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Isolation of High-Molecular-Weight Activation Complexes of Initiator Caspases in DNA Damage

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Initiation of apoptosis by chemotherapeutic drugs is one of the most effective approaches to the treatment of cancers. Caspases, the main enzymes of apoptosis, undergo activation to initiate cell death. Activation of initiator caspases requires their binding to special protein complexes. For elucidation of the mechanisms of apoptosis, these complexes should be isolated. However, their purification is challenging because they are formed in the cell in negligible amounts and rapidly degrade. We have developed an effective way to isolate caspase activation complexes formed in tumor cells in response to DNA damage. The method is based on combination of gel filtration with immunoprecipitation. The first stage is aimed at the separation of the high-molecular-weight caspase activation complexes and their monomeric forms, which allows increasing the efficiency of isolation of complexes at the second stage.

Key Words: *apoptosis; initiator caspase; DNA damage; cisplatin*

Apoptosis, one of the most important types of programmed cell death, is responsible for different biological processes occurring in the tissues and organs of multicellular organisms. It provides morphogenesis and adequate functioning of various systems in multicellular organisms and elimination of damaged cells. For instance, during treatment of cancer with chemotherapeutic agents, the death of cancer cells occurs in many cases via apoptosis. For understanding of the pathophysiology of these diseases, comprehensive study of the mechanism of apoptosis activation and the role of all factors in this process is required.

Caspases, proteins of the cysteine protease family, are the main enzymes involved in the initiation and progression of apoptosis. The process of apoptotic death is triggered by extracellular or intracellular factors, such as hypoxia, hyperoxia, subnecrotic lesions caused by chemical or physical factors, activation of "death receptors", disordered cell cycle signaling, removal of growth factors and metabolites [6]. Normally, caspases are present in cells in the form of inactive proenzymes and are activated via proteolytic processing [4]. Caspases are usually classified in three groups. Among caspases involved in apoptosis, initiatory (CASP2, CASP8, CASP9, and CASP10) and effector (CASP3, CASP6, and CASP7) caspases are distinguished; a special group of caspases participates in the innate immune response (CASP1, CASP4, CASP5, and CASP11) [8,13,18]. It should be noted that CASP2, is a unique caspase that exhibits properties of not only initiator, but also effector caspases [1].

There are two principal pathways of apoptosis triggering: internal (mitochondrial) and external (receptor-mediated). Both pathways are realized via activation of caspase cascades. The external pathway is triggered upon activation of death receptors united in one family, which, in turn, is a subfamily of TNF receptor superfamily [10]. Up to date, CD95/Fas/Apo-1, TRAIL-R1/R2, and TNF-R1 are best characterized death receptors; their activation can lead to different types of programmed cell death, *e.g.* apoptosis and necroptosis. The internal or mitochondrial

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pathway of apoptosis starts from permeabilization of the external mitochondrial membrane (PVMM) and the release of cytochrome C into the cytoplasm, which triggers the assembly of the high-molecular-weight CASP9-apoptosome activation complex [11,18,19]. Once activated, CASP9 acquires the ability to activate the effector CASP3, which leads to cleavage of substrate proteins, morphological changes in the cell, and its death [3].

The key role in initiation of apoptosis in response to genotoxic stress is played by the initiator caspases: CASP2, CASP8, and CASP9. Of these, CASP2 is most important for triggering the caspase cascade, but the mechanism of its activation remains unknown. In 2004, it was shown that activation of CASP2 in response to DNA damage occurs in a complex PIDDosome that includes adaptor protein RAIDD and C-terminal fragment of PIDD protein [15]. RAIDD protein (RIP-associated homolog ICH1) contains death domain (DD) and a caspase activation recruitment domain (CARD), which allows interaction with the C-terminal fragment of PIDD protein and procaspase-2. Later, it was reported in a series of papers that CASP2 can be activated independently of both PIDD and RAIDD proteins and of PIDDosome complex assembly, but in high-molecular-weight (HMW) platform(s) not described in the literature [5,7,12]. However, the composition of these complexes remains unknown.

It was also demonstrated that another initiator caspase, CASP8, in some cell lines can trigger apoptosis upon DNA damage [2]. Genotoxic stress triggers the formation of an HMW CASP8 activation complex containing RIPK1, RIPK3, FADD, and c-FLIP proteins [14].

The most important substrates of CASP2 and CASP8 are procaspase-3 and Bid, a Bcl-2 family protein. Procaspase-3 cleavage leads to its activation and triggers the effector phase of apoptosis, while cleaved form of Bid (tBid) couples the external and mitochondrial pathways of cell death. tBid interacts with proapoptotic proteins Bax or Bak and induces their structural modification. As a result, conformationally altered Bax or Bak incorporate into the mitochondrial membrane and cause PVMM, which leads to the formation of an apoptosome and activation of CASP9.

