Apoptosis Induces Efflux of the Mitochondrial Matrix Enzyme Deoxyguanosine Kinase*

Mia Jüllig‡ and Staffan Eriksson

From the Department of Veterinary Medical Chemistry, University of Agricultural Sciences, The Biomedical Centre, Box 575, S-751 23 Uppsala, Sweden

Deoxyguanosine kinase (dGK) initiates the salvage of purine deoxynucleosides in mitochondria and is a key enzyme in mitochondrial DNA precursor synthesis. The active form of the enzyme is a 60-kDa protein normally located in the mitochondrial matrix. Here we describe the subcellular distribution of dGK during apoptosis in human epithelial kidney 293 cells and human lymphoblast Molt-4 cells. Immunological methods were used to monitor dGK as well as other mitochondrial proteins. Surprisingly, dGK was found to relocate to the cytosolic compartment at a similar rate as cytochrome c, a mitochondrial intermembrane enzyme known to enter the cytosol early in apoptosis. The redistribution of dGK from the mitochondria to the cytosol may be of importance for the activation of apoptotic purine nucleoside cofactors such as dATP and demonstrates that mitochondrial matrix proteins may selectively leak out during apoptosis.

Apoptosis (programmed cell death) is a highly regulated cellular suicide machinery that enables cells to die without causing an inflammatory response. A key event in apoptosis is the relocation of cytochrome c from the mitochondrial intermembrane space to the cytosol (1). The presence of cytochrome c in the cytosol initiates the formation of a cytosolic apoptosome (a multimeric complex consisting of cytochrome c, apoptosis protein-activating factor Apaf-1, and dATP (2, 3)). This complex is capable of binding and activating the caspase-9zymogen, which leads to activation of the caspase-3zymogen, which catalyzes the proteolytic activation of several downstream caspases responsible for degradation of structural components of the cell.

A recent study describes the ability for various adenine nucleotide analogs to supplant dATP in the assembly of the apoptosome (4). The relative efficiencies of the tested nucleotides were Ara-ATP > F-Ara-ATP > dATP > CdATP > Ara-GTP > dADP > ATP. The non-phosphorylated forms of these nucleoside analogs (Ara-A, F-Ara-A, CdA, and Ara-G) are of pharmacological importance and are used as cytosstatic or antiviral agents (5). Much of their therapeutic effects have traditionally been attributed to their ability to terminate replication when the resulting triphosphate forms are incorporated into the DNA of target cells and thus stop cell division (reviewed in Ref. 6). However, nucleoside analogs that interfere with DNA synthesis can also induce cell death by apoptosis (7, 8).

Deoxyguanosine kinase (dGK) is a 60-kDa enzyme localized in the mitochondria, and it catalyzes the phosphorylation of the natural substrates dGuo, dAdo, and dIno to the corresponding monophosphate nucleotides (9, 10). The recombinant enzyme has been studied and shown to accept various cytosstatic nucleoside analogs as substrates (11, 12). Examples of such analogs are F-Ara-A, CdA, and Ara-G, all precursors of the apoptogenic adenine deoxynucleotide analogs mentioned above (7). The observation that the substrate specificity of dGK coincides with the nucleotide requirement for caspase-9 activation raises the possibility of a role for dGK during apoptosis. However, the assembly of the apoptosome takes place in the cytosol, and dGK is located in the mitochondrial matrix (13) where its deoxynucleotide products have limited access to the cytosol due to the impermeable nature of the inner mitochondrial membrane that surrounds the matrix compartment. To efficiently assist in dATP/adenosine deoxynucleotide analog production for the caspase-9 activating apoptosome, dGK would have to be present in the cytosol.

dGK has been proposed to mediate apoptosis of T-cells in SCID disease by causing elevated intramitochondrial levels of dGTP in T-cells lacking purine nucleoside phosphorylase (PNP) (14, 15). Thus, dGK plays a vital role in the early chain of reactions that eventually leads to dGuo-induced apoptosis, and the fact that dGK is present inside the mitochondria is a prerequisite for the proposed mechanism of T-cell toxicity. It was therefore important to monitor the subcellular location of dGK during apoptosis.

