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Degradation of glyceraldehyde-3-phosphate dehydrogenase induced by acetylleucine chloromethyl ketone in U937 cells

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Abstract

We examined whether any changes were induced in cellular proteins by an inhibitor of acylpeptide hydrolase (ACPH) (EC 3.4.19.1), acetylleucine chloromethyl ketone (ALCK), which was shown in our previous report to induce apoptosis of human U937 cells. Extract prepared from U937 cells in 0.05% Triton X-100–PBS was incubated with ALCK at 37°, and then analyzed using SDS–PAGE. A 36 kDa protein in the cell extract was decreased markedly during the incubation period. This protein was purified and identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) by its specific enzyme activity, N-terminal amino acid sequence, and Western blotting. Incubation of purified GAPDH with ALCK resulted in a decrease of GAPDH activity, but not in a decrease in the amount of GAPDH. The ALCK-induced GAPDH decrease in the cell extract was abrogated by co-incubation with a serine protease inhibitor, diisopropyl fluorophosphate, suggesting that GAPDH was first inactivated by ALCK, and subsequently degraded by a serine protease(s). GAPDH degradation was also observed in U937 cell cultures in the presence of ALCK. The significance of GAPDH inhibition in the apoptotic process is discussed.

Keywords: Acetylleucine chloromethyl ketone; U937 cells; Glyceraldehyde-3-phosphate dehydrogenase; Serine protease

1. Introduction

In our previous work, ALCK, an inhibitor of ACPH (EC 3.4.19.1), was shown to induce apoptosis in monoblastic U937 cells [1]. The mechanism by which ALCK triggers apoptosis has not been elucidated. ACPH, which catalyzes the hydrolysis of an *N*-acylated peptide to an acylamino acid and a residual peptide, is widely distributed in various kinds of cells, although there are differences depending on the species and tissue examined [2–5]. Recently, ACPH was shown to have a novel function, that of hydrolyzing oxidized proteins [6]. *N*-Acetylation is a ubiquitous structural modification of eukaryotic proteins, which has been suggested to regulate the metabolism of cellular proteins [7,8]. Therefore, we expected that protein profiles would be changed in U937 cells by ALCK exposure.

Using SDS–PAGE, we compared the protein bands of a U937 cell extract after incubation with or without ALCK, and found that a 36 kDa protein identified as GAPDH (EC 1.2.1.12) was decreased. Since the activity of purified GAPDH was inhibited directly by ALCK, modification of GAPDH by ALCK might target it for digestion by a serine protease(s).

2. Materials and methods

2.1. Chemicals

ALCK was synthesized from L-leucine chloromethyl ketone HCl (LCK, Sigma), as described previously [1]. Etoposide, human erythrocyte GAPDH, and glyceraldehyde-3-phosphate (GAP) were obtained from Sigma. β-NAD was purchased from the Oriental Yeast Co.

2.2. Culture of U937 cells and preparation of extracts

A human leukemia cell line, U937, was a gift from the Cell Resource Center for Biomedical Research, Institute of

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Abbreviations: ACPH, acylpeptide hydrolase; ALCK, acetylleucine chloromethyl ketone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAP, glyceraldehyde-3-phosphate; LCK, L-leucine chloromethyl ketone HCl; DFP, diisopropyl fluorophosphate.

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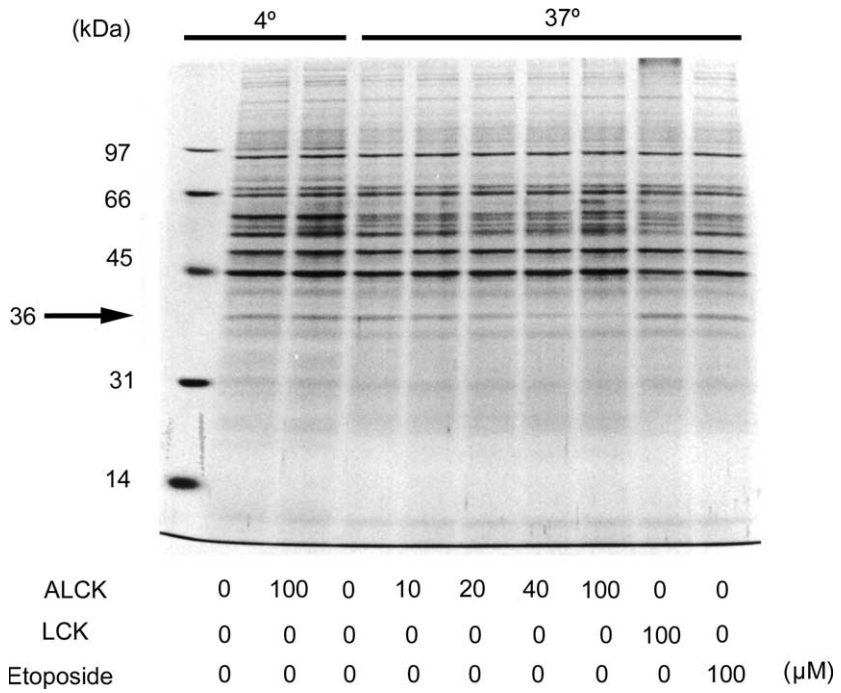

Fig. 1. Effect of ALCK on the protein profile of U937 cell extracts. U937 cell extracts were incubated with either ALCK, LCK, or etoposide at 37 or 4° for 3 hr. Triton X-100 soluble cellular proteins were separated on 10% SDS–PAGE and visualized by silver staining. Similar results were obtained in multiple separate experiments.

Development, Aging and Cancer, Tohoku University. U937 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine at 37° in a 95% air and 5% CO_2 atmosphere. U937 cells were seeded at a concentration of 1×10^6/mL and cultured for 3 days. To prepare the cell extracts, 5×10^6 U937 cells were collected, washed twice in 0.5 mL of ice-cold calcium- and magnesium-free PBS, and treated at 37° for 1 min in PBS containing 0.05% (v/v) Triton X-100. The cell lysates were centrifuged at 10,000 *g* for 10 min at 4°, and the resultant supernatants were used for analysis. Protein concentration was determined by the method of Bradford [9], using BSA as a reference standard.

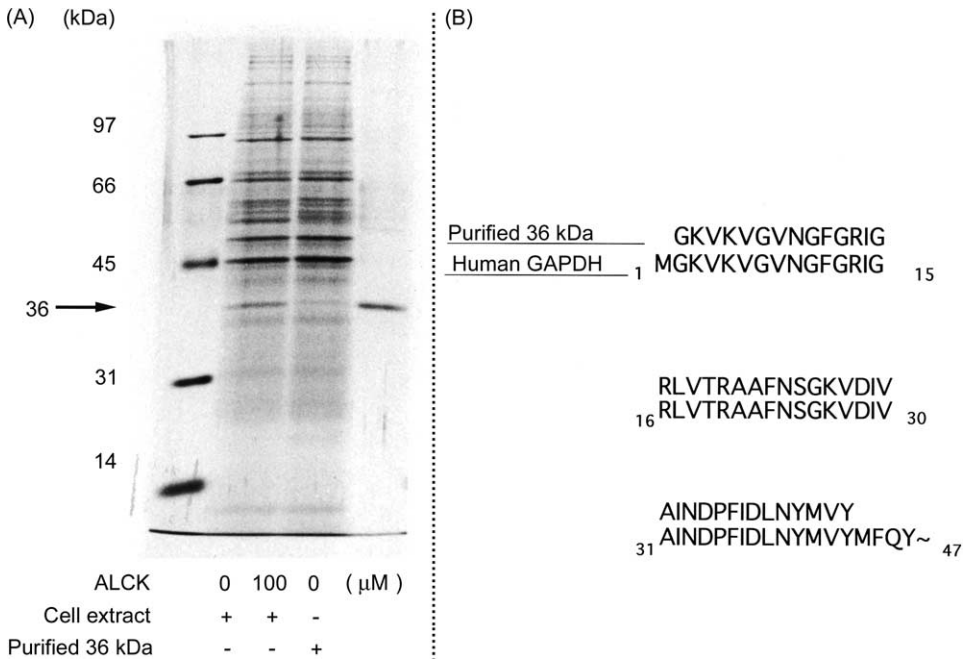

Fig. 2. Analysis by SDS–PAGE of purified 36 kDa protein and its N-terminal amino acid sequence. The 36 kDa protein was purified from a U937 cell extract as described in Section 2 and applied to 10% (v/v) SDS–PAGE. (A) Protein bands visualized by silver staining. (B) Analysis of the N-terminal amino acid sequence of the 36 kDa protein and alignment with that of human GAPDH.

2.3. Electrophoresis and Western blotting of U937 cell extracts treated with ALCK, LCK, or etoposide

U937 cell extracts (0.2 mg/mL) were incubated with ALCK, LCK, or etoposide at 4 or 37° for 3 hr, and aliquots of samples (0.75–1.00 μg protein) were subjected to analysis by SDS–PAGE on duplicate 10% (w/v) gels. After electrophoresis, one gel was stained with silver, while the counterpart gel was blotted on a polyvinylidene difluoride membrane at 80 mA for 1 hr. The blotted membrane was blocked for 30 min in PBS containing 0.5% (w/v) non-fat dry milk, and then probed for 1.5 hr with 1.0 μg/mL of a monoclonal antibody against GAPDH (6G5, Biogenesis). The bound antibody was visualized with an Immunoblotting ABC-POD Kit (Wako Pure Chemical) and diaminobenzidine.

2.4. Purification of a 36 kDa protein and sequencing of its amino acids

A U937 cell extract prepared from 10^8 U937 cultured cells was diluted in 100 mL of 20 mM phosphate buffer (pH 7.4) and passed through a DEAE-5PW column (21.5 mm × 150 mm, Tosoh) equilibrated with 20 mM phosphate buffer (pH 7.4). The unbound fractions were collected and concentrated in an Amicon microconcentrator with a 10 kDa cut-off. The concentrate was applied to a CM-5PW (7.5 mm × 75 mm, Tosoh) column equilibrated with 20 mM phosphate buffer (pH 7.4), and eluted with a NaCl (0–0.5 M) linear gradient. The fraction containing the 36 kDa protein was eluted through Protein-Pak 300 (7.8 mm × 300 mm, Nihon Waters). The 36 kDa

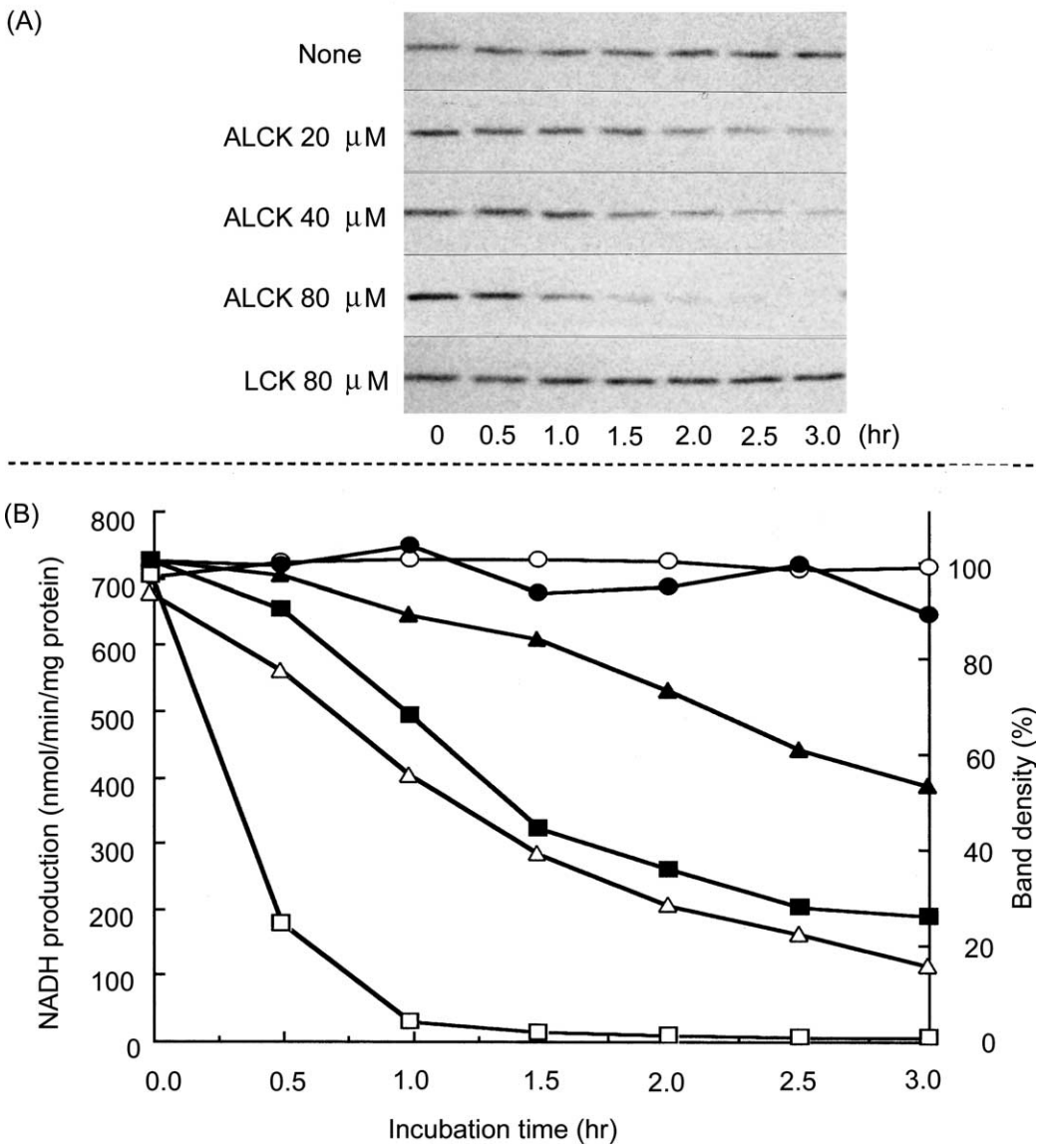

Fig. 3. Concentration- and time-dependent decrease in GAPDH activity and GAPDH protein in a U937 cell extract following incubation with ALCK. A U937 cell extract was incubated with ALCK (20, 40, and 80 μM) and LCK (80 μM) at 37°. (A) Analysis by Western blotting of the amount of GAPDH, using an anti-human GAPDH monoclonal antibody. (B) Analysis of immunoblots using NIH image software (●, control; ▲, 20 μM ALCK; ■, 80 μM ALCK). In this same panel, GAPDH activity is expressed as NADH production (○, control; △, 20 μM ALCK; □, 80 μM ALCK). Values of NADH production are means for three separate experiments performed in quadruplicate. Values of band density are means for, and immunoblots are representative of, these three experiments.

protein-enriched fraction was analyzed by an N-terminal amino acid sequencer, Procise491 (PE Biosystems).

2.5. Assay for GAPDH activity in U937 cell extracts treated with ALCK

GAPDH activity was determined according to the procedure of Dimmeler *et al*. [10] with minor modifications. The U937 cell extract (30 μg protein/assay) was treated with 20 μM ALCK in 20 mM phosphate buffer (pH 7.4) in a total volume of 100 μL at 37° for 3 hr. Then it was added to 400 μL of pre-warmed 50 mM triethanolamine buffer (pH 7.5) containing 50 μM arsenate, 2.4 mM glutathione, 0.4 mM NAD, and 150 μg/mL of GAP. GAPDH activity was determined by NADH production measured at 340 nm for 3 min at 37°.

3. Results

3.1. Decrease of the 36 kDa protein in U937 cell extracts by incubation with ALCK

Freshly cultured U937 cells were extracted with PBS containing 0.05% (v/v) Triton X-100 and centrifuged. The supernatants were incubated with 100 μM ALCK at 37 or 4° for 3 hr, and proteins were separated by SDS–PAGE and visualized by silver staining. The 36 kDa protein band was shown to be selectively decreased by ALCK in a concentration-dependent manner, at 37° but not at 4° (Fig. 1). The reduction of the 36 kDa protein band was not caused by LCK, a precursor in the synthesis of ALCK, which neither inhibits ACPH activity nor induces apoptosis of U937 cells [1], or by etoposide, which induces apoptosis of U937 cells without inhibiting ACPH activity [1].

3.2. Sequence of the 36 kDa protein decreased by incubation with ALCK

The 36 kDa protein was purified from a U937 cell extract prepared from 10^8 cells, using conventional chromatographic techniques as described in Section 2, to one band on SDS–PAGE (Fig. 2A). The N-terminal amino acid sequence of the purified 36 kDa protein was analyzed using BLAST (Protein Database Search Programs). The N-terminal amino acids (1–44) were shown to be identical to those deduced from human liver GAPDH cDNA [11] (Fig. 2B).

3.3. Effect of ALCK on GAPDH activity and GAPDH amount in a U937 cell extract and a purified GAPDH preparation

The cell extract or a purified GAPDH preparation (seen as one band on SDS–PAGE) was tested for GAPDH activity and GAPDH amount after incubation with and without ALCK. Both the activity and the amount of GAPDH in the U937 cell extract were decreased by ALCK treatment in a concentration- and time-dependent manner (Fig. 3). The inhibition of GAPDH appeared to precede the decrease of GAPDH protein. In contrast, in the case of the purified GAPDH sample, GAPDH activity was inhibited (Fig. 4A), but the amount of GAPDH was not decreased by ALCK treatment (Fig. 4B). Neither GAPDH activity nor GAPDH amount in U937 cell extracts was affected by LCK treatment (Fig. 4A and B). The data suggest that GAPDH might be modified directly by ALCK to lose its activity. As a result, GAPDH may be digested by a proteolytic enzyme(s) contained in the U937 cell extract.

3.4. Effects of various protease inhibitors on the decrease of GAPDH in U937 cell extracts treated with ALCK

U937 cell extracts were incubated with ALCK in combination with various protease inhibitors. Among the protease inhibitors used, diisopropyl fluorophosphate (DFP), a serine protease inhibitor, completely prevented the GAPDH decrease in the presence of ALCK, while chymostatin only partially abrogated it. However, the other types of serine protease inhibitors (leupeptin, antipain, and elastatinal), metalloproteinase inhibitors (EDTA, EGTA, and phosphoramidon), and an aspartic protease inhibitor

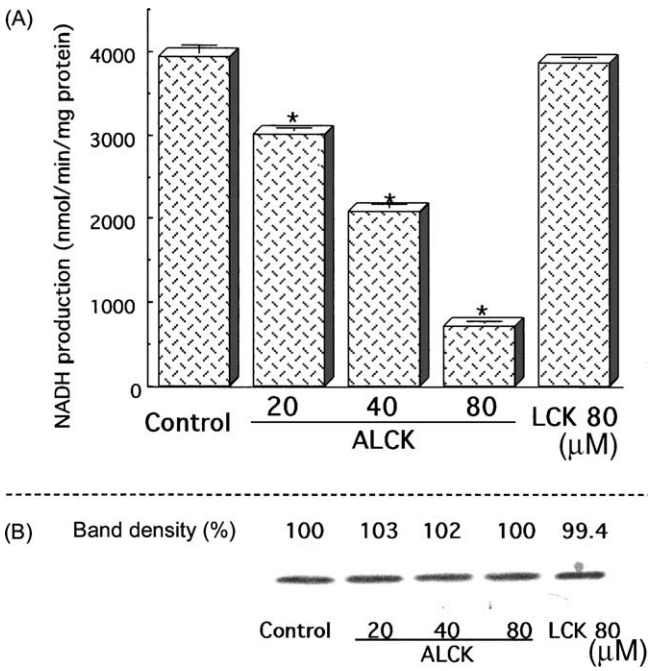

Fig. 4. Change in the activity and amount of the purified GAPDH sample by incubation with ALCK. GAPDH purified from U937 cells was incubated with ALCK (20, 40, and 80 μM) and LCK (80 μM) at 37° for 3 hr. (A) GAPDH activity was expressed as NADH production. (B) The amount of GAPDH was analyzed by Western blotting, using an anti-human GAPDH monoclonal antibody, and immunoblots were analyzed using NIH image software. NADH production is expressed as mean ± SEM for three separate experiments performed in quadruplicate. Band density values are means for, and immunoblots are representative of, these three experiments. Key: (*) significantly different from control, $P < 0.001$.

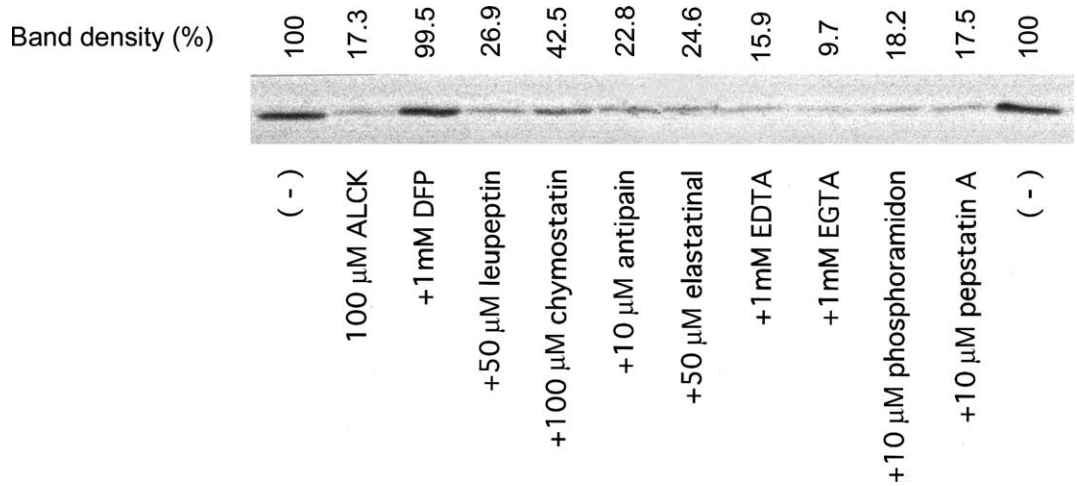

Fig. 5. Effects of various protease inhibitors on the reduction of GAPDH protein in U937 cell extracts in the presence of ALCK. U937 cell extracts were incubated with 100 μM ALCK in combination with various protease inhibitors at 37° for 3 hr. Each cell extract was analyzed by Western blotting using anti-human GAPDH monoclonal antibody, and immunoblots were analyzed using NIH image software. Band density values are means for, and immunoblots are representative of, three separate experiments.

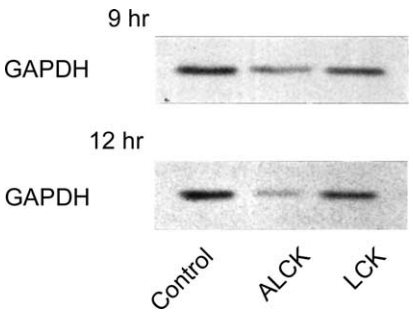

Fig. 6. Effect of ALCK on GAPDH in U937 cell culture. U937 cells were cultured with ALCK or LCK (80 μM) at 37° for 9 and 12 hr in a humidified 5% CO_2 incubator. Extracts prepared from the cells were analyzed by Western blotting, using an anti-human GAPDH monoclonal antibody. Similar results were obtained in two separate experiments.

(pepstatin A) did not prevent the degradation of GAPDH (Fig. 5). These data suggest that a serine protease(s) is responsible for the digestion of ALCK-modified GAPDH in the U937 cell extract.

3.5. Loss of GAPDH protein in U937 cells treated with ALCK in culture

We tested whether ALCK induced a reduction of GAPDH in the U937 cell culture system. U937 cells were cultured in the presence of ALCK or LCK, and then were extracted with 0.05% (v/v) Triton X-100–PBS and analyzed by Western blotting. The amount of GAPDH was decreased by culturing U937 cells with ALCK for 9 and 12 hr (Fig. 6).

4. Discussion

Incubation of U937 cell extracts in the presence of an ACPH inhibitor, ALCK, resulted in a decrease of GAPDH activity and GAPDH protein. The inhibition of GAPDH activity occurred prior to the decrease of GAPDH protein during the incubation period. When purified GAPDH was incubated with ALCK, GAPDH activity was decreased, but GAPDH protein was not. Reduction of GAPDH by ALCK was also induced in cultured U937 cells. Therefore, it is possible that ALCK directly modifies the GAPDH molecule causing it to lose its activity and, simultaneously targets it for subsequent degradation by a protease(s) by altering its conformation. ALCK is known as a very effective irreversible inhibitor of ACPH [12]. An active site residue attacked by ALCK was identified as histidine-707, and, furthermore, the modification of a cysteine residue by ALCK was also suggested to be responsible for the inhibition of ACPH [12]. Human GAPDH is known to have these histidine and cysteine residues [13]. The residues in GAPDH modified by ALCK should be analyzed in a subsequent study. It is unlikely that the GAPDH degradation by ALCK occurs due to inhibition of ACPH, because an inhibitor of ACPH, ebelactone A [12], did not induce GAPDH degradation in the U937 cell extract (data not shown). The proteolytic enzyme, which participates in the degradation of GAPDH in the cell extract, may be a member of the serine protease family, because the decrease of GAPDH was abrogated by co-incubation with DFP, but not with other specific serine protease inhibitors such as leupeptin, antipain, or elastatinal.

Intracellular proteins in mammalian cells are degraded through different pathways. The *in vitro* inactivation mechanisms of GAPDH, an integral enzyme in the glycolytic pathway, have been studied [14–16]. GAPDH is degraded, at least in part, by a selective lysosomal pathway [15,16], different from the classical non-specific macroautophagy. Chymostatin, which was reported to effectively inhibit the intralysosomal proteolysis of GAPDH [16], could partially prevent ALCK-induced GAPDH degradation (Fig. 5). It remains unclear what kinds of lysosomal or

non-lysosomal serine proteases are involved in ALCK-induced GAPDH degradation.

Recent evidence demonstrates that GAPDH has a number of diverse roles such as in membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication, and DNA repair [17], in addition to the classical role in glycolysis for energy production. With regard to apoptosis, there is contradictory evidence. In normal cerebellar granule cells, GAPDH is increased markedly during age-induced apoptosis, and the antisense DNA for GAPDH suppressed age-induced neuronal apoptosis, suggesting that overexpression of GAPDH promotes programmed cell death [18,19]. In contrast, a strong GAPDH inhibitor, koningic acid [20], and antisense DNA for GAPDH [21] induce apoptosis in neuroblastoma × glioma hybrid cells and cervical carcinoma cell lines, respectively, suggesting that inhibition of GAPDH activity induces apoptosis, at least in part. Nonetheless, these data were interpreted to mean that overexpression of, or deficiency in, GAPDH may lead to apoptosis, depending on the cell type.

In our previous study [1], we reported that ALCK causes apoptosis in U937 cells, suggesting that inhibition of ACPH activity led to apoptosis. In the present study, it was found that ALCK inhibits GAPDH activity and, as a result, GAPDH is subject to being digested by intracellular serine protease(s). Apoptosis may be triggered by loss of GAPDH activity as well as loss of ACPH activity. It remains unclear whether inhibition of both ACPH and GAPDH, or of either one, is a prerequisite for the triggering of apoptosis of U937 cells by ALCK.

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References

[1] Yamaguchi M, Kambayashi D, Toda J, Sano T, Toyoshima S, Hojo H. Acetylleucine chloromethyl ketone, an inhibitor of acylpeptide hydrolase, induces apoptosis of U937 cells. Biochem Biophys Res Commun 1999;263:139–42.

[2] Marks N, Lo E-S, Stern F, Danho W. Observations on *N*-deacylation of model amino acids and peptides: distribution and purification of a specific *N*-acyl amino acid releasing enzyme. J Neurochem 1983;41: 201–8.

[3] Jones WM, Mannering JM. Acylpeptide hydrolase activity from erythrocytes. Biochem Biophys Res Commun 1985;126:933–40.

[4] Kobayashi K, Smith JA. Acylpeptide hydrolase from rat liver. Characterization of enzyme reaction. J Biol Chem 1987;262:11435–45.

[5] Scaloni A, Barra D, Jones WM, Manning JM. Human acylpeptide hydrolase. Studies on its thiol groups and mechanisms of action. J Biol Chem 1994;269:15076–84.

[6] Fujino T, Watanabe K, Beppu M, Kikugawa K, Yasuda Y. Identification of oxidized protein hydrolase of human erythrocytes as acylpeptidase. Biