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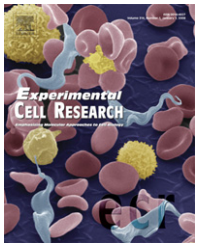

Research Article

Identification of a novel phosphorylation site of acyl-CoA binding protein (ACBP) in nodularin-induced apoptotic hepatocytes

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ABSTRACT

The liver specific protein phosphatase inhibiting toxin nodularin (from *Nodularia spumigena*) rapidly induces hepatocyte apoptosis. Incubation of freshly isolated hepatocytes with this toxin results in hyperphosphorylation of cellular proteins before any morphological signs of apoptosis appear. These phosphorylated proteins may play key roles in the early stage of apoptosis. Here, we identified one of the phosphoproteins to be acyl-CoA binding protein (ACBP), a highly conserved and ubiquitously expressed protein. Phosphorylation-site analysis by matrix-assisted laser desorption ionization time-of-flight MS/MS revealed that the observed phosphorylation is positioned on Ser1 in the N-terminal tryptic peptide Ac-SQADFDKAAE EVKRLK of the rat liver protein. Additionally, we observed a translocation of ACBP towards the cellular membrane in the apoptotic hepatocytes. Moreover, nodularin-induced apoptosis was highly dependent on calpain activation, an event that has previously been shown to be regulated by ACBP. Our findings introduce the possibility that reversible phosphorylation of ACBP regulates its ability to activate calpain in phosphatase inhibitor-induced apoptosis and controls the cellular accessibility of long-chain fatty acid-CoAs for cellular signaling.

Introduction

Bloom forming cyanobacteria produces hepatotoxins, named nodularin and microcystins, which are able to induce apoptotic cell death in primary hepatocytes. The ability of these toxins to ultrarapidly induce apoptosis in hepatocytes and also, if microinjected, in other cell types, indicate that they may short-cut a conserved apoptogenetic pathway [1,2]. Nodularin and microcystins inhibit the general serine/threonine protein phosphatases (PP1 and PP2A), thereby resulting in hyperphosphorylation of a number of proteins [1,3]. The reversible nature of protein phosphorylation favors it as a key player in the early phase of apoptosis when the cells are sorting out different cellular signals in order to make the decision whether to commit to apoptotic cell death or not [4]. By identifying the phosphorylated target proteins in our apoptotic model, we

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believe new clues for understanding the early regulation of cell death may be found [5].

Nodularin-treated hepatocytes become apoptotic within minutes, providing us with a unique amount of synchronized cells, thus increasing the possibility to identify the low amount phosphoproteins that are involved in apoptotic cell signaling. We identified acyl-CoA binding protein (ACBP) as a phosphorylated target protein in the initial phase of apoptosis. ACBP is a 10 kDa highly conserved protein that binds and induces synthesis of long-chain acyl-CoA esters [6,7]. ACBP is found in the cytosol, endoplasmatic reticulum, golgi and nucleus, but has also been localized to the mitochondrial intermembrane space from where it is released during apoptosis [7–10]. In addition to induction of acyl-CoA ester synthesis, ACBP plays a role in the distribution of the cellular long-chain fatty acyl-CoA pool. The protein is suggested to have a dual function in creating a cytosolic pool of acyl-CoA protected against acyl-CoA hydrolases and protecting vital cellular processes from being affected by LCFA-CoA esters [11]. It has also been found to modulate central benzodiazepine receptors (GABA), enhance steroid biosynthesis in various tissues via modulation of pheripheral benzodiazepine receptors, decrease glucose induced insulin secretion and modulate cell growth [12–18]. Recently, ACBP was described to be required for Bid-induced activation of μ-calpain and cleavage of full-length Bid to tBid, thus facilitating the release of apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space and the onset of apoptosis [19]. Although several proteins bind LCFA-CoAs [20], only ACBP exclusively binds C12-C22 LCFA-CoAs [21,22]. These LCFA-CoAs act as intracellular messengers and apoptosis inducers by mediating calcium flux and/or via the generation of ceramide as well as some other not yet elucidated mechanisms [23–25]. So far, relatively little is known about factors that regulate the cellular LCFA-CoA pool size and LCFA-CoA acyl-chain composition. However, an accumulating number of experiments can demonstrate a direct link between lipid metabolism and apoptosis. Our finding that ACBP is phosphorylated in the early phase of apoptosis and its observed translocation, suggests that the availability of the cellular LCFA-CoA pool as well as its size and acyl-chain composition might be regulated by reversible phosphorylation of ACBP. Additionally, our results may contribute to a further understanding of the regulative role of ACBP in calpain activation in cell death and fatty acid metabolism.