EXPERIMENTAL PROCEDURES

Materials—The anti-dGK antibodies were prepared as described (13). Anti-cytochrome c antibodies were obtained from CLONTECH. An anti-adenine nucleotide translocase (ANT) antibody preparation was a gift from Dr. Patrik Andrée, Clinical Research Center, Div. of Medical Cell Biology, Karolinska Institute, NOVUM, Huddinge, Sweden. Cell Culture and Treatments—Human epithelial kidney (HEK) 293 cells were cultivated in Dulbecco’s Modified Eagle Medium with GLUTAMAX™ supplemented with 10% (v/v) fetal calf serum, 1 mM sodium pyruvate, and 1% (v/v) antibiotic/antimycotic solution (Life Technologies, Inc.). Human lymphoblast Molt-4 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 1% (v/v) antibiotic/antimycotic solution (Life Technologies, Inc.). Cells grown to late log-phase were treated with daunorubicin (Rhone-Poulenc Rorer, Bristol, England) (1–10  μM for 6 h) or CdCl₂ (20

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‡ To whom correspondence should be addressed: Dept. of Veterinary Medical Chemistry, Univ. of Agricultural Sciences, The Biomedical Center, Box 575, S-751 23 Uppsala, Sweden. Tel.: 46-18-4714101; Fax: 46-18-550762; E-mail: Mia.Jullig@vmk.slu.se.

1 The abbreviations used are: dGK, deoxyguanosine kinase; F-Ara-ATP, 2-fluoroarabinosyladenine triphosphate; CdATP, 2-chloro-2'-deoxyadenosine triphosphate; dFdG, 2',2'-difluorodeoxyguanosine; SCID, severe combined immunodeficiency; PNP, purine nucleoside phosphorylase; ANT, adenine nucleotide translocase; HEK, human embryonic kidney; dCK, deoxycytidine kinase; CS, citrate synthase; DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin.
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μM in serum-free growth medium for 3–48 h. Mitochondrial and cytosolic fractions were isolated immediately after each incubation using the ApoAlert Cell Fractionation Kit (CLONTECH) according to the description provided by the manufacturer. Proteinase inhibitors were present throughout the isolation procedure, and all samples were stored at −20 °C for a maximum of 5 days before analysis. The protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin (fraction V) as standard.

Enzyme Assays—Caspase-3 activation was determined using the DEVD-AMC cleavage assay (16). Cells were resuspended in lysis buffer (100 mM Hepes, pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol, and 0.01% Nonidet P-40) in a 96-well microtiter plate at room temperature. After rapid freezing of the microtiter plate, 1 μL of the fluorogenic peptide substrate DEVD-AMC (Bachem) was added and liberation of AMC by cleavage of DEVD-AMC was monitored using a fluoroscope plate reader (BioTech FL 600, Boule) using a 360-nm excitation and a 460-nm emission wavelength. Fluorescence was measured every 60 s during a 60-min period. The activity of citrate synthase was assayed as described (17) in samples incubated with 0.1% (v/v) Triton X-100/mg protein for 10 min on ice. To evaluate inhibition of the enzyme by CdCl₂, the assay was also performed with mitochondria obtained from control cells with CdCl₂ present in the reaction mixture.

Quantification of Apoptosis—Flow cytometry analysis was carried out as described (18) on HEK 293 cells after incubation with CdCl₂. The area of the peak with low DNA content, representing apoptotic cells, was calculated for each time point.