The study of the molecular platforms for activation of initiator caspases, including CASP2, requires a systematic approach aimed at identification of protein components and elucidation of their role in apoptosis. As the caspase cascade is a key mechanism of apoptosis, identification of unknown participants provides new insight into the regulation of this type of programmed cell death and helps to understand the principles of cancer pathogenesis. Modulation of activity of these complexes can be used for triggering apoptosis in cancer cells and thereby for the treatment of different types of malignant neoplasms.

Creation of approaches for purification of initiator caspase activation complexes from cancer cells is an intricate scientific and technical problem, because the proteins can dissociate from the complex during isolation, form aggregates, and lose their native conformation. Isolation of caspase activation complexes is also complicated by negligible amounts and rapid degradation of these molecular platforms. These HMW complexes are studied over the last two decades and complete description of this or that complex is a time-consuming process.

To solve this problem, we developed a protocol for obtaining HMW signal complexes controlling the processes of programmed cell death. A macromolecular CASP2 activation complex assembled in response to DNA damage in ovarian carcinoma Caov-4 cells served as the model.

MATERIALS AND METHODS

Cell culturing. The studies were carried out on ovarian adenocarcinoma cell line Caov-4 and modified line Caov-4 lacking CASP2 generated using the CRISPR/Cas9 genome editing system. Wild-type Caov-4 cell line was kindly provided by the Department of Toxicology of the Karolinska Institute (Stockholm, Sweden). Along with wild-type cells, the following Caov-4 cell lines were used: shRNA-caspase-2 Caov-4 line expressing small hairpin RNA that suppresses the expression of the *CASP2* gene by the mechanism of RNA interference; non-targeting CRISPR/Cas9 Caov-4 (control) obtained by using control plasmid CRISPR/Cas9 sc-418922 (Santa Cruz); caspase-2 knockout Caov-4 (CRISPR/Cas9), a *CASP2* gene knockout cell line obtained by using plasmid CRISPR/Cas9 KO caspase-2 sc-401079 (Santa Cruz).

The cells were cultured in DMEM high glucose (Gibco) nutrient medium supplemented with 10% fetal calf serum (Gibco), 1 mM sodium pyruvate (PanEco), and a mixture of antibiotic-antimycotic (Gibco): 100 U/ml penicillin+100 μg/ml streptomycin. All procedures with live eukaryotic cells were carried out under sterile conditions, the cells were grown in a CO_2 incubator (5% CO_2) at 37°C. For subculturing, the cells were washed with Versene (PanEco) and harvested from plastic with 0.15% trypsin (Gibco)

Experiments. Experiments were carried out on cells in the phase of logarithmic growth. Conditioned medium was removed, the cells were washed with PBS (PanEco), and fresh medium and necessary reagents in working concentrations were added. For induction of cell death, cisplatin (Teva) in a concentration of 70 μM

and doxorubicin (Sigma) in a concentration of 600 nM and 2 μM were added to the cultures.

Transfection of cells with small interfering RNA (siRNA). For transfection, Caov-4 cells attaining 70% confluence were placed in an antibiotic-free OPTI-MEM medium (Gibco). RNAiMAX kit (Invitrogen) was used for introduction of 100 nM siRNA targeted to CASP2, CASP8, and control miRNA (all were from Dharmacon) into cells according to manufacturer's instructions. In 18-20 h, OPTI-MEM medium was changed to complete DMEM medium and an inductor of cell death was added.

Western-blot analysis. After appropriate culturing, the cells were harvested from Petri dishes with 0.15% trypsin and transferred to a conditioned medium. Then, the cells were centrifuged (1000*g*, 5 min, 4°C), the supernatant was discharged and the cells were washed with cold PBS. The cell pellet was resuspended in 20-100 μl lysing buffer and incubated on ice for 20 min. After centrifugation (13,200*g*, 15 min, 4°C), an aliquot of the supernatant was taken for measuring protein concentration in the lysates using a Pierce BCA Protein Assay Kit (Thermo Scientific). The rest supernatant was used for Western blotting. The cell lysate was mixed with 5× buffer for sample application, heated at 95°C for 5 min, separated in PAAG as described elsewhere [9], and transferred to a nitrocellulose membrane (Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad) or Mini Trans-Blot transfer chambers (Bio-Rad). The membranes were blocked for 40 min with 5% skimmed milk in a Tris buffer (TBS), washed four times in TBS, and incubated with primary antibodies diluted in an appropriate buffer for 16 h at 4°C. Then, the membrane was washed 3 times in TBS containing 0.05% Tween-20 (TBST), incubated with secondary antibodies diluted in 2.5% skimmed milk (or 1.25% BSA) for 1 h, then washed three times in TBST. Membranes were developed using ECL reagents (Promega) or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) on a Molecular Imager Chemidoc system (Bio-Rad).

Flow cytofluorometry. The cells were harvested from Petri dishes with 0.15% trypsin, precipitated, and washed with PBS. The cell pellet was resuspended in PBS (100 μl per ~1 million cells). About 100,000 cells were transferred to 200 μl annexin-binding buffer (BD Biosciences), 2 μl annexin V-FITC (Invitrogen) was added, and the samples were incubated in a dark place at room temperature for 15 min. Then, PI was added to the samples to a final concentration of 0.5 μg/ml. After 5-min incubation in a dark place at room temperature, the cell population was analyzed on a FACSCanto II flow cytofluorimeter (BD Biosciences) using FACSDiva software (BD Biosciences).

Gel filtration. For exclusion chromatography, ~50×10^6 Caov-4 cells (four 100-mm dishes) were treated with cisplatin in the media specified in the description of the experiment. After incubation, the cells were collected with a scraper, the cell suspension was centrifuged at 1000*g* and 4°C for 5 min. The cells were washed three times with cold PBS, incubated with 1 ml lysing buffer for 30 min at 4°C, the lysate was centrifuged (13,200*g*, 15 min, 4°C); 20 μl lysate was samples for measuring protein concentration and 20 μl was used for Western blotting. The rest lysate was fractionated by gel filtration on an AKTA FPLC explorer system (GE Healthcare) with Superose 6 10/300 GL column (GE Healthcare) at a flow rate of 1 ml/min using a buffer for gel filtration: 20 mm tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 5% glycerin, 1% Triton-X100, and 1% PMSF. Proteins were detected by light absorption at 280 nm. The column was calibrated using a set of gel filtration standards (Bio-Rad).

Immunoprecipitation. Rabbit polyclonal antibodies to CASP2 (sc-625, 3-5 μg, Santa Cruz) were added to the gel filtration fractions incubated overnight at 4°C; then, 20 μl protein-A sepharose was added and incubation was continued at 4°C for 3-4 h. After incubation, the samples were centrifuged (1000*g*, 1 min, 4°C) and the supernatant was collected. Protein-A-sepharose was then washed 2 times with 1 ml lysis buffer and 2 times with 1 ml PBS, and dried with Hamilton. For Western blotting, 2× buffer for sample application to the gel was added to protein-A sepharose.

RESULTS

CASP2 is a key enzyme triggering apoptosis in ovarian carcinoma cells Caov-4 under conditions of genotoxic stress. To study caspase activation complexes in apoptosis induced by DNA damage, experimental cell model should be chosen and the caspase playing the key role in this process should be identified. As was noted above, CASP2 and CASP8 can initiate apoptosis upon DNA damage. Ovarian carcinoma Caov-4 cells were transfected with the corresponding CASP2- and CASP8-targeting miRNA. Non-target siRNA (not specific to any protein) was used as the control. In 18 h after transfection, the cells were treated with 70 μM cisplatin for 24 h. Apoptotic cell death was analyzed by Western blotting by assessing the level of procaspase cleavage indicating their activation. It was shown that addition of cisplatin to cells treated with non-target siRNA was followed by cleavage of initiator CASP2, CASP8, and CASP9 and effector CASP3, which was associated with a decrease in the content of the corresponding proenzymes (Fig. 1, *a*). Addition of CASP2-targeted siRNA decelerated cleavage of CASP9 and CASP3 and preserved the lev-

els of the corresponding proenzymes in the cell. Suppression of CASP8 synthesis by siRNA had a similar effect: the decrease in the level of this protein in the cell decelerated cleavage of initiator CASP9 and effector CASP3. However, suppression of CASP2 synthesis significantly reduced CASP8 processing, but not *vice versa*. *CASP8* gene knockdown had little effect on CASP2 cleavage and activation (Fig. 1, *a*). In addition, *CASP2* gene knockdown suppressed CASP3 processing to a greater extent.

The intensity of cell death was quantitatively assessed by flow cytofluorometry with AnnV-FITC and PI staining. Annexin V binds to phosphatidylserine on the outer surface of the plasma membrane of apoptotic cells, thus visualizing the population of early apoptotic cells [16,20]. Intercalating dye PI cannot penetrate through the membranes of live cells and stains only cells with impaired plasma membranes. Thus, AnnV-FITC$^+$ and PI$^+$ cells correspond to apoptotic and necrotic populations, respectively; cells stained with both dyes constitute the population of late apoptotic and necrotic cells.

