CPP32/Yama/apopain cleaves the catalytic component of DNA-dependent protein kinase in the holoenzyme

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Abstract DNA-dependent protein kinase (DNA-PK) is composed of a 460-kDa catalytic component (p460) and a DNA-binding component Ku protein. Immunoblot analysis after treatment of Jurkat cells with anti-Fas antibody demonstrated the cleavage of p460 concomitantly with an increase in CPP32/Yama/apopain activity. Recombinant CPP32/Yama/apopain specifically cleaved p460 in the DNA-PK preparation that had been purified from Raji cells into 230- and 160-kDa polypeptides, the latter of which was detected in anti-Fas-treated Jurkat cells. The regulatory component Ku protein was not significantly affected by CPP32/Yama/apopain. DNA-PK activity was decreased with the disappearance of p460 in the incubation of DNA-PK with CPP32/Yama/apopain. These results suggest that the catalytic component of DNA-PK is one of the target proteins for CPP32/Yama/apopain in Fas-mediated apoptosis.

Key words: DNA-dependent protein kinase; Apoptosis; CPP32/Yama/apopain; DNA-PK catalytic component (p460); Ku protein; Jurkat cell

1. Introduction

DNA-dependent protein kinase (DNA-PK) is composed of a 460-kDa catalytic subunit tentatively termed DNA-PKcs [1] and a DNA-binding component Ku protein (p70/p80) [2–4]. DNA-PK requires double-stranded DNA for the phosphorylation of a variety of replication/transcription factors, such as Sp1, p53, c-Myc and RP-A [5–13], which likely contain X-S/T-Q or P-S/T-X as minimal requirement for the recognition sequence [4]. It has recently been revealed that DNA-PK is involved at least in DNA double-strand break repair and V(D)J recombination [14–20], the molecular mechanism of which is not fully understood. DNA-PKcs is a member of the PI3-kinase superfamily and DNA-PK activity is sensitive to wortmannin, a potent inhibitor of PI3-kinase. DNA-PK, however, has no lipid kinase activity [1]. Take et al. [21,22] reported a specific inhibitor for DNA-PK, which is competitive to ATP and structurally different from wortmannin.

Based on these attractive properties of DNA-PK, DNA-PK has been considered to function in regulating cell growth, cell differentiation or cell maintenance. We detected preliminarily limited degradation products of DNA-PKcs in peripheral blood lymphocytes or T lymphocytes after growth stimulation (Suwa et al., unpublished results). Casciola-Rosen et al. [23] recently reported that several nuclear autoantigens including poly(ADP-ribose) polymerase, U1 snRNA-70 kDa and DNA-PKcs are cleaved early during apoptosis in UV-irradiated HeLa cells. Poly(ADP-ribose) polymerase that seems to be involved in DNA repair has recently been reported to be a potential substrate for apopain, also called CPP32 or Yama, under the apoptotic process [24–26]. Since the human Jurkat T cell line exhibits typical apoptosis after treatment with Fas ligand or an agonistic anti-Fas antibody [27], we examined the possibility that DNA-PKcs is one of the death substrates during Fas-mediated apoptosis in the Jurkat T cell line.

Here we show that DNA-PKcs was cleaved on incubation with recombinant apopain in vitro as well as during Fas-mediated apoptosis in Jurkat cells.

2. Materials and methods

2.1. Antibodies and chemicals

Anti-human Fas antibody (clone CH-11) was purchased from Immunotech, Marseille, France. Rabbit anti-DNA-PKcs antibody was from Serotech, Oxford, UK. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from NEN (Wilmington, DE, USA). Ac-DEVD-AMC, a substrate for apopain and Ac-DEVD-CHO, an inhibitor of apopain [26] were from the Peptide Institute, Inc., Osaka, Japan.