Western Blot Analysis—Protein was precipitated as previously described (19) and incubated in an electrophoresis sample buffer containing 25% glycerol, 400 mM Tris-base, 10 mM dithiothreitol, 1% (w/v) SDS, and 0.01% (w/v) bromphenol blue for 10 min at room temperature after which 1/10 volume of 1 M HCl was added. Equal amounts of protein were separated on 12% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes and probed for 1–2 h with anti-ANT, anti-cytochrome c, or anti-dGK antibodies. The antibodies were diluted 1:1000, 1:200, and 1:600, respectively, in TBS/0.5% Tween 20 supplemented with 3% (w/v) bovine serum albumin (fraction V). The secondary horseradish peroxidase-conjugated anti-rabbit IgG was diluted 1:2500 in TBS/0.5% Tween 20. The protein bands were detected by the enhanced chemiluminescence immunodetection system (Amersham Pharmacia Biotech). All films were scanned using an UMAX Astra 2400S scanner and analyzed using the Molecular Analyst software (Bio-Rad) whereby a numerical value, based on size and density, was obtained for each band.

Immunohistochemistry—All steps were carried out at 25 °C unless otherwise stated. One hundred thousand HEK 293 cells were seeded/well in a fibronectin-coated 8-well culture slide and allowed to attach and expand for 12 h at 37 °C. Apoptosis was induced by a 6-h incubation at 37 °C with 20 μM CdCl₂ in serum-free Dulbecco’s modified Eagle’s medium. The wells were then incubated for 45 min at 37 °C with 200 nm MitoTracker® Red CM-H₂XRos (Molecular Probes, Leiden, the Netherlands) in growth medium. Fixation, permeabilization, and incubation with purified anti-dGK antibodies were performed as previously described (15). The cells were then incubated for 1 h with anti-rabbit Cy2 (Jacksons Laboratories) diluted 1:20 in phosphate-buffered saline supplemented with 0.1% azide. After extensive washing, the chamber walls were removed, and the slides were mounted using the ProLong™ Antifade reagent (Molecular Probes). The slides were then examined in an Olympus BX60 fluorescence microscope connected to a Sony Digital Photo Camera DKC-5000.

RESULTS AND DISCUSSION

HEK 293 cells are well suited for this dGK study because they provide a rich source of mitochondria and are ideal for immunohistochemistry. It has been shown that Cd²⁺ induces apoptosis in cultured proximal tubule cells (20) and that incubation with 20 μM CdCl₂ raises the apoptotic index of HEK 293 cells at least 15 times compared with untreated cells (21).

Western blot analysis of extracts of HEK 293 cells treated with CdCl₂ showed that the intramitochondrial protein levels of dGK and cytochrome c decreased in a time-dependent manner and at similar rates. After incubation for 6 h, the intramitochondrial concentrations of dGK and cytochrome c were reduced to half that of the untreated control cells (Fig. 1A). In the corresponding cytosolic fractions, cytochrome c (Fig. 1B) and dGK (Fig. 1C) levels increased after the addition of CdCl₂ to the medium. After 6 h of treatment, the detected level of cytochrome c in the cytosol was >40% higher than in the untreated control. dGK was detected in the cytosolic fraction after 24 and 48 h.

Similar observations were made by Western blot analysis of mitochondrial and cytosolic fractions from Molt-4 cells treated with daunorubicin. After treatment with 1 μM daunorubicin, 40% of the dGK had relocated to the cytosol, whereas the cytochrome c level was largely unaffected (Fig. 2A). After incubation with 10 μM daunorubicin ~50% of both dGK and cytochrome c had left the mitochondria (Fig. 2A). Elevated levels of both dGK and cytochrome c were detected in the cytosolic fraction at both tested concentrations (Fig. 2B).

ANT, a mitochondrial inner membrane protein, was used as a marker to ensure that equal amounts of mitochondrial material had been loaded on the gel. The levels of ANT remained constant throughout the experiments (Figs. 1A, 2A, and 3).

Flow cytometry showed that 25% of the HEK 293 cells had undergone apoptotic DNA fragmentation after 24 h of treatment with CdCl₂ (Fig. 1D). The DEVD-AMC cleavage assay used to monitor apoptosis in Molt-4 cells showed a 6-fold increase of the apoptotic index when the cells were incubated with 5 μM daunorubicin for 6 h (Fig. 2, A and B).

