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Review

An Apoptotic 'Eat Me' Signal: Phosphatidylserine Exposure

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Apoptosis and the clearance of apoptotic cells are essential processes in animal development and homeostasis. For apoptotic cells to be cleared, they must display an 'eat me' signal, most likely phosphatidylserine (PtdSer) exposure, which prompts phagocytes to engulf the cells. PtdSer, which is recognized by several different systems, is normally confined to the cytoplasmic leaflet of the plasma membrane by a 'flippase'; apoptosis activates a 'scramblase' that quickly exposes PtdSer on the cell surface. The molecules that flip and scramble phospholipids at the plasma membrane have recently been identified. Here we discuss recent findings regarding the molecular mechanisms of apoptotic PtdSer exposure and the clearance of apoptotic cells.

Phosphatidylserine Exposure during Apoptosis

Every day, billions of senescent or damaged cells in the body undergo **apoptosis** (see Glossary) (Box 1) and are cleared by macrophages. Macrophages engulf apoptotic but not healthy cells [1], suggesting that apoptotic cells expose an 'eat me' signal to **phagocytes**. Many 'eat me' signals have been proposed, including **phosphatidylserine** (**PtdSer**), carbohydrates (amino sugars or mannose), intercellular adhesion molecule-3 (ICAM3), and calreticulin [2]. Of these, PtdSer is the most studied and the most likely 'eat me' signal candidate. PtdSer normally localizes to the inner leaflet of the plasma membrane, but is exposed to the cell surface during apoptosis [3]. Red blood cells that are heavily loaded with PtdSer are recognized and engulfed by macrophages [4]. However, apoptotic cells are not engulfed by macrophages either *in vitro* or *in vivo* when PtdSer is masked by PtdSer-binding proteins or competed with PtdSer-bearing liposomes [5,6]. Although PtdSer exposure and PtdSer-dependent apoptotic cell engulfment are evolutionarily conserved from lower organisms (*Caenorhabditis elegans* and *Drosophila*) to mammals [7,8], the molecular mechanism that externalizes PtdSer to the plasma membrane during apoptosis has only recently been identified.

Flippases in the Plasma Membrane

In healthy cells, phospholipids are maintained asymmetrically in the plasma membrane [3]. The amine-containing lipids, PtdSer and phosphatidylethanolamine (PtdEtn), are localized to the cytoplasmic leaflet of the plasma membrane, while phosphatidylcholine (PtdCho) and sphingomyelin are concentrated in the exoplasmic leaflet. ATP-dependent aminophospholipid translocase(s) or flippase(s) are proposed to establish this asymmetrical phospholipid localization by transporting PtdSer and PtdEtn from the outer to the inner leaflet of the lipid bilayer (Box 2).

Candidate flippases include type IV **P-type ATPases** (P4-ATPases); this large family has 15 murine and 14 human members of membrane proteins carrying ten transmembrane segments [9]. CDC50 functions as a chaperone for the proper subcellular localization of P4-ATPases, and is necessary for their lipid transport activity [10]. Flippase activity was originally found in bovine chromaffin granules, and ATPase II, a P4-ATPase (now called ATP8A1), was surmised to function as a flippase [11]. It was also reported that Drs2p, a yeast ortholog of ATP8A1,

Trends

Flippases translocate phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) from outer to inner leaflets of the plasma membrane, keeping these lipids in the inner leaflet. Recent data indicate that ATP11C, a member of P4-type ATPases, together with CDC50A (cell division cycle protein 50A), functions as a flippase.

Scramblases nonspecifically and bidirectionally translocate phospholipids between inner and outer leaflets of the plasma membranes. Members of TMEM16 and Xkr families were shown to support Ca^{2+}- and caspase-dependent scrambling of phospholipids, respectively.

When cells undergo apoptosis, PtdSer is exposed to work as an 'eat me' signal. Recent data indicate that for apoptotic PtdSer exposure, caspases inactivate the flippase, and activate the scramblase by cleavage.

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Box 1. Caspase Activation during Apoptosis

Apoptosis is executed by two distinct intrinsic and extrinsic pathways [97]. Caspases, which are cysteine proteases, are activated by both intrinsic and extrinsic pathways to execute apoptosis. In the intrinsic pathway, which is activated during animal development or by genotoxic stimuli (antitumor drugs or γ-rays), the upregulation of BH-3 proteins of the Bcl-2 family triggers Bax/Bak oligomerization. The oligomerized Bax/Bak causes mitochondria to release cytochrome c, which forms a complex with Apaf-1 to activate caspase 9. This leads to the sequential activation of the downstream effector caspases, caspase 3 and 7. The extrinsic pathway is initiated by death ligands such as Fas ligand (FasL), tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL). The engagement of FasL with its receptor (Fas) assembles the death-signaling complex (DISC), which is composed of Fas, an adaptor protein (FADD), and procaspase 8. Procaspase 8, which is processed to its active form in the DISC, transduces death signaling via two different pathways. Type I cells (e.g., thymocytes) produce large amounts of active caspase 8, which directly activates caspase 3. By contrast, caspase 8 activation is weak in type II cells such as hepatocytes, in which active caspase 8 cleaves a BH3-only protein Bid to activate Bax/Bak and trigger the activation of downstream effector caspases. More than 500 substrates are cleaved by effector caspases [98], which are responsible for DNA fragmentation [99], membrane blebbing [100], nucleotide release [101], and PtdSer exposure.

was responsible for flipping PtdSer in plasma membranes [12]. However, Drs2p mainly localizes to intracellular vesicles of the trans-Golgi network [13]; therefore, ATP8A1 and Drs2p likely flip PtdSer in intracellular vesicles rather than in plasma membranes. By contrast, Dnf1p and Dnf2p predominantly localize to the plasma membrane in yeast, and promote translocation of PtdSer, PtdEtn, and PtdCho [14]. The exposure of PtdEtn on the plasma membrane is increased in the mutants lacking both *dnf1* and *dnf2*, supporting their essential roles as flippases [14].

By genetic screening with the human near-haploid cell line KBM7, the P4-ATPase family member ATP11C and its chaperone **CDC50A** were found to be required for flipping PtdSer and PtdEtn at the plasma membrane [15]. ATP11C localizes to the plasma membranes in a CDC50A-dependent manner, and ATP11C deficiency severely reduces the PtdSer and PtdEtn flippase activity in WR19L (mouse T-lymphoma cell line) and KBM7 (human myeloma cell line) cells. However, ATP11C-null cells maintain an asymmetrical PtdSer distribution even though flippase activity is reduced by approximately 80%, suggesting that factors other than ATP11C help generate PtdSer asymmetry. Consistent with this possibility, asymmetrical PtdSer distribution is normal in the plasma membrane of ATP11C-deficient mouse pro-B cells and thymocytes, where PtdSer flippase activity is significantly reduced [16].

By contrast, CDC50A-deficient KBM7 and WR19L cells lose their PtdSer flippase activity at plasma membranes, and constitutively expose PtdSer to the cell surface [15]. CDC50A functions as a chaperone for multiple P4-ATPases, from yeast to mammals [17,18], and KBM7 and WR19L cells express several P4-ATPases. It is likely that ATP11C directly flips PtdSer at the plasma membrane and is responsible for most of the PtdSer flippase activity in WR19L and KBM7 cells, while other molecules have weak, but enough, flippase activity to maintain asymmetrical PtdSer distribution. These findings suggest that CDC50A-regulated molecules with weak flippase activity exist. Furthermore, these molecule(s) may not localize to the plasma membrane, since a flippase located at trans-Golgi network might still regulate asymmetrical PtdSer distribution at the plasma membrane [19]. CDC50A-overexpressing ATP11C-deficient human and mouse cell lines in which the flippase activity is strongly reduced are available [15]. The question of whether other P4-ATPases have flippase activity can be addressed by introducing the 14 human P4-ATPases individually into these cell lines, and by assaying their ability to incorporate fluorescent labeled PtdSer.

ATP11C belongs to the P4-ATPase subfamily of the P-type ATPases [9]. Among P-type ATPases, the tertiary structures of P2-type ATPases, including sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), Na^+,K^+-ATPase, and H^+, K^+-ATPase, have been studied the most [20–22]. Na^+,K^+-ATPase and H^+,K^+-ATPase are heterodimers consisting of a P-type ATPase catalytic α-subunit and a β-subunit that functions as a chaperone to stabilize the newly synthesized

Glossary

Apoptosis: an evolutionarily conserved cell death process that removes unwanted or harmful cells. This process involves cell shrinkage, chromatin condensation, DNA fragmentation, and blebbing of plasma membranes. Apoptotic cells are engulfed by macrophages before they rupture. This differs from necrosis, in which cells swell and plasma membranes are ruptured, spilling out cellular contents.
Caspase: a family of cysteine proteases that carry a cysteine residue at the active site, and cleave after aspartic acid. At least 12 members exist in the human caspase family. Some of them are involved in inflammation, while others are involved in apoptosis.
CD47: a type I membrane protein that belongs to the Ig superfamily, also called integrin-associated protein (IAP). It is expressed ubiquitously, in particular, in human tumor cells.
CD300: a type I membrane protein that belongs to the Ig superfamily, and carries ITIM motifs in the cytoplasmic region. It is expressed by natural killer (NK) cells, T cell subsets, B cells, dendritic cells, mast cells, granulocytes, and monocytes.
CDC50A: cell division cycle protein 50A, also called TMEM30A (Transmembrane protein 30A). It carries two transmembrane regions with cytosolic N and C termini. Identified as one of cold-sensitive cell division cycle (cdc) mutants in yeast, it associates with Type IV P-type ATPases, and is involved in lipid transport. Humans and mice carry three genes: CDC50A, -50B, and -50C.
Efferocytosis: a process in which apoptotic cells are phagocytosed. It comes from '*effere*' in Latin, which means 'bury' or 'take to the grave'.
ITIM: immunoreceptor tyrosine-based inhibitory motif composed of a conserved sequence of 'S/I/V/LxYxxI/V/L' that mediates inhibitory signals. Upon ligand engagement, its tyrosine residue is phosphorylated by src kinase, which recruits tyrosine phosphatases to downregulate the signal.
Phagocytes: cells that engulf and digest dead cells and foreign pathogens. In mammals, professional phagocytes such as macrophages and immature dendritic cells aggressively engulf apoptotic cells.