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Commentary

Cytoskeleton and apoptosis

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ABSTRACT

Apoptosis is a genetically programmed and physiological mode of cell death that leads to the removal of unwanted or abnormal cells. Cysteine-proteases called caspases are responsible for the apoptotic execution phase which is characterized by specific biochemical events as well as morphological changes. These changes, which lead to the orderly dismantling of the apoptotic cell, include cell contraction, dynamic membrane blebbing, chromatin condensation, nuclear disintegration, cell fragmentation followed by phagocytosis of the dying cell. They involve major modifications of the cytoskeleton which are largely mediated by cleavage of several of its components by caspases. For example, dynamic membrane blebbing is due to the increased contractility of the acto-myosin system following myosin light chain (MLC) phosphorylation. MLC phosphorylation is a consequence of the cleavage of a Rho GTPase effector, the kinase ROCK I, by caspase-3. This cleavage induces a constitutive kinase activity by removal of an inhibitory domain. Chromatin condensation is facilitated by the processing of lamins by caspases. Collapse of the cytokeratin network is mediated by cleavage of keratin 18. On another hand, the actin cytoskeleton rearrangement needed in the phagocyte for engulfment of the dying cell is due to the activation of the small GTPase Rac, a GTPase of the Rho family that induces actin polymerisation and formation of lamellipodia. In addition to mediating the morphological modifications of the apoptotic cell, several proteins of the cytoskeleton such as actin and keratins are also involved in the regulation of apoptotic signaling.

Apoptosis is a physiological mode of cell death central to the maintenance of tissue homeostasis. In the dying cell, a family of cysteine-proteases called caspases is responsible for the apoptotic signaling during which the cell undergoes several morphological and biochemical changes. In the early stages the cell volume decreases and contact with the neighboring cells is interrupted while the plasma membrane undergoes dynamic membrane blebbing [1]. Ultimately, the dying cell forms buds and fragments into apoptotic bodies that are phagocytosed by macrophages or neighboring cells. This engulfment of apoptotic cells ensures that this form of cell death does not lead to inflammatory reactions.

Apoptotic cells orderly remodelling is a crucial aspect of the process. It involves reorganization of the cytoskeleton and whereas polymerized actin is the major player, intermediate filaments and microtubules also play a role.

1. Generalities on apoptosis

Caspases have an aspartase specificity and must themselves be cleaved twice at aspartic acid residues to generate the active caspase: the first cleavage separates the large and the small subunits whereas the second cleavage liberates the pro-domain [2].

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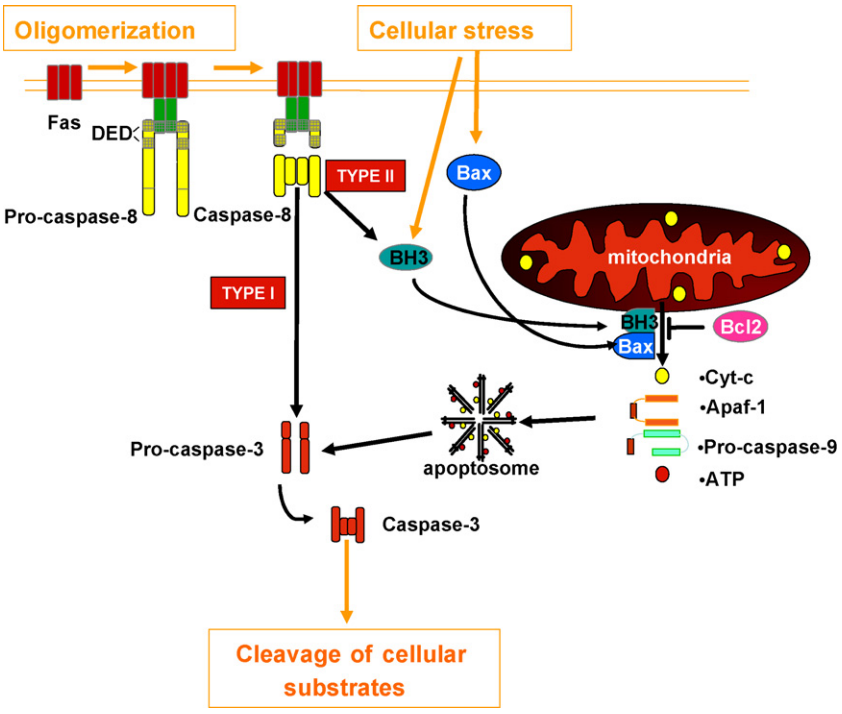

Fig. 1 – The two major pathways of apoptotic signalling.