Because induction of apoptosis in HEK 293 cells was carried out by treatment with CdCl₂ in serum-free medium, the effects of serum removal itself had to be investigated to interpret the results with CdCl₂. The intramitochondrial levels of cytochrome c, dGK, and the integral inner membrane protein ANT were monitored in mitochondrial isolates throughout a 72-h incubation in serum-free medium. Western blot analysis showed no decrease in protein concentration for either of the tested proteins. Instead, the levels of cytochrome c and dGK increased (50 and 150%, respectively) as a response to serum depletion (Fig. 3). This observation strongly indicates an increased synthesis of the two proteins upon serum starvation. Recent studies demonstrate that inhibition of DNA synthesis in human lymphocytes increases the activity of the cytosolic deoxynucleoside salvage enzyme deoxyctydine kinase (dGK) (22). It is possible that serum depletion invokes an increase in dGK levels similar to the activation of dGK. The mechanisms behind these effects remain to be determined.

Citrinate synthesize (CS), a mitochondrial matrix enzyme with a molecular mass of 49 kDa, was assayed in the mitochondrial fractions to monitor leakage of mitochondrial matrix material. No inhibition of CS activity was detected when CdCl₂ was added to previously untreated mitochondria (not shown), indicating that the moderate decrease in CS activity observed in mitochondria obtained from CdCl₂-treated HEK 293 cells reflects a true loss of CS from the mitochondria (Fig. 1E). This finding probably represents CS efflux from necrotic cells in which the mitochondrial membranes are known to disintegrate. Due to interference with the assay by daunorubicin, citrate synthesize activity could not be determined in apoptotic Molt-4 cells.

In an immunohistochemical experiment, the dGK antibody was used with HEK 293 cells treated with 20 μM CdCl₂ for 6 h followed by incubation with MitoTracker® Red CM-H₂XRos to visualize respiring mitochondria. In non-treated cells, dGK labeling colocalized with MitoTracker® Red CM-H₂XRos in practically all the examined cells (Fig. 4A). The punctate labeling pattern around the nuclei was identical to that previously reported for HEK 293 cells (13) and similar to that obtained when a mouse dGK was fused to green fluorescent protein and overexpressed in Cos-1 cells (23). The majority of the CdCl₂-treated cells showed a diffuse cytosolic staining pattern with the dGK antibody (Fig. 4, B and C). Six h after addition of CdCl₂ to HEK 293 cells, dGK was localized extra-
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mitochondrially in many cells. The localization pattern was similar to that reported for the cytosolic isoform of mouse dGK (23) and Smac/DIABLO 4 h after induction of apoptosis (24). In many CdCl2-treated cells, the overall dGK labeling was more intense compared with the control cells. The most likely explanation for this finding is that relocated, cytosolic dGK, compared with mitochondrial, was more easily available for detection with antibodies. However, it can not be excluded that induction of the enzyme was contributing to the increased fluorescence observed in apoptotic cells. The CdCl2-treated cells showed varying degrees of apoptotic characteristics. Although some cells appeared largely unaffected by the treatment, most exhibited early morphological changes associated with apoptosis such as spherical shape and substantial size reduction (Fig. 4C). In many CdCl2-treated HEK 293 cells that appeared clearly apoptotic, labeling with MitoTracker® Red CM-H2XRos was still detected indicating that the mitochondrial

Fig. 1. Correlation between apoptosis and the levels of various mitochondrial proteins in mitochondrial and cytosolic extracts from HEK 293 cells incubated with CdCl2. A, protein levels in mitochondrial extracts detected by Western blotting. Open circles, dGK; open triangles, cytochrome c; crosses, ANT; closed circles, apoptotic cells. B, protein levels in cytosolic extracts detected by Western blotting. Open triangles, cytochrome c; closed circles, apoptotic cells. C, Western blot of cytosolic extracts from HEK 293 cells treated with CdCl2 using the anti-dGK antibody. The number over each lane indicates h of incubation with CdCl2. D, apoptosis, as measured by flow cytometry of HEK 293 cells. Left panel, untreated control cells; right panel, cells treated with CdCl2 for 24 h. E, citrate synthase activity (17) in % as compared with untreated cells (0 h). The fraction of apoptotic cells was measured by flow cytometry as shown in Fig. 1D.
drial membrane potential was not disrupted (Fig. 4, B and C).