Flow cytometry showed that after 24-h incubation of Caov-4 cells with cisplatin, the number of apoptotic cells appreciably increased and necrotic cells appeared (Fig. 1, *b*). Cisplatin treatment induced less pronounced cell death in cells transfected with siRNA targeting CASP2 and CASP8 than in cells transfected with non-target siRNA: 7.5 and 13.1%, respectively, *vs*. 28% in the control. It should be noted that *CASP2* knockdown was associated with more pronounced suppression of apoptosis in Caov-4 cells. Thus, the data of Western blotting and cytofluorometric analysis showed that the death of Caov-4 ovarian carcinoma cells in response to DNA damage depended on the level of CASP2 and CASP8 in cells and their activa-

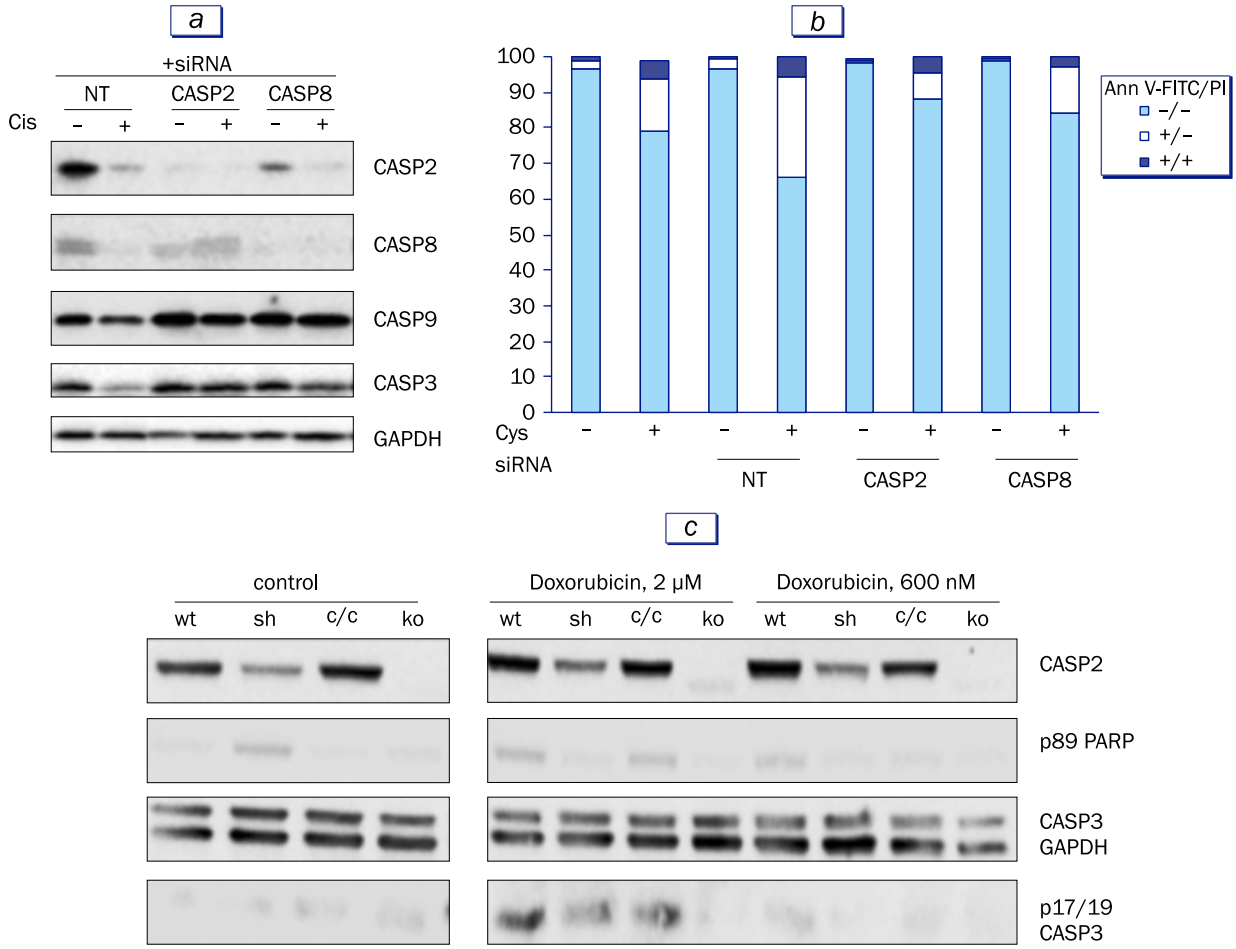

Fig. 1. Analysis of the role of CASP2 and CASP8 in induction of cell death under conditions of genotoxic stress. *a*) Western blot of cell death markers after 24-h treatment with the corresponding siRNAs and cisplatin (Cis, 70 μM). NT: non-target siRNA. GAPDH (glyceraldehyde-3-phosphate dehydrogenase): protein marker of gel loading. *b*) Flow cytofluorometry. Caov-4 cells treated with the specified siRNAs and stained with AnnV-FITC conjugate and PI: -/-, viable cells; +/-, apoptotic cells; +/+, late apoptotic/necrotic cells. *c*) Western blot of cell death markers in normal and CASP2-deficient Caov-4 cells treated with doxorubicin for 24 h. wt: wild-type line; sh: Caov4-shRNA caspase-2 line; c/c: control non-targeting CRISPR/Cas9 Caov-4 line (control); ko: caspase-2 knockout Caov-4 line (CRISPR/Cas9).

tion. However, in the experimental model used by us, CASP2 is the key element of the apoptotic cascade.