2.2. Cell culture and preparation of cell-free extracts

A human leukemic T cell line, Jurkat, was kindly provided by Professor K. Yamamoto in our Institute and was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum as described previously [28]. The cells were incubated with anti-Fas antibody (100 ng/ml) at a density of 4×10^5 cells/ml. Cell viability was examined by the trypan blue dye-exclusion test. At the indicated times, cells were collected and washed once with phosphate-buffered saline. To 5×10^6 cells was added 100 μl buffer B (20 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, 0.2 mM EDTA, 10% glycerol and 1 μg ml^{-1} each of aprotinin, leupeptin A and pepstatin) containing 0.4 M KCl as described [4]. After standing on ice for 10 min, the mixture was centrifuged in an Eppendorf tube at 15000 rpm for 15 min to obtain whole cell extracts.

2.3. Analysis of DNA fragmentation

The analysis of oligonucleosomal DNA fragments was performed essentially as in [29]. Briefly, the pellet of 10^6 cells in a Eppendorf tube was suspended in 20 μl of 50 mM Tris-HCl (pH 8), 10 mM EDTA, 0.5% sodium lauryl sarkosinate and 10 μg proteinase K. The mixture was incubated at 50°C for 1 h, and after addition of 5 μg RNase A the incubation continued for a further 1 h. To the lysate heated to 70°C was added 10 μl of pre-heated 1% low-melt agarose containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, 0.25% bromophenol blue and 40% sucrose. The mixed sample was loaded into a dry well on a 2% agarose gel containing 1 μg ml^{-1} ethidium bromide and 0.5×TBE. After electrophoresis in 0.5×TBE, DNA was visualized under UV light.

2.4. Assay of apopain

Apopain activity was fluorometrically determined with Ac-DEVD-AMC as a specific substrate [26]. An aliquot of whole cell extracts (2.5

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and 5 μl) was incubated in a 100 μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 250 μM Ac-DEVD-AMC and 10% glycerol at 30°C for 20 min. After addition of 2.9 ml H_2O, the product was determined in a Hitachi F-2000 fluorescence spectrophotometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

2.5. Purification of DNA-PK

DNA-PK was partially purified from Raji nuclear extracts as described previously [4] and subsequently subjected to glycerol gradient (15–30%) centrifugation. The DNA-PK preparation containing DNA-PKcs and Ku protein was separated from free Ku. These purification procedures will be published in detail elsewhere.

2.6. Assay of DNA-PK

DNA-PK activity was determined with either a synthetic peptide 15, EPPLSQEAFADLWKK, or α-casein as described previously [4] except that 300 μM ATP (0.1 μCi [γ-^{32}P]ATP) was used for peptide 15 and 50 μM ATP (0.2 μCi [γ-^{32}P]ATP) for α-casein. Mutant peptide 15, EPPLSEEAFADLWKK, was used as a negative control [21]. The enzyme activity was expressed as pmol P_i incorporated into substrate per min at 30°C.

2.7. Treatment of DNA-PK with recombinant apopain

The purified DNA-PK was incubated with recombinant apopain [30] at 30°C for 30 min in a reaction mixture (20 μl) containing 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol and 10% glycerol. When necessary, 0.2 μl 10 mM Ac-DEVD-CHO was added to the reaction mixture. For assay of DNA-PK, 5-μl portions were taken from the reaction mixtures, and residues were subjected to SDS-polyacrylamide gel electrophoresis.

2.8. Western blot/enzyme immunoassay of DNA-PK

The samples were subjected to electrophoresis on 0.1% SDS/7.5% polyacrylamide gel and then blotted onto a nitrocellulose paper. The paper was blocked with 1% casein in TBS/Tween 20 (20 mM Tris-HCl, pH 7.4/0.15 M NaCl/0.05% Tween 20). The paper was incubated with either 1000-fold diluted rabbit anti-DNA-PKcs serum or anti-Ku serum from a patient (OM) [31] for 1 h, and then with 1000-fold diluted peroxidase-conjugated second antibody against rabbit IgG (Bio-Rad) or human IgG (Sigma) in TBS/Tween 20 containing 1% casein for 30 min. After washing with TBS/Tween 20, the antigen-antibody complex was visualized with a Konica immunostain assay kit (Konica, Tokyo, Japan).

2.9. Protein determination

Protein was determined by the method of Bradford [32] with bovine serum albumin as a standard. Silver staining after SDS-polyacrylamide gel electrophoresis was performed with a kit from Wako Pure Chemicals, Osaka, Japan.

3. Results

Whole cell extracts from Jurkat cells treated with anti-Fas antibody for 0, 2, 4 and 6 h were analyzed by Western blot/enzyme immunoassay with rabbit anti-DNA-PKcs antibody (Fig. 1a). In Jurkat cells 4 and 6 h after treatment with anti-Fas antibody, a 160-kDa polypeptide (p160) was observed in addition to 460-kDa DNA-PKcs (p460). Since a faint immunostain band of p160 also appeared 2 h after the treatment, the appearance of p160 seems to be earlier than the detection of the DNA ladder (Fig. 1b). Apopain, a thiol proteinase involved in apoptosis, was induced slightly at 2 h and drastically at 4–6 h under treatment with anti-Fas antibody (Fig. 1c). These results suggest that p460 is cleaved by apopain during Fas-mediated apoptosis in Jurkat cells. DNA-PK activity in the crude extracts from Jurkat cells was essentially constant 0–4 h after treatment with anti-Fas antibody, and slightly decreased at 6 h (Fig. 1d). Since the cleavage of DNA-PKcs resulted in decrease of the catalytic activity in vitro (see below), this may reflect the fact that a small proportion of DNA-PK in apoptotic Jurkat cells is susceptible to digestion with apopain.

To determine whether DNA-PKcs is one of the death substrates during Fas-mediated apoptosis, we directly tested whether DNA-PKcs is sensitive to digestion with apopain. As the DNA-PK holoenzyme consists of DNA-PKcs, Ku protein and double-stranded DNA, the DNA-PK preparation purified from Raji cells was incubated in the presence of calf thymus DNA with different amounts of recombinant apopain. Silver staining of the digests showed 230- (p230) and 160-kDa polypeptides (p160) derived from p460 in a dose-dependent manner (Fig. 2a). In the absence of DNA, a similar result was obtained (data not shown). In this preparation of DNA-PK containing approximately equimolar DNA-PKcs (p460) and Ku protein (p70/p80), p80 and p70 were hardly detectable during the silver staining probably because of the great difference in their molecular masses. To confirm that apopain itself cleaves p460, recombinant apopain was preincubated with a potent peptide aldehyde inhibitor, Ac-DEVD-CHO. Under these conditions, cleavage of p460 was completely inhibited (Fig. 2b), indicating the direct involvement of apopain in the digestion of p460 in vitro.

We next investigated the relationship between the cleavage of DNA-PKcs with apopain and DNA-PK activity. As indicated in Fig. 2b, DNA-PK activity was decreased in proportion to the digestion of p460, and the reduction in activity was restored by preincubation with Ac-DEVD-CHO. Since the DNA-PK activity is largely dependent on the Ku DNA-binding component that recruits DNA-PKcs to double-stranded DNA, it is important to establish whether DNA-PKcs, Ku protein or both are responsible for the decrease in DNA-PK activity on incubation with apopain. Immunostaining with anti-DNA-PKcs and anti-Ku antibodies after incubation of DNA-PK with recombinant apopain revealed that DNA-PKcs (p460) was cleaved into p230 and p160 (Fig. 3a), and Ku (p70/p80) was not significantly affected (Fig. 3b). These observations revealed that only DNA-PKcs is sensitive to digestion with apopain in DNA-PK holoenzyme composed of DNA-PKcs, a regulatory component Ku protein (p70/p80) and a cofactor double-stranded DNA.

4. Discussion

DNA-PK activity was substantially decreased with disappearance of DNA-PKcs after incubation of DNA-PK holoenzyme with a thiol proteinase apopain (Fig. 2b). In contrast, we observed only a slight decrease in activity of DNA-PK in crude extracts from apoptotic Jurkat cells (Fig. 1d). This is likely to imply that a small proportion of DNA-PKcs was cleaved in apoptotic Jurkat cells (see Fig. 1a). Human cells and tissues contain a large number of DNA-PKcs as in the case of Ku protein abundant in higher organisms, so that DNA-PK activity in human cells has been reported to be approximately two-orders higher than that in rodent cells exhibiting a quite low activity of DNA-PK (see [33]). A small proportion of p460 may be functional in Jurkat cells derived from human T lymphocytes, which is presumably susceptible to digestion with apopain under apoptosis.