Caspases involved in apoptosis are separated into two groups: initiator caspases: caspase-8, -10, and -9 (may be also caspase-2) and executioner caspases: caspase-3, -7, and -6. Schematically, two main pathways lead to the activation of caspases. One pathway is initiated at the level of death receptors of the Fas/TNF family. Ligation of these receptors leads to their oligomerization and to the recruitment of the adaptor protein FADD (Fas associated protein with death domain) that then complexes with pro-caspase-8 through homotypic interactions of their death effector domain (DED). This induced proximity of several pro-caspase-8 molecules leads to their auto-activation (Fig. 1). In type I cells active caspase-8 then cleaves and activates pro-caspase-3 which then target the various proteic substrates that are processed during apoptosis. In type II cells caspase-8 is not cleaved enough to activate pro-caspase-3 and triggers what is called the mitochondrial amplification loop. Lymphoid tumor cell lines can be either type I (ex: H9, SKW6.4) or type II (ex: CEM, Jurkat) whereas within solid tumor cell lines those of type I tend to express mesenchymal-like genes whereas those of type II preferentially express epithelium-like markers. It has been suggested that type I and II tumor cells represent different stages of carcinogenesis that resemble the epithelial–mesenchymal transition [3].

The mitochondrial events are under the control of members of the Bcl-2 family (Bcl-2, Bax and BH3-only proteins) [4]. The ultimate pro-apoptotic event in this pathway is the release of cytochrome *c* from the mitochondria. This release is triggered by Bax molecules which oligomerize and form pores in the outer membrane of the mitochondria. This activation of Bax is under the dependence of other members of the Bcl-2 family called BH3-only proteins. In contrast, Bcl-2 prevents the oligomerization of Bax. In type II cells, caspase-8 cleaves a member of these BH3-only proteins called Bid and truncated Bid activates Bax. Cytosolic cytochrome *c* then triggers the formation of a large complex called the apoptosome that contains several Apaf-1 (apoptosis protease-activating factor 1) adaptor molecules which recruit several units of pro-caspase-9. Caspase-9 is autoactivated inside this complex and then activates pro-caspase-3. The second pathway of caspase activation is initiated directly at the mitochondria where Bax is activated following several forms of cellular stress (irradiation, chemotherapeutic drugs). More than 400 proteins have been identified as cleaved by executioner caspases during apoptosis and cell death results from the multiple dysfunctions caused by these cleavages. Among these substrates are found several cytoskeleton proteins whose processing ensures the orderly dismantlement of the dying cell.

2. Actin reorganisation during apoptosis

Actin forms short, polar filaments that bundle up to increase their tensile strength. Members of the Rho family of GTPases coordinate organization of these filaments: Rho induces stress fibers, Rac promotes the formation of lamellipodia and membrane ruffles and Cdc42 drives the formation of filipodia [5]. The first step in cells undergoing apoptosis is to partially detach from the extracellular matrix, round up, contract and disassemble peripheral focal adhesion complexes with reconcentration of new complexes ventrally underneath the rounded cell body. This is accompanied by caspase-mediated cleavage of focal adhesion kinase pp125FAK [6] as well as other structural proteins that link actin to focal adhesions: α-actinin, talin and p130-CAS (Crk-associated substrate).