To confirm the key role of CASP2 in programmed cell death, CASP2-deficient Caov-4 cells and cells with normal levels of this enzyme were treated with another genotoxic agent doxorubicin. In these experiments, shRNA-caspase-2 Caov-4 (sh) and caspase-2 knockout CRISPR/Cas9 Caov-4 (ko) lines were used

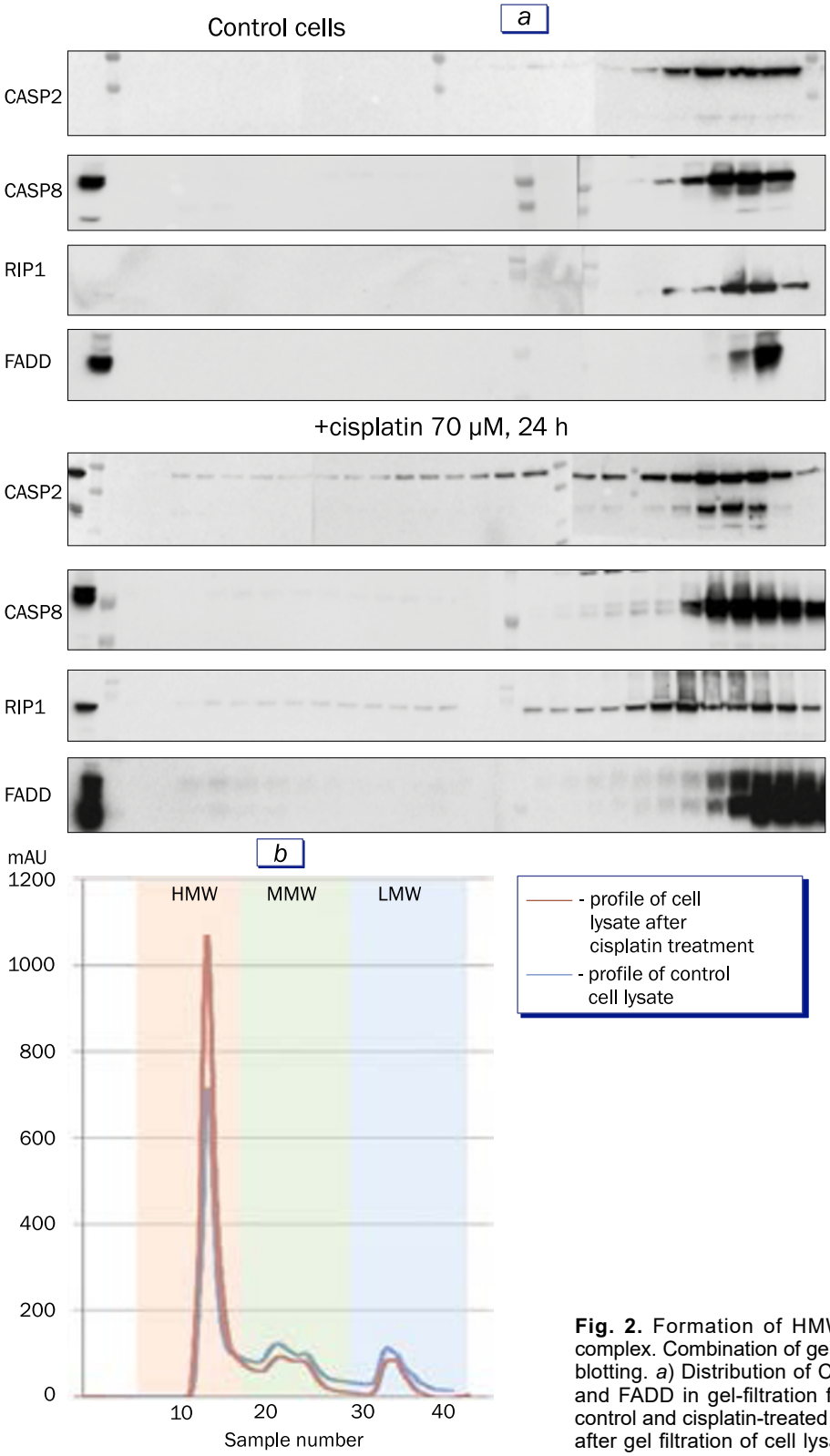

Fig. 2. Formation of HMW CASP2 activation complex. Combination of gel filtration and Western blotting. *a*) Distribution of CASP2, CASP8, RIP1, and FADD in gel-filtration fractions of lysates of control and cisplatin-treated cells; *b*) elution profile after gel filtration of cell lysate.

as CASP2-deficient cells and control by the genome editing procedure by the CRISPR/Cas9 method Caov-4 line with normal CASP2 expression (non-targeting CRISPR/Cas9) (c/c). The four cell lines were incubated with 600 nM and 2 μM doxorubicin for 18 h; then, CASP3 processing and cleavage of the effector caspase substrate poly(ADP-ribose) polymerase (PARP) and appearance of its fragment p89 were analyzed by Western blotting. It was shown that accumulation of enzymatically active fragment p17/p19 of CASP3 and fragment P89 of PARP upon doxorubicin treatment was significantly lower in Caov-4 cells with CASP2 deficiency (sh and ko lines) compared to cells with normal levels of CASP2 (wt and c/c lines) (Fig. 1, *c*). Thus, CASP2 deficiency markedly attenuated apoptotic processes caused by doxorubicin-induced double DNA breaks.