Two polypeptides with molecular masses of approx. 230 kDa (p230) and 160 kDa (p160) derived from DNA-PKcs (p460) were detected by silver staining. In apoptotic Jurkat cells, however, p160 alone was observed and p230 was hardly

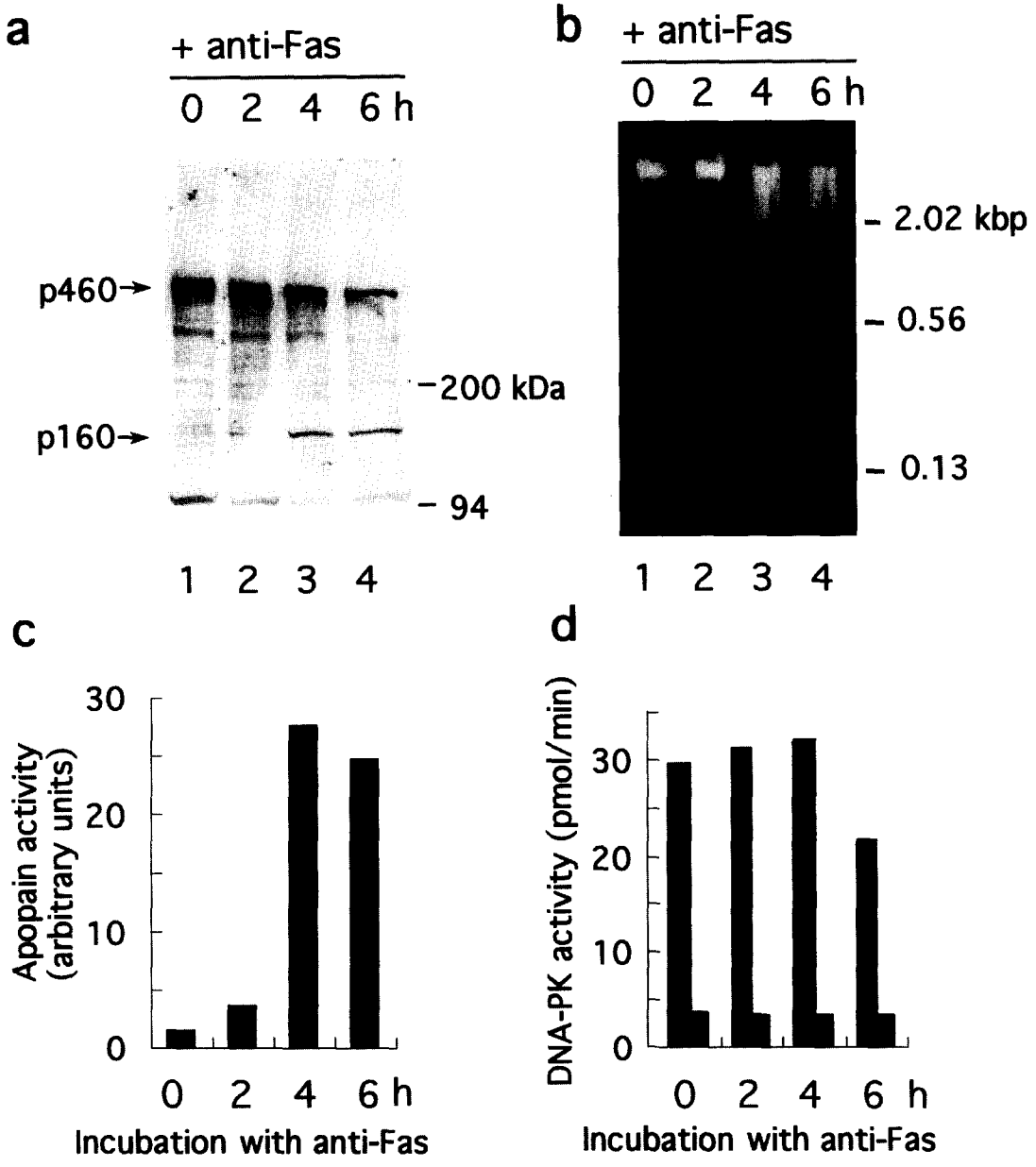

Fig. 1. Cleavage of DNA-PKcs by treatment of Jurkat cells with anti-Fas antibody. Jurkat cells were treated with anti-Fas antibody (100 ng/ml) for 0 (lane 1), 2 (lane 2), 4 (lane 3) and 6 h (lane 4). (a) The whole cell extracts from 4×10^5 cells were subjected to electrophoresis on 0.1% SDS/4–20% polyacrylamide gradient gel, followed by immunostaining with anti-DNA-PKcs antibody. Myosin (200 kDa) and phosphorylase *b* (94 kDa) are indicated as marker polypeptides. DNA-PKcs (p460) and the cleaved polypeptide (p160) are indicated by arrows. (b) DNA from the anti-Fas treated Jurkat cells was subjected to electrophoresis on 2% agarose gel/0.5×TBE/1 μg ml^{-1} ethidium bromide. λDNA digested with *Hin*dIII was used as a marker. (c) The activity of apopain in 2.5- and 5-μl aliquots of the whole cell extracts was determined with Ac-DEVD-AMC as a substrate. The released AMC was fluorometrically detected. (d) DNA-PK activity in a 3 μl portion of the whole cell extracts was determined with a substrate peptide 15 (black bars) and mutant peptide 15 (shaded bars).