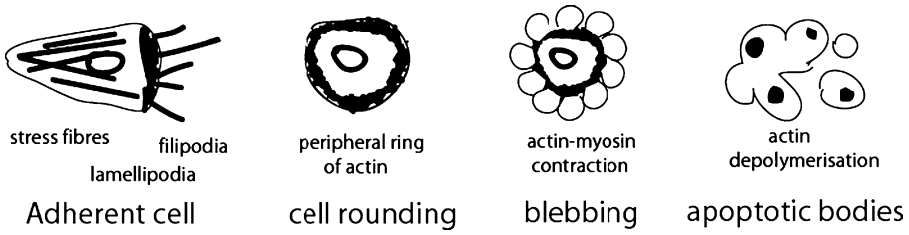

Fig. 2 – Actin reorganization during apoptosis.

Concomitantly, there is a loss of stress fibers and actin is reorganized into a peripheral ring (Fig. 2). Then, myosin II is stimulated by phosphorylation of its light chain (MLC) leading to the contraction of the cortical actin ring and formation of dynamic membrane protrusions (blebs). Bleb protrusion is due to a pressure gradient between the interior and the exterior of a cell and occurs in areas where the strength of interaction that tethers the plasma membrane to the actin cytoskeleton is weakened, for example by caspase-cleavage of fodrin (nonerythroid spectrin) [7]. MLC phosphorylation is under the dependence of MLC kinase (MLCK) and of the kinases ROCK I and ROCK II (Rho-associated coiled-coil-forming protein kinase), effectors of Rho, that either directly phosphorylate MLC [8] or phosphorylate and inhibit MLC phosphatase [9]. The involvement of ROCK in apoptotic blebbing can be shown with the use of the specific inhibitor Y27632 whereas paradoxically the Rho inhibitor C3 transferase is unable to block blebbing [10,11]. Thus this phenomenon appears dependent on ROCK but without the involvement of Rho. In fact, during apoptosis, ROCK I but not ROCK II is cleaved by caspase-3 at a site that removes an autoinhibitory C-terminal domain leading to a constitutively active kinase. Transfection of cells with truncated ROCK I is sufficient to induce membrane blebbing independently of apoptosis [10,11]. Exclusive ROCK I cleavage has been found in all cell types and with all inducers of apoptosis tested with one exception, apoptosis induced by cytotoxic lymphocytes using the granule-mediated pathway. In this case, ROCK II is cleaved at a similar site, not by a caspase but by granzyme B and the consequence of this deregulated kinase activity is also induction of membrane blebbing [12]. The contractile force generated by ROCK probably involves not only MLC but additional ROCK targets. In particular, ROCK activating phosphorylation of LIM kinase (LIM is an acronym of the three gene products Lin-11, Isl-1 and Mec-3) results in the phosphorylation and inactivation of cofilin and thus stabilization of polymerized actin [13].

A question remains: what are blebs for? The destiny of the apoptotic cell is to undergo phagocytosis and it has been observed that blebs are chemotactic for monocytes [14]. Moreover, phosphatidylserine is translocated to the outer leaflet of the plasma membrane of these blebs and this molecule is a flag for recognition by phagocytes. Among the substrates of caspases, several are autoantigens and their cleavage might expose immunogenic neoepitopes. Interestingly these autoantigens, which are mainly nuclear, are redistributed and aggregated in membrane blebs during apoptosis [15]. These observations have lead to the hypothesis that phagocytosis of these vesicles could participate to the maintenance of a state of tolerance to self antigens [16]. Indeed, defective phagocytosis of apoptotic cells is known to contribute to the development of autoimmune and inflammatory diseases.

In most cases, if they are not phagocytosed, cells will eventually stop blebbing after about an hour. It is not clear what causes the cessation of blebbing but this may be due to the depolymerisation of actin filaments [17,18]. Signaling by Rho GTPases effectors may contribute to this second step of actin reorganisation. The Rho effector PRK1 (protein kinase C-related kinase) is cleaved by caspases leading to a constitutively active kinase fragment [19] and PRK1 can induce actin disassembly [20]. Likewise, the kinase PAK2 (p21-activated kinase), a Rac effector, can be activated by caspases [21,22] and active PAK has been shown to promote stress fibers dissolution [23]. The end of blebbing is followed by chromatin condensation and nuclear fragmentation. Like bleb formation, apoptotic nuclear disintegration requires ROCK activity and actin–myosin contractile force [24]. The final step is the dismantlement of the cell into apoptotic bodies after actin depolymerisation.

3. Intermediate filaments

Intermediate filaments (IF) are encoded by more than 60 different genes and are classified into five classes. Type I and type II are acid keratins and basic keratins, respectively, and are present in all epithelial cells. The type III intermediate filaments are distributed in a number of cell types: vimentin in mesenchymal cells, endothelial cells and leukocytes, desmin in muscle, glial fibrillary acidic protein in glial cells and peripherin in peripheral nerve fibers. Neurofilaments and internexin (type IV) are found in neurons and cells of the peripheral neuroendocrine system. The type V are the three nuclear lamins which form a filamentous support at the inner layer of the nuclear membrane. Each intermediate filament monomer consists of an alpha helical rod domain which connects the N-terminal and C-terminal globular domains [25]. IF are polymerized and cross-linked to the other members of the cytoskeleton via proteins of the plakin family (periplakin, plectin and desmoplakin). A common functional feature of IF is their resilience to mechanical stress [26].

The keratins K8 (type II) and K18 (type I) have been shown to modulate apoptosis induced by Fas/TNF family receptors. Deficiency in K8/K18 sensitizes normal and malignant epithelial cells to TNF/cycloheximide-induced apoptosis about a 100 times [27]. In addition to the adaptor FADD mentioned above for apoptosis mediated by the Fas receptor, apoptosis mediated by TNF requires another adaptor called TRADD (TNF

Receptor-Associated Death Domain). One possible mechanism for K18 modulation of TNF-induced apoptosis is the fact that K18 has been shown in various epithelial cells to sequester TRADD and thus prevent the interaction of TRADD with TNF-R1 [28]. A role for K8 in protection from Fas-mediated apoptosis in hepatocytes has also been reported and attributed to a reduced targeting of the receptor to the cell membrane [29]. In addition, further work in hepatocytes and mammary cells has shown that the loss of K8 is associated with a down-regulation of c-FLIP (Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein) expression [30]. In as much as c-FLIP prevents the recruitment of caspase-8 to the Fas receptor, cells expressing K8 would be less sensitive to Fas-induced apoptosis.

Most type I keratins contain a caspase-6 (or caspase-3) cleavage consensus sequence in the rod domain whereas type II keratins are resistant to proteolysis by caspases [31]. In addition to this cleavage site in the linker region, K18 displays a second cleavage site, specific for caspase-9, in the C-terminal domain at aspartate^{397}. This second cleavage creates a neo-epitope that is specifically targeted by the M30 CytoDeath antibody which permits to monitoring of K18 cleavage during apoptosis [32]. Cleavage of K18 C-terminal domain is a very early event in apoptotic signalling preceeding phosphatidylserine externalization but with no consequences on keratin filaments organization. Subsequent cleavage of K18 in the linker region of the central rod domain results in the collapse of the cytokeratin scaffold into large aggregates [33]. A mechanism has been proposed that may explain how K18 is targeted by caspases. A protein called death effector domain containing DNA binding protein (DEDD) has been identified that contains nuclear localization signals and binds DNA but predominantly resides in the cytoplasm. It has been shown in HeLa, MCF-7 and A549 cell lines that cytosolic DEDD is expressed in a diubiquitinated form which associates with K18, procaspase-3 and procaspase-9 [34,35]. Upon induction of apoptosis, a small amount of caspase-3 is activated causing, via unknown mechanisms, a conformational change in DEDD which forms filamentous structures associated with K18 and procaspases. This DEDD-mediated accumulation of procaspase-9 at the cytokeratin scaffold could lead to an increase in its local concentration allowing oligomerization and autoactivation. Active caspase-9 then targets K18 as well as downstream caspases. Downstream caspase activation results in the second cleavage of K18 and the final collapse of the cytokeratin network into aggregates and intracellular inclusions which migrate into cytoplasmic blebs. Thus, the cleavage of cytokeratin during apoptosis may induce the orderly packaging and disposal of insoluble structural proteins.