Materials and methods

Materials

Nodularin was either purified from the cyanobacteria *Nodularia spumigena* as described [26] and generously given by L. Herfindal and S.O. Doeskeland, University of Bergen, Norway or was purchased from Calbiochem (La Jolla, CA). [^{32}P]-orthophosphate (10 mCi/ml), IPG-buffer, linear-immobilized pH 5.3–6.5 gradients, CHAPS and Coomassie Brilliant Blue R250 were from Amersham Biosciences, Inc. The matrix 2, 5-dihydroxybenzoic acid was purchased from Bruker Daltronics GmbH. Sequencing grade modified trypsin was obtained from Promega Co. (Madison, WI). Bromopenol blue was obtained from J.T. Baker (Deventer, Holland). Ammonium bicarbonate, trifluoroacetic acid (TFA), *ortho*-phosphoric acid (PA) and all other biochemicals were purchased from Sigma Chemical (St. Louis, MO). Calpain inhibitor (PD150606) was from Calbiochem (La Jolla, CA). Karsten Kristiansen, University of Southern Denmark, generously provided the polyclonal rabbit anti-ACBP.

Cell culture and labeling of cellular phosphoproteins

Hepatocytes were isolated from male Wistar rats (120–200 g) by *in vitro* collagenase perfusion as previously described [27,28]. In the non-radioactive experiments hepatocytes were incubated as previously reported [1], except that nodularin concentration was 200 nM. Calpain inhibitor was added 20 min prior to apoptosis induction. For [^{32}P]-labeling, hepatocytes (1.8×10^6 cells/ml) were incubated for 35 min in a low-phosphate RPMI 1640 medium (Eurobio, Les Ublis Cedex, Fr) supplemented with 0.5 mCi/ml [^{32}P]-orthophosphate prior to the addition of nodularin (200 nM). All incubations were in capped vials with gyratory-shaking (175 cycles/min, 37 °C).

Evaluation of apoptosis

Apoptosis was evaluated in an invert phase microscope by observing the morphology of cells fixed in 2–4% formaldehyde in PBS (pH 7.4). Apoptotic cells were easily discriminated from normal and necrotic cells by the appearance of cell surface buds.

For ultrastructural analysis, the hepatocytes were fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) and post-fixed in 2% OsO_4 containing 0.1 M Na-cacodylate buffer. Ultrathin sections from embedded cells were stained in uranyl acetate and lead citrate followed by electron microscopy using a JeolS100 electron microscope.

Fractionation of hepatocytes

Following apoptosis induction, the hepatocytes were carefully spun down (200 ×*g*, 2 min), added ice-cold 10 mM Na-Hepes buffer, pH 7.4 (0.5 mM sucrose, 15 mM KCl, 10 mM $MgCl_2$, 50 mM NaF, 5 mM $NaPP_i$, 1 mM Na_3VO_4 and Complete protease inhibitor cocktail) and mixed up and down 10 times on ice using a 24 G needle. By centrifuging the suspension at 500 ×*g* for 10 min at 4 °C, the nuclei and remaining whole cells were separated from the supernatant to which EDTA was added to a final concentration of 10 mM. The organelle fraction was separated by a further centrifugation of the supernatant at 16,000 ×*g* for 10 min at 4 °C. Protein concentration was measured in the remaining supernatant using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc, Hercules, CA).

2-D gel electrophoresis and autoradiography

A protein amount corresponding to 1 mg of our fractionated protein supernatant, was precipitated by four volumes of ice-cold acetone, left at −20 °C o/n, and then centrifuged at 16,000 ×*g* at 4 °C for 20 min. The acetone was removed and the protein pellet was resolved in 300 μl of a solution containing 8 M urea, 20 mM, 1,4-dithioerythreitol, 1% v/v IPG-buffer (pH 4–7), 2% w/v