detectable in immunoblot analysis of the whole cell extracts (Fig. 1a). This does not seem to be incompatible because p230 was less immunoreactive with the rabbit anti-DNA-PKcs antibody than p160 (see Fig. 3a). DNA-PKcs seems to be a death substrate susceptible to proteolytic cleavage by apopain during the Fas-mediated apoptotic process. A DNA-binding component Ku protein composed of 70- and 80-kDa polypeptides was not significantly susceptible to the digestion with apopain. No cleavage of Ku protein was observed in anti-Fas-treated Jurkat cells (data not shown). This is consistent with the observation with UV-irradiated HeLa cells [23], but seems to stand in contrast to the result demonstrating decreased levels of Ku protein during apoptosis in HL-60 and peripheral blood lymphocytes [34].

Apopain specifically induces cleavage of poly(ADP-ribose) polymerase, which occurs at the onset of apoptosis, and a potent peptide aldehyde inhibitor of apopain prevents apoptosis in vitro [24–26]. The cleavage site in poly(ADP-ribose) polymerase is DEVD216-G217, and there are six D-G sites within p460 [1]: PCLD797-G798, GDSD2020-G2021, QTAD2650-G2651, DWVD2982-G2983, VDQD3210-G3211 and GQYD3705-G3706. In order to determine the cleavage