Based on these findings we conclude that initiatory CASP2 and CASP8 play an important role in apoptosis induction in response to DNA damage by chemotherapeutic agents in ovarian carcinoma Caov-4 cells. Once activated, these proteases provide processing and activation of CASP9 that, in turn, activates effector caspases. It should be noted that CASP2 plays the key role in induction of apoptosis by cisplatin and doxorubicin in the used cell model.

Genotoxic stress induces the formation of high-molecular-weight complexes that include CASP2 and CASP8. The formation of caspase activation complexes was analyzed by gel filtration of the lysate of control and cisplatin-treated Caov-4 cells. To this end, cisplatin-treated or control cells were removed from plastic, washed, lysed in a buffer containing 1% nonionic detergent Triton X-100, and separated by gel filtration. Superose 6 carrier effectively separated HMW (≥670 kDa), medium-molecular-weight (MMW, 70-500 kDa) and low-molecular-weight (LMW, ≤70 kDa) protein fractions (Fig. 2, *a*). Gel filtration profile of the lysate of untreated cells was similar to that of cisplatin-treated cells: the greater part of protein material was concentrated in the HMV fractions, which attested to the presence of appreciable content of protein complexes in the cells (Fig. 2, *b*).

Western blot analysis of gel-filtration fractions detected CASP2 in HMW fractions in cisplatin-treated

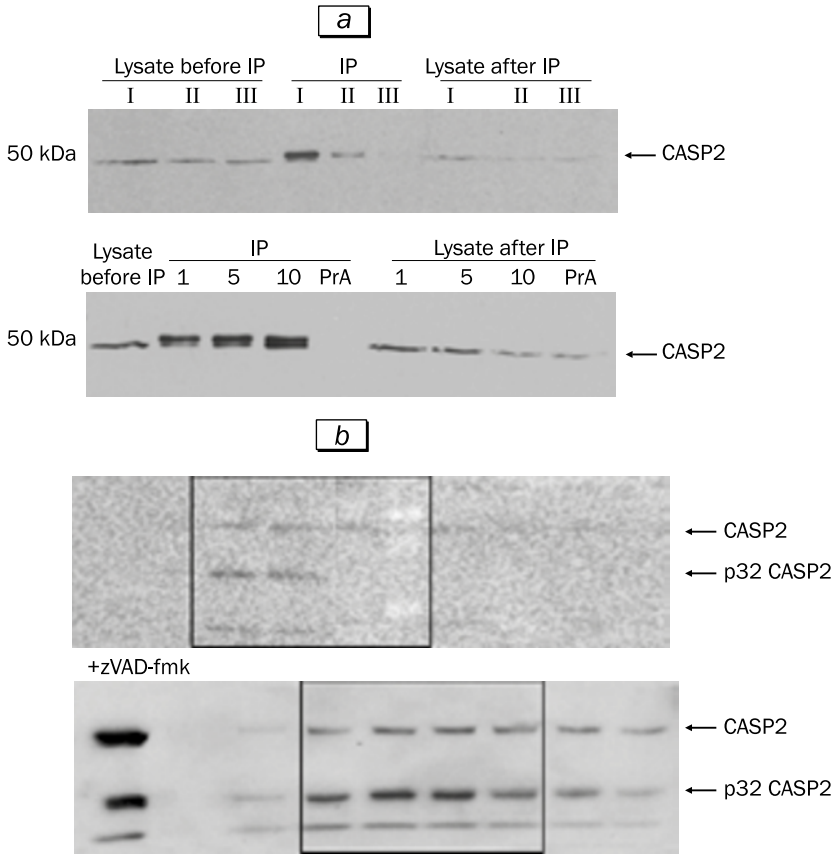

Fig. 3. Choosing optimal conditions for isolation of HMW CASP2 activation complex. *a*) Western blot analysis of CASP2 after IP with different detergents for cell lysis (I — Triton X-100, II — NP-40, III — Chaps) and different amounts of antibodies for IP (1, 5, and 10 μg). PrA: protein-A-sepharose for control of nonspecific sorption; *b*) analysis of the effect of pan-caspase inhibitor Z-VAD-FMK on stability of CASP2 activation complex. The cells were treated with cisplatin (70 μM, 24 h). In the frame: HMW fraction containing CASP2. Arrows show full-length procaspase-2 and its cleavage product p32.