A role for dGK in apoptosis has recently been proposed (15). Individuals suffering from SCID disease have an inherited deficiency of either adenosine deaminase or PNP, both enzymes crucial for the degradation of deoxyribonucleosides. Complete PNP deficiency in mice leads to accumulation and elevated urine contents of the PNP substrates (dGuo, Guo, dIno, and Ino), but it is also reported that the intramitochondrial levels of dGTP are increased. Arpaia et al. (15) propose that dGK is responsible for the latter by catalyzing the formation of dGMP from accumulated dGuo, after which dGTP is formed by deoxynucleoside mono- and diphosphate kinases. The abnormal levels of dGTP thus produced causes an imbalance of the deoxynucleotide pools, which damages the mitochondrial DNA and leads to apoptosis. In this scenario dGK is present inside the mitochondria when catalyzing dGMP formation, which is not in conflict with the finding that dGK relocates to the cytosol during apoptosis because the dGTP-mediated damage on the mtDNA in SCID disease is an event that proceeds cytochrome c relocation and caspase activation.

Zhu et al. have suggested that dGK may be involved in the cytotoxicity observed during treatment with the anti-proliferative nucleoside analogs CdA, dFdG, and Ara-G (25). Overexpression of dGK was reported to lead to increased sensitivity to the above-mentioned drugs in human pancreatic cancer cell lines. It was suggested that the analogs, after intramitochondrial phosphorylation catalyzed by dGK, were exported to the cytosolic compartment where they could interfere with nuclear DNA replication. Whether the observed effect on cell viability in this report was due to apoptotic cell death is not clear. However, evidence suggest that mitochondrial deoxynucleotides are not exported to the cytosol. Already in 1982, Bestwick et al. showed that depletion of cytosolic deoxynucleotide pools is not accompanied by a similar depletion of the corresponding mitochondrial pools (26). A mitochondrial deoxynucleotide carrier was recently reported and initial studies of the protein indicate that it is primarily involved in the mitochondrial uptake of various deoxynucleotides including several analogs in exchange for internal ATP (27). In the light of this, it seems reasonable to assume that intramitochondrially phosphorylated nucleoside analogs are confined to the mitochondrial compartment and cause damage to mtDNA rather than the nuclear DNA, provided that the inner mitochondrial membrane is not disrupted.

In a more recent publication the importance of the cellular site of phosphorylation of CdA and other nucleoside analogs is discussed (28). dCK, normally a cytosolic enzyme, was overex-
pressed with a leader sequence targeting the enzyme to different cellular compartments. When CdA was administered, mitochondrial expression of dCK resulted in less cell death compared with when dCK was targeted to the cytosol or the nucleus. This was most likely due to entrapment of the intramitochondrially phosphorylated analogs inside the mitochondria and further strengthen the assumption that dGK, as well as dCK, must be present in the cytosol to efficiently contribute to caspase activation.

Our results show that dGK relocates to the cytosolic compartment during apoptosis in the two cell types tested. dGK was recently localized in the mitochondrial matrix in human placenta mitochondria (13), and to the best of our knowledge, no other matrix protein has previously been demonstrated to leave the mitochondrial matrix in apoptotic cells. It was recently reported that mitochondria in apoptotic Jurkat cells release the intermembrane form of adenylate kinase (AK3) remains inside the mitochondria (29). The question of how dGK crosses the virtually impermeable mitochondrial membrane remains to be answered. One intriguing possibility is that the enzyme could be transported into the cytosol to assist in amplifying the apoptotic cascade in the proximity of the caspase-9 activating apoptosome by being involved in the formation of dATP.

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