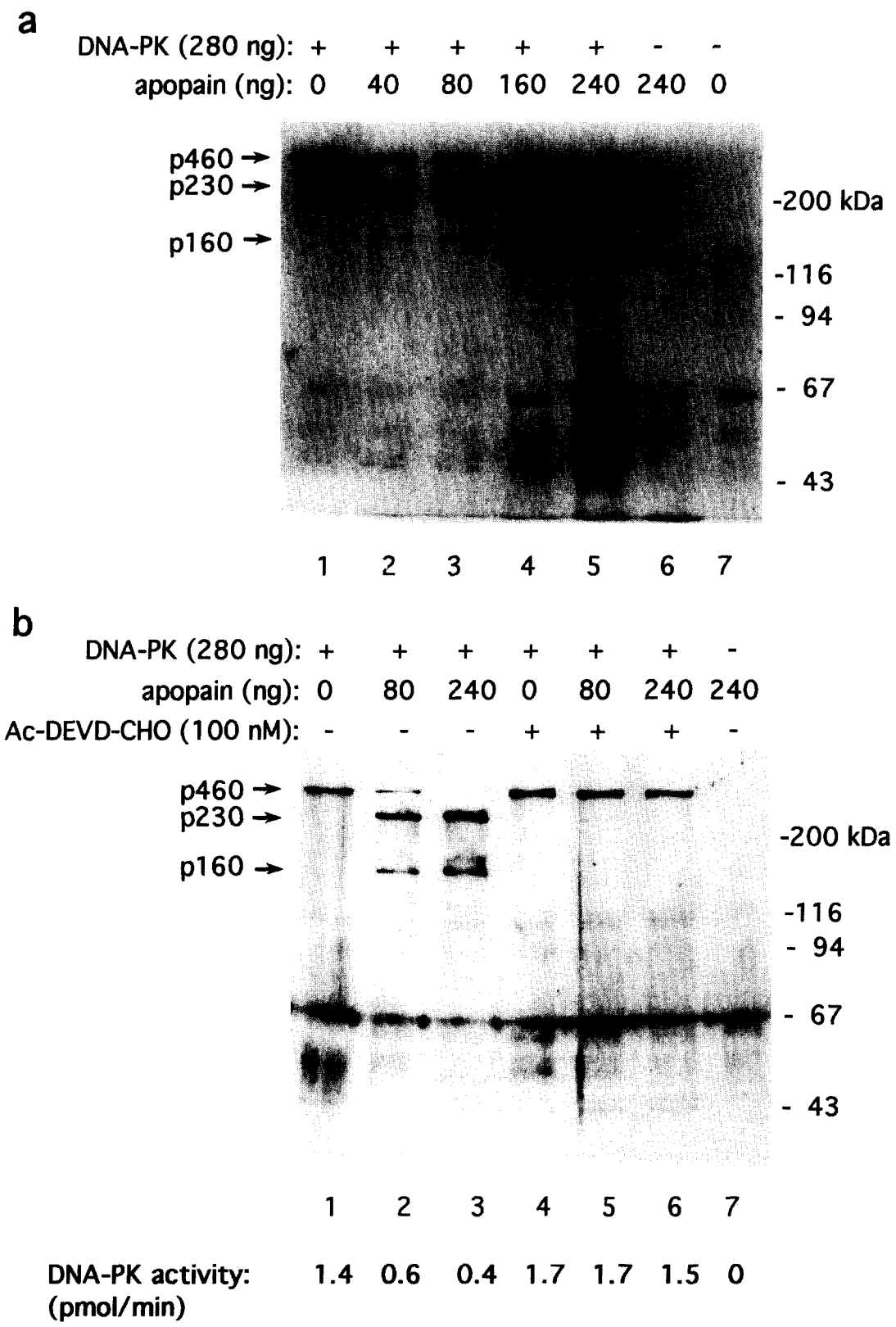

Fig. 2. Treatment of DNA-PK with recombinant apopain. After incubation of DNA-PK with apopain in a 20 μl reaction mixture containing 0.4 μg calf thymus DNA, 5-μl portions of the reaction mixture were used for determination of DNA-PK activity, and the residues were subjected to electrophoresis on 0.1% SDS/7.5% polyacrylamide gel with subsequent sliver staining. DNA-PKcs (p460) and its limited-degradation products (p230 and p160) are indicated by arrows. Myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) were used as marker polypeptides. (a) The purified preparation of DNA-PK (280 ng) was incubated with the indicated amount of recombinant apopain (lanes 1–5) at 30°C for 30 min. Lane 6, without DNA-PK; lane 7, buffer alone. (b) Apopain was preincubated with Ac-DEVD-CHO (100 μM) at 30°C for 10 min (lanes 4–6), followed by addition of DNA-PK. After incubation of DNA-PK in a total volume of 20 μl, 0.2 μl of 10 mM Ac-DEVD-CHO was added to the reaction mixture to inactivate apopain (lanes 1–3). Lane 7, apopain alone. DNA-PK activity expressed as pmol P_i incorporated into peptide 15 per min at 30°C is indicated below the lanes.