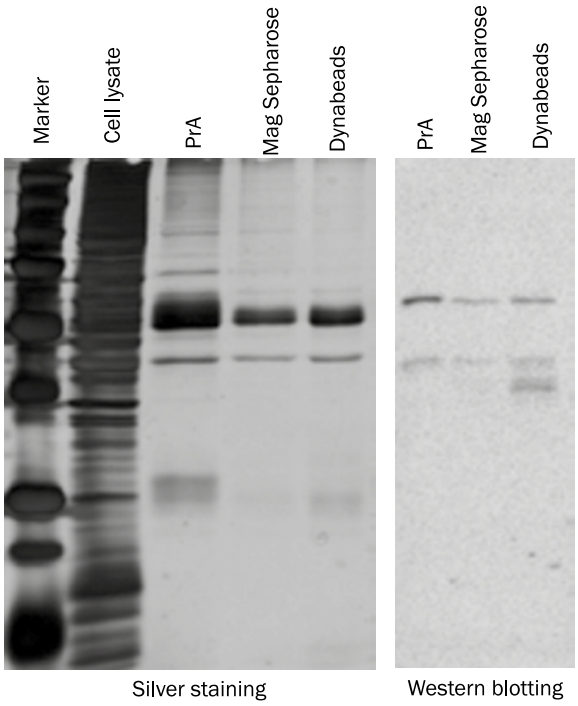

Fig. 4. Comparison of different sorbents for IP. After electrophoresis, the gel was stained with silver (SilverQues Silver Staining Kit) or Western blotting was performed using antibodies to CASP2.

cells, but not in control cells. Interestingly, not only CASP2, but also CASP8 was found in the same fractions of lysates of cisplatin-treated cells. Along with CASP8, RIP1 and FADD proteins, the components of CASP8 activation complex formed in response to DNA damage were also detected in HMW fractions [14]. It was previously shown that CASP8 activation platform does not contain CASP2 and *vice versa* [17]. Moreover, it was demonstrated that CASP2 is an apical initiator caspase in this experimental model. Thus, our findings drew us to a conclusion that cisplatin treatment of ovarian carcinoma cells induced the formation of an HMW complex where CASP2 activation occurs. In parallel, an HMW complex that includes CASP8 is formed, but according to our data, this process is not initiatory.

To further isolate the CASP2 activation complex, its components were immunoprecipitated from gel filtration fractions. The isolation conditions were optimized to increase the yield of the purified complex.

Selection of conditions for immunoprecipitation (IP). To optimize the isolation conditions, IP was performed using different amounts of antibodies to CASP2 (1, 5, and 10 μg/ml) and different detergents were used for cell lysis: 1% Triton X-100, 1% NP-40, 1% Chaps.