Cytolinkers proteins of the plakin family, plectin, desmoplakin and periplakin, are also cleaved by caspases [36]. This disruption of the IF-binding domain of plakins from their actin-binding domain enables the formation of IF aggregates without affecting the microfilaments.

In addition to cytokeratin, other IF proteins, such as vimentin and lamins, are also caspase substrates. Vimentin is cleaved by several caspases at distinct sites disrupting its cytoplamic network [37]. A-type and B-type lamins are polymerized underneath the nuclear membrane to form the lamina which is important for the maintenance of nuclear organization. Phosphorylation of lamins by protein kinase C-delta precedes their cleavage by caspases [38]. Caspases being cytoplasmic must first enter the nucleus in order to process lamins. It has been shown that caspase-9, through an undefined mechanism, increases the diffusion limit of nuclear pores allowing the caspases to enter the nucleus [39]. Cleavage of lamin-A is due to caspase-6 whereas that of lamin-B appears to be also mediated by caspase-3 [40]. Lamina degradation is a rather ubiquitous event in apoptosis and facilitates chromatin condensation [41,42].

Thus IF have two types of functions during apoptosis: they decrease the apoptotic signalling triggered by activation of TNF-family receptors and, after their cleavage by caspases, they participate in the organized dismantling of the dying cell.

4. Microtubules

Microtubules are depolymerized at the onset of the execution phase of apoptosis concomitant with disruption of pericentriolar components (pericentrin, ninein and γ-tubulin) of the centrosomal region [43–46]. The cleavage of the motor protein dynein might facilitate this depolymerisation by dispersing centrosomal nucleating complexes thus releasing labile microtubules minus end [43]. Membrane movement and motor-based organelle trafficking rely on microtubules [47]. It has thus been suggested that interphase microtubules depolymerisation might contribute to apoptotic events such as Golgi fragmentation [48], clustering of mitochondria [49] or secretory membrane traffic arrest [50].

However, subsequently, extensive non-centrosomal novel arrays of densely packed, dynamic microtubules rapidly assemble throughout the cytoplasm [51]. What stimulates the reassembly of microtubules at this stage is not known. Caspase cleavage of the regulatory C-terminus of α-tubulin [52] might contribute because this cleavage increases the capacity of tubulin to assemble into polymers [43]. Several roles have been proposed for these microtubules in the late phase of apoptosis in some cell types and await verification in a broader cell panel. At this stage, large blebs have been described that tend to accumulate chromatin and endoplasmic reticulum and may be the progenitors of apoptotic bodies [53]. It has been shown in HeLa cells that transport of condensed apoptotic chromatin in these blebs can be inhibited by the microtubule poison nocodazole [51]. The function of microtubules appears to be to maintain chromatin at the periphery by resisting some form of retractile pressure. A second role of the apoptotic microtubule network would be to preserve plasma membrane integrity during the execution phase of apoptosis [54]. Colchicine-mediated disruption of apoptotic microtubule network in H-460 cell line results in enhanced plasma membrane permeability and secondary necrosis. A third role of microtubules, but this seems restricted to the A431 cell line, is in apoptotic body formation which in this cell line depends on the extension of rigid microtubule spikes. Apoptotic bodies remain attached along these spikes or in clusters at their tips and nocodazole prevents cell fragmentation in this cell line [51].

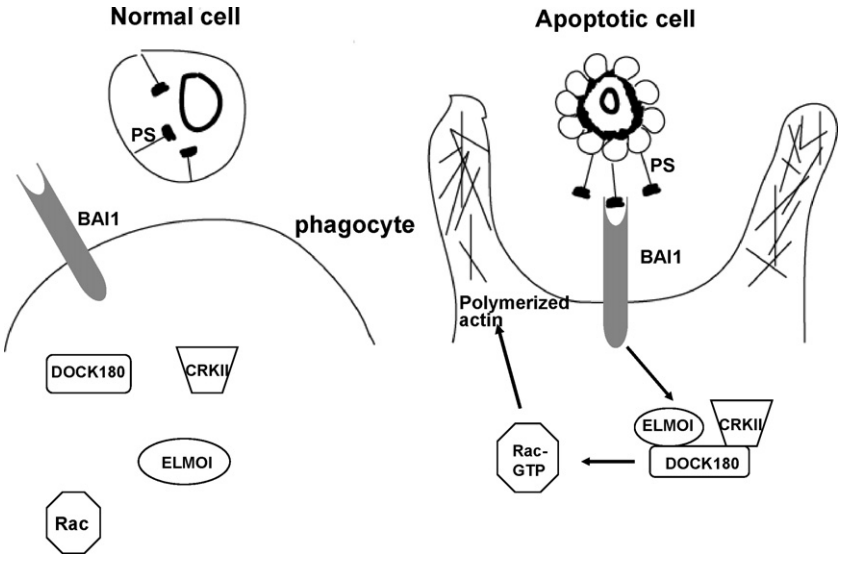

Fig. 3 – Activation of Rac and polymerisation of actin in the phagocyte during apoptotic cell engulfment. PS: phosphatidylserine; BAI1: brain specific angiogenesis inhibitor 1.

5. Phagocytosis of apoptotic cells

''Eat-me'' signals are displayed on the surface of dying cells very early in the apoptotic process [55]. The most studied signal is the ubiquitous translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane [56]. A variety of engulfment receptors on the phagocyte have been identified that recognize these ''eat-me'' signals [57]. Phagocytosis of the apoptotic cell requires major cytoskeleton rearrangements in the phagocyte which are mediated at least in part by both activation and inactivation of specific members of the Rho-GTPase family. RhoA itself has an inhibitory role on engulfment [58,59]. This seems to be due to the activation of ROCK by Rho and the subsequent increase in cell contractility [58]. Such contractility would impair the ability of a phagocyte to extend its pseudopods around the dying cell. In contrast to RhoA inhibition at early stages of engulfment, the level of active Rac increases in the phagocyte (Fig. 3). Studies in both mammals and *C. elegans* have led to the identification of an evolutionary conserved pathway that results in Rac activation during the phagocytosis of apoptotic cells and is distinct from the usual pathways [60]. Two proteins act directly upstream of Rac: ELMO1 (engulfment and cell motility 1) [61] and DOCK180 (dedicator of cytokinesis) [62]. ELMO1 is an adaptor protein that facilitates the activation of DOCK180. DOCK180 mediates the transition of Rac from its unbound form to its active form (bound to GTP). Such an activity is usually due to GTPase exchange factors (GEF) but DOCK180 contains no discernible Dbl-homology domain usually found in GEFs [62]. Recently, a new GEF domain within DOCK180 was identified and called the Docker domain [63]. In its inactive state, DOCK180 is in a closed conformation where the Docker domain is bound by the SRC homology 3 (SH3) domain and thus inaccessible to Rac. The binding of ELMO1 to the SH3 domain of DOCK180 induces the opening of DOCK180 and allows the binding of the Docker domain to Rac [64]. The adaptor protein CRKII (CT10 regulator of kinase II) participates to this pathway of Rac activation but its role is not clearly defined [65]. The increased Rac activity leads to the generation of membrane ruffles that allow the formation of the phagocytic cup necessary for the uptake of apoptotic cells. Among the various receptors that have been described to directly or indirectly recognize externalized phosphatidylserine, BAI1 (brain specific angiogenesis inhibitor 1) has recently been identified as an engulfment receptor upstream of the ELMO/DOCK180/Rac module [66].

The situation appears different in epithelia where apoptotic cells are generally not phagocytosed but are extruded out of the plane of the epithelium [67]. A ring of actin and myosin forms within the apoptotic cell and in the cells surrounding it. The signal to form actin cables in the live cells comes from the dying cell and involves activation of the RhoA GTPase. Contraction of the ring formed in the live neighbors leads to the extrusion of the apoptotic cell and resealing of the epithelium.