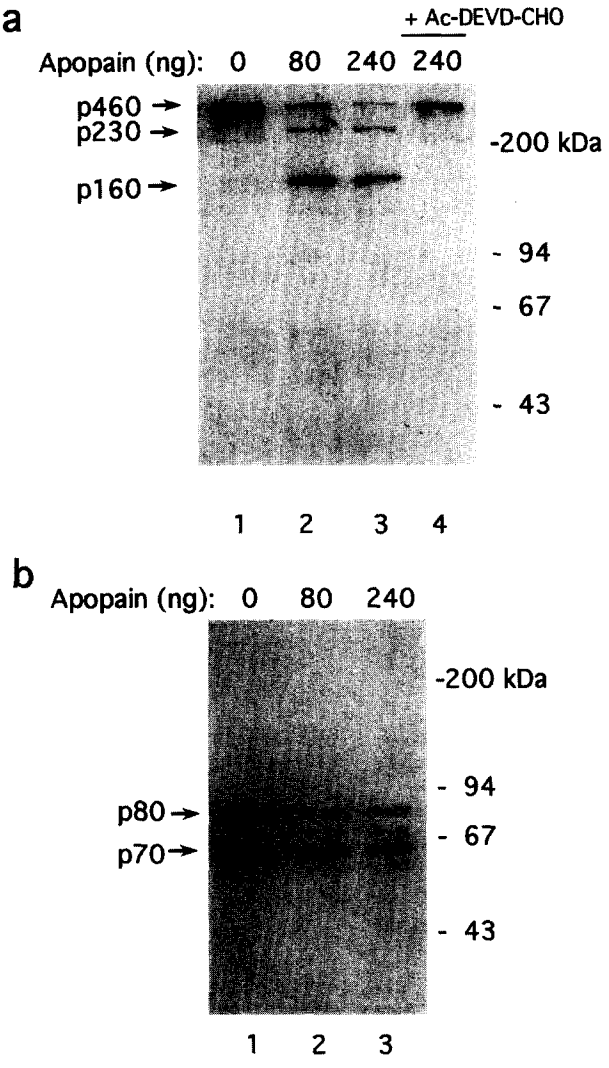

Fig. 3. Western blot/enzyme immunoassay of DNA-PK after treatment with apopain. (a) The purified DNA-PK (280 ng) was incubated with the indicated amount of apopain at 30°C for 30 min in a 20 μl reaction mixture containing 0.4 μg calf thymus DNA (lanes 1–4). Apopain was preincubated with Ac-DEVD-CHO (100 μM) at 30°C for 10 min (lane 4). The reaction products were subjected to electrophoresis on 0.1% SDS/7.5% polyacrylamide gel. Immunoblot analysis was performed with rabbit anti-DNA-PKcs antibody. (b) The purified DNA-PK (280 ng) was incubated with the indicated amount of apopain (lanes 1–3) and the reaction products were analyzed as in (a) with human anti-Ku serum (OM) instead of anti-DNA-PKcs antibody.

sites within DNA-PKcs, it may be necessary to perform micro-sequencing of p230 and p160. Considering the molecular mass of p230, we now estimate GDSD2020-G2021 as one of the cleavage sites in p460. Enari et al. [30] recently reported sequential activation of ICE-like proteinase and CPP32-like proteinase (=apopain) during Fas-mediated apoptosis in mouse transformant T cells expressing exogenous Fas, and induction of apoptosis in intact nuclei from mouse liver by addition of apopain together with the cytoplasmic component(s). Accumulating lines of evidence including this report to date demonstrate that apopain is responsible for the specific cleavage of poly(ADP-ribose) polymerase and DNA-PKcs early during the apoptotic process. It is especially interesting that poly(ADP-ribose) polymerase and DNA-PK have common characteristics including the requirement of DNA for enzymatic activity, involvement in the repair process, early cleavage during the apoptotic process and a target for autoantibody in autoimmune disease [23,31].

Since DNA-PK appears to be involved at least in double-strand break repair as well as V(D)J recombination [14–20], a functional significance in apoptotic degradation of DNA-PKcs would be to prevent rejoining fragmented DNA during the apoptotic process. Specific inhibitors of DNA-PK, such as OK-1035 [21,22], or mutant cells devoid of DNA-PK activity, such as mouse *scid* cells, may be useful for confirming the significance of DNA-PK in the apoptotic process.

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