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Research Article

Rho kinase regulates phagocytosis, surface expression of GlcNAc, and Golgi fragmentation of apoptotic PC12 cells

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ABSTRACT

Apoptotic cells undergo a number of changes to prepare for phagocytosis; most occur during the execution phase of apoptosis, when dying cells undergo shrinkage and/or fragmentation into apoptotic bodies and express phagocytic markers on their surface. Although events during the execution phase are important to prepare corpses for phagocytosis, the mechanisms that control most execution phase events are unknown. To understand regulation of execution events we focused on Rho kinase (ROCK), because one isoform of ROCK, ROCK-I, is constitutively activated by caspases during execution. Using apoptotic PC12 cells as a model, we find that inhibition of ROCK activity during apoptosis decreases surface expression of GlcNAc, a carbohydrate known to function as a phagocytic marker. In addition, inhibition of ROCK blocks Golgi fragmentation in apoptotic cells, and constitutively active ROCK induces Golgi fragmentation in the absence of apoptosis. Importantly, PC12 cells dying in the presence of a ROCK inhibitor are less efficiently phagocytized than those dying without the inhibitor. These data highlight the role of ROCK in multiple processes in the execution phase of apoptosis, and suggest that ROCK plays an important role in controlling the outcome of apoptosis, that is, preparation of corpses for phagocytosis.

Abbreviations:
ROCK, Rho kinase
MLC, myosin light chain
GlcNAc, N-acetyl glucosamine
WGA, wheat germ agglutinin
PMA, phorbol 12-myrisate 13-acetate
PS, phosphatidylserine
caROCK-I, constitutively active ROCK-I
BFA, brefeldin A

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Introduction

Apoptosis and necrosis are both cell death processes; however, there are key differences. During necrosis, intracellular contents are released, resulting in local damage and inflammation. In contrast, during apoptosis a cell packages itself into apoptotic bodies so that its contents are not released, and the resulting apoptotic bodies are removed from the surrounding tissue through phagocytosis [1–6]. Most of the key processes that prepare an apoptotic cell for phagocytosis occur during the execution phase, which consists of three stages: release, membrane blebbing, and condensation [7]. During the release stage, a dying cell loses contact with the extracellular matrix and surrounding cells, and begins to round up. During the membrane blebbing stage, actin–myosin contraction, coupled with focal loss of contact between the cytoskeleton and the plasma membrane, causes blebs to form [8–10]. After a period of membrane blebbing, cells move into the condensation stage; where cells either condense into a single apoptotic body or fragment into multiple apoptotic bodies [7]. At the end of execution, an apoptotic corpse is formed which can be removed from the surrounding tissue through phagocytosis. The removal of apoptotic corpses is important for all multicellular organisms; if the corpses are not phagocytized efficiently, they undergo secondary necrosis, burst and release intracellular contents. Although the morphological changes that occur during the execution phase of apoptosis are evident, the underlying mechanisms that drive these changes are not fully understood. Since the events that prepare a dying cell for phagocytosis occur during execution, it is important to understand regulation of these events.

One execution phase event, membrane blebbing, is regulated by phosphorylation of the regulatory myosin light chain (MLC), which drives actin–myosin contraction [8,9]. MLC phosphorylation is regulated by Rho kinase (ROCK), which controls MLC phosphorylation either directly or by inhibiting myosin phosphatase [11–13]. ROCK contains an N-terminal kinase domain and a C-terminal Rho binding domain; when ROCK is inactive, the C-terminal domain binds and inhibits the kinase domain. Rho binding results in a conformational change that releases the C-terminal domain, allowing the kinase domain to become active [14]. During apoptosis, one isoform of ROCK, ROCK-I, is cleaved at a consensus caspase cleavage site, DETD1113^G, removing the inhibitory domain and creating a constitutively active kinase [15,16]. Expression of this truncated form of ROCK-I in cells drives membrane blebbing [15,16]. This raises the possibility that ROCK is a key regulator of other execution events, such as preparing corpses for phagocytosis. We recently showed that ROCK controls phagocytosis by regulating fragmentation during the execution phase [17]. However, in cells that do not fragment during apoptosis, it is not known whether ROCK has any affect on the preparation of corpses for phagocytosis. To determine whether this is the case, we used PC12 cells as a model to identify execution events that are controlled by ROCK. We determined that activation of ROCK during apoptosis controls phagocytosis of corpses. In addition, ROCK regulates surface expression of GlcNAc, a phagocytic marker, during apoptosis. Finally, ROCK regulates Golgi fragmentation during apoptosis. These results underscore the importance of ROCK in execution phase events, and highlight the importance of ROCK in the preparation of corpses for phagocytosis.

Materials and methods

Materials and chemicals

Hoechst 33342, wheat germ agglutinin, N-acetyl-glucosamine (GlcNAc), Brefeldin A (BFA), and phorbol 12-myrisate 13-acetate (PMA) were obtained from Sigma (St. Louis, MO). ZVAD-fmk, Y-27632, latrunculin B, and blebbistatin were obtained from CalBioChem (San Diego, CA). Streptavidin-Alexa 488, Amplex® Red Sialidase Assay Kit, and BODIPY FL C_5-ceramide, DiI and DiO were obtained from Molecular Probes (Eugene, OR). Anti-ROCK-I antibody was from BD Biosciences (San Jose, CA), and anti-caspase-cleaved ROCK-I antibody was obtained from Imgenex (San Diego, CA). The TGN38 antibody was from Affinity BioReagents (Golden, CO), and the TGN46 antibody was from Serotec (Raleigh, NC). The caROCK-I vector (pCAG-myc-p160 ROCKΔ4) was obtained from Shuh Narumiya, and the RFP vector (pDS Red2-C1) was obtained from Clontech.

Cell culture, apoptotic induction and quantitation of apoptotic cells

PC12 cells were grown in RPMI with 10% horse serum, 5% FBS and penicillin/streptomycin. COS-7 cells were grown in DMEM with 10% FBS containing penicillin/streptomycin. THP-1 cells were grown in RPMI with 10% FBS, 2 mM glutamine, and gentamicin. Unless stated otherwise, apoptosis was induced in PC12 cells by washing cells three times in serum-free RPMI medium, and plating into serum-free medium for 18 h in the presence or absence of inhibitors. The percent of apoptotic cells was determined by counting total cells and condensed cells. The percent of blebbing cells was determined by counting total and blebbing PC12 cells. At least 200 cells were counted for each condition, under blinded conditions.

ROCK-I cleavage

Apoptosis was induced in PC12 cells in the absence or presence of 100 μM ZVAD-fmk and/or 20 μM Y-27632; apoptotic cells were scraped, centrifuged at 15,000×*g*, and resuspended in lysis buffer (40 mM Tris–HCl, pH 8, 0.2% NP-40, 300 mM NaCl, 2 mM EDTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, 5 ng/ml aprotinin, 5 ng/ml leupeptin, and 1 μg/ml pepstatin). Samples were sonicated, centrifuged at 15,000×*g*, and an equal amount of protein was loaded onto a 7.5% SDS-PAGE gel to visualize full-length ROCK-I, or 12% gel to visualize the 30 kDa portion of caspase-cleaved ROCK-I. Protein was transferred to PVDF membrane (NEN Life Science Products), and blocked with 5% milk in PBS containing 0.1% Triton X-100 (PBST) for 1 h. Membranes were incubated overnight with primary antibodies in milk at 4°C, washed with PBST, incubated for 40 min with HRP-conjugated secondary antibodies, and visualized by enhanced luminescence (Western Lightning).