6. Role of the cytoskeleton in Fas signaling

In activated lymphocytes, Fas polarization to the uropod seems necessary for the sensitization to Fas-mediated apoptosis [68]. Uropod formation is dependent on actin cytoskeleton remodelling and depolymerisation of actin with cytochalasin D inhibits Fas-mediated signaling. Indeed, Fas was found to be linked with actin via the ERM protein ezrin [68]. ERM proteins (ezrin, radixin, and moesin) are a family of closely related proteins that link transmembrane proteins to the cortical actin cytoskeleton [69]. This association with actin is important for Fas signaling as downregulation of ezrin inhibits Fas-mediated apoptosis [68]. Fas translocates into membrane rafts following Fas-triggering [70] and ezrin, moesin, RhoA and RhoGDI are conveyed into these Fas-enriched rafts [71]. Our own recent results (submitted) show that, in Jurkat cells, not only ezrin but also moesin bind to Fas

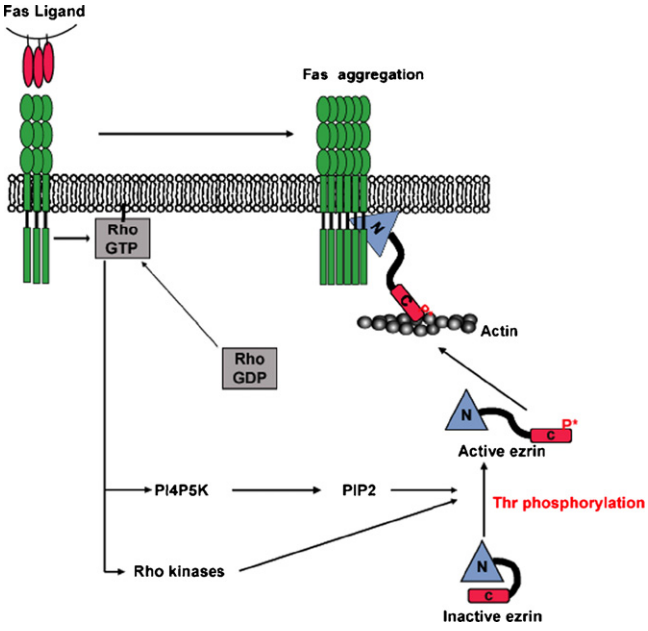

Fig. 4 – Activation and binding to Fas of ezrin during the early stages of Fas signaling. PI4P5K: phosphatidylinositol-4-phosphate 5-kinase; PIP2: phosphatidylinositol 4, 5-biphosphate.

and that downregulation of moesin with specific siRNA inhibits Fas-mediated apoptosis. In addition, we found that Rho is activated a few minutes after Fas-triggering. Rho-GTP is known to activate phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) that produces phosphatidylinositol 4, 5-bisphosphate (PIP2) which permits the opening of the inactive, folded form of ERM proteins. We found that Rho activation after Fas ligation also leads to the activation of ROCK that phosphorylates ezrin and moesin on a conserved threonine residue (Fig. 4). This phosphorylation is necessary for the stabilisation of the opened form of ezrin and moesin, their interaction with Fas and the formation of Fas aggregates.

In conclusion, the cytoskeleton is involved in several steps of apoptotic signaling: inhibition of Fas/TNF signaling by keratins, sensitisation to Fas-induced apoptosis by polymerized actin, triggering of membrane blebbing by acto-myosin contraction, uptake of apoptotic cells by phagocytes through the activation of Rac. In addition, the cleavage of several cytoskeleton components by caspases ensures the orderly dismantlement of the dying cell. The three cytoskeleton components are interconnected in live cells but such is not the case during apoptosis. The cytolinker proteins of the plakin family that link polymerized actin to IF are cleaved during apoptosis and thus the collapse of the cytokeratin network has no consequences on actin microfilaments. Likewise, after the depolymerisation step of interphase microtubules, the non-centrosomal novel microtubules arrays that assemble during apoptosis most likely have little connection with the other components of the cytoskeleton. Therefore, each of the three members of the cytoskeleton network undergoes modifications in an independent manner in the apoptotic cell.

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