al.*, 1975). Thus the aggregation machinery is very rapidly turned on to full capacity. After aggregation, cAMP oscillations continue. The cells are now very close together and higher (micromolar instead of nanomolar) cAMP concentrations can accumulate. µM cAMP triggers expression of the prespore genes (Wang *et al.*, 1988) and the prespore cells in turn synthesize DIF (Thompson and Kay, 2000). DIF then induce the expression of a subclass of prestalk genes. In a later stage of development cAMP inhibits expression of the stalk genes (Berks and Kay, 1988). The prestalk to

stalk transition is also inhibited by ammonia (NH_3). Loss of ammonia occurs locally when the tip extends upwards during early culmination. This allows stalk cells to differentiate at the tip (Wang and Schaap, 1989). The prestalk cells secrete the spore differentiation factor SDF-2 which causes the prespore cells to mature into spores (Anjard *et al.*, 1998).

1.3.2. *D. discoideum* as a model organism

Because of the simple life cycle of *D. discoideum*, it is commonly used as a model organism to study various aspect of the cell. One of the incredible strengths of this model system is the capacity to track the dynamic behavior of individual cells, yet can function as a collection of cells. This makes *D. discoideum* the best of both the worlds – an easily studied single cell organism for one half of its life cycle, and a multicellular organism during the other half, such that it can be used to understand the fundamental principles of cell behavior. It can be observed at organismic, cellular, and molecular levels primarily because of their restricted number of cell types, behavior, and rapid growth. The genome repertoire of *D. discoideum* allows the expression of features like cell type determination, spatial patterning, chemotaxis, altruistic cell death and other fundamental aspects essential for multicellular organisms (Eichinger and Noegel, 2003, Ennis *et al.*, 2003). It is also used to study other aspects of development including cell sorting, pattern formation, phagocytosis, motility, and signal transduction. These processes and aspects of development are either absent or too difficult to view in other model organisms. *D. discoideum* is closely related to higher metazoans. It carries similar genes and pathways making it a good candidate for gene knockout studies (Landree and Devreotes, 2004). *D. discoideum* makes a valuable model also because of a small genome compared to the higher eukaryotes. The *D. discoideum* genome is approximately 40 Mbp, with approximately 10,000–13,000 genes on six chromosomes. Genome haploidy makes it relatively easy to generate and select mutants. Advantages of *D. discoideum* as a model organism include its genetic, biochemical, and cell biological accessibility. Genetic tools such as gene targeting by homologous recombination; structure function analysis by random mutagenesis; insertional mutagenesis, which allows easy cloning of the gene disrupted; and multicopy suppression libraries are available (Landree and Devreotes, 2003). The vegetative growth and developmental forms in *D. discoideum* are temporally separated; the developmental mutants can be propagated under vegetative conditions thus behaving like conditional mutants. The discoveries made in this model organism will

provide insights into the working of other organisms. We have mainly exploited this organism to study a typical cell death i.e. paraptosis.

1.3.3. *D. discoideum* and Programmed Cell Death

Cell death occurs in many places along the phylogenetic tree other than in animals. Investigation of cell death in other organisms may reveal phenomenological convergence or molecular conservation and thus yield invaluable comparative information (Golstein, 1998). The slime mold *D. discoideum* is an early conditional multicellular organism that shows developmental cell death. It has been observed by many workers that *D. discoideum* demonstrates cell death mechanism similar to that seen in some of the higher eukaryotes. Another ontogenic reason being the relatively simple pattern of development in this organism facilitates the study of cell death that occurs during development. There are methods that allow triggering *in vitro* differentiation without morphogenesis and thus facilitate the isolation of dying cells for study (Kay *et al.*, 1987).

D. discoideum during starvation induced developmental process exhibits PCD in the 20% stalk cell population. *D. discoideum* exhibits caspase independent type of cell death (paraptosis) during its development which occurs even in the presence of caspase inhibitors. Paraptosis is characterized by the absence of oligonucleosomal DNA fragmentation. Developmental cell death requires starvation and presence of DIF (Cornillon *et al.*, 1994). Also *Dictyostelium* cells in conditioned medium undergo cell death that shares essential features with mammalian cell apoptosis. This involves loss of mitochondrial membrane potential (Ψm), resulting in the release of AIF from the mitochondria (Arnoult *et al.*, 2001). Stationary phase cells also exhibit similar kind of features (Tatischef *et al.*, 2001). It has been documented that *D. discoideum* undergoes an 'apparent' caspase independent programmed cell death (Olie *et al.*, 1998). The stalk cells show massive vacuolization, prominent cytoplasmic condensation and focal chromatin condensation (Olie *et al.*, 1998). The *D. discoideum* vacuolar cell death pathway does not require cellulose synthesis and includes early actin rearrangements (F-actin segregation, then depolymerization); contemporary with irreversibility, corresponding to the emergence and demise of highly polarized paddle cells (Levraud *et al.*, 2003). Contradictory observations have been made for the cell death in stalk cell in the presence of caspase inhibitors. Simbulan *et al.*, (1999) showed no effect on stalk cell death while on the other hand though caspase inhibitors did not inhibit cell death they were observed

to impair development in *D. discoideum*. Also these inhibitors show dose dependent increase in percent of stalkless fruiting bodies (Olie *et al.*, 1998).

According to Kawli *et al.*, (2002) there is no internucleosomal cleavage of DNA in *D. discoideum*. However, nuclear condensation and peripheralization does occur in stalk cells. It was also shown that the fraction of cells showing caspase 3 like activity increases and reaches a maximum of around 25 % in the slug stage correlating with proportion of stalk cells (Kawli *et al.*, 2002). Thus, cell death in *D. discoideum* shows some, but not all, features of apoptotic cell death as recognized in other multicellular systems. The molecular mechanism underlying this kind of cell death is yet to be understood (Kawli *et al.*, 2002).

Blast search results suggested that *D. discoideum* has a paracaspase gene, no metacasapase and a caspase gene. The paracaspase null mutants showed undiminished cell death *in vivo* and *in vitro*, in addition paracaspase inactivation led to no alteration in development. Thus programmed cell death does not require paracaspase (Bouffay *et al.*, 2004). In this way *D. discoideum* shares ancestry in some of the molecular mechanisms of cell death with mammalian cells and is a good model system to characterize paraptotic cell death. Thus the social amoeba *D. discoideum*, a powerful paradigm provides clear insight into the regulation of growth and development.

In view of the above *D. discoideum* is an excellent model system to study the role of PARP and AIF in caspase independent cell death.

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Objectives

- Role of Poly {ADP-Ribose} Polymerase (PARP) in UV-C induced cell death in *D. discoideum.*
- Role of Poly {ADP-Ribose} Polymerase (PARP) during starvation induced cell death in *D. discoideum.*
- PARP in staurosporine induced cell death in *D. discoideum.*
- Response of *Dictyostelium discoideum* to UV -C and involvement of PARP.
- cAMP mediated chemotaxis during UV-C induced *D. discoideum* developmental changes.
- Down-regulation of AIF in *D. discoideum.*