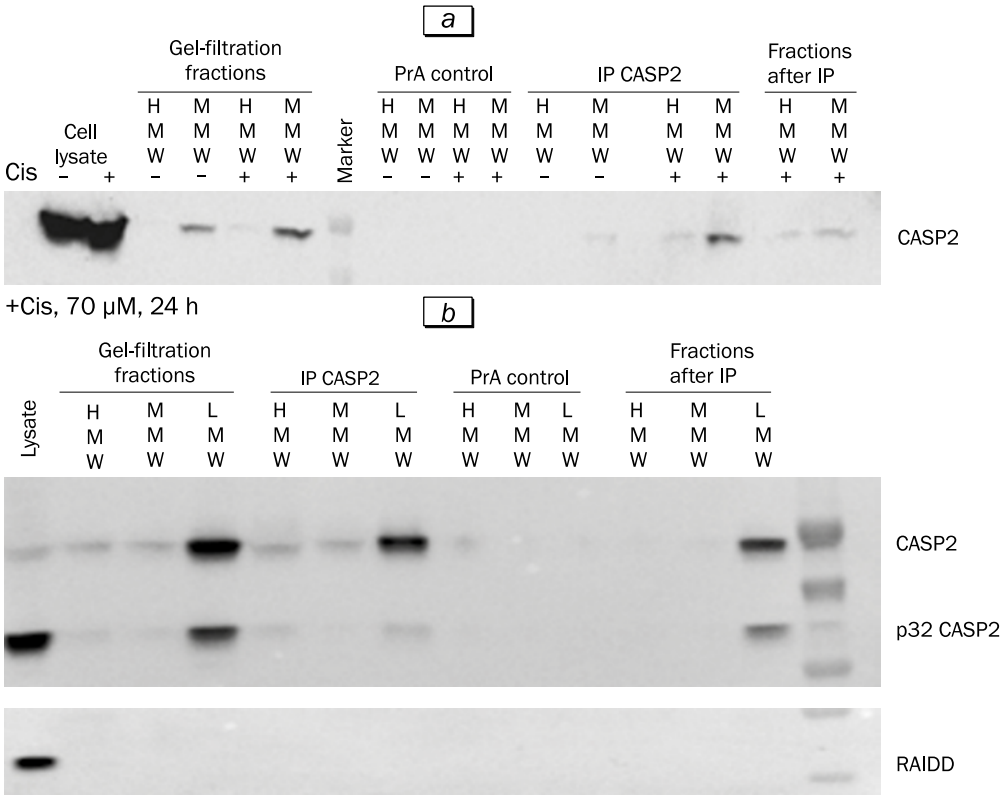

Fig. 5. Isolation of HMW CASP2 activation complex. *a*, *b*) IP of CASP2 from fractions with different molecular weight followed by Western blotting under non-optimized (*a*) and optimized (*b*) conditions. Fractions were obtained by gel filtration of lysates of control cells and cells treated with cisplatin (Cis, 70 μM, 24 h). Full-length procaspase-2 and RAIDD protein are shown. PrA control: samples incubated with protein-A sepharose without specific antibody.

Total cell lysates or fractions obtained by gel filtration were used for IP. For obtaining total lysates, the control and stimulated cells were processed by the protocol used for lysates for gel filtration. Nonspecific sorption was prevented by preliminary purification as follows: gel filtration fractions or cell lysates were incubated with nonspecific polyclonal antibodies (Santa Cruz) for 1 h at 4°C; then, protein-A-sepharose (Sigma) was added, the samples were incubated at 1 h at 4°C at constant agitation, centrifuged, and the supernatant was collected. This preliminary purification step ensured removal of a significant amount of nonspecifically binding proteins.

The results of IP were assessed by gel electrophoresis and Western blotting. This analysis showed that Triton X-100 was the best detergent for the lysing buffer used in IP, and the optimal amount of antibodies was 5-10 μg/ml lysate at protein concentration of 3-5 mg/ml (Fig. 3, *a*). Nonspecific binding of proteins to protein-A sepharose was controlled using a sample containing no antibodies (protein-A control). The analysis of this sample revealed no nonspecific sorption (Fig. 3, *a*). Thus, optimal conditions were selected for IP to obtain samples for further analysis.

The next step in selection of optimal conditions for successful IP of HMW activation platforms was addition of caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl] fluoromethylketone) for stabilization of the initiator complexes and more effective precipitation of caspases. Inhibitor Z-VAD-FMK added to cell lysate promoted stabilization of CASP2 activation complex (Fig. 3, *b*). As the proteins involved in CASP2 activation most likely do not form covalent bonds in the studied complex, this complex gradually dissociates in solution, which makes it difficult to isolate and identify its components. The use of inhibitor Z-VAD-FMK, apparently, blocked the dissociation of CASP2 from the HMW platforms, which substantially improved the content of this protein in HMW fractions and, hence, increased the lifetime of the studied complex (Fig. 3, *b*).

To maximize the yield of the studied complex from HMW fractions and to reduce nonspecific binding of proteins to protein-A sepharose, additional optimization of the IP Protocol was carried out. To prevent nonspecific sorption, protein-A sepharose was replaced with magnetic particles with protein-A/G (Thermo Scientific Pierce Protein A/G Magnetic Beads) and Dynabeads magnetic particles (Thermo Scientific Pierce Protein A Dynabeads) (Fig. 4). This replacement significantly reduced in the proportion of nonspecifically bound proteins (silver staining, Fig. 4).

Isolation of high-molecular-weight CASP2 activation complex. For further isolation of the macromolecular CASP2 activation complex, we used combination of gel filtration and IP. To this end, the lysates of control and cisplatin-treated cells were fractionated by gel filtration. Then, IP of CASP2 under the above described conditions was performed.

Western blot analysis of the obtained samples showed the presence of CASP2 in IP from HMW fractions of cisplatin-treated, but not control cells (Fig. 5, *a*). CASP2 was also successfully isolated from MMW fractions of both control and cisplatin-treated cells. However, due to the use of suboptimal conditions for isolation of CASP2, a part of the protein remained in the gel filtration fractions after IP (Fig. 5, *a*). The use of conditions chosen at the previous stage made it possible to completely precipitate CASP2 from HMW fractions (Fig. 5, *b*).

During analysis of CASP2 activation complex, it was of great interest to check the presence of PIDDosome platform components in this complex. None of the post-IP samples contained RAIDD adaptor protein, the key component of the PIDDosome complex (Fig. 5, *b*), which corresponded to the previous results confirming the existence of a PIDDosome-independent pathway of CASP2 activation under conditions of genotoxic stress [7].

Thus, we propose an effective method of purification of the HMW CASP2 activation complex. This method allows isolation of endogenous caspases in the composition of HMW platforms. The developed method can be used to isolate other HMW that regulate the processes of programmed cell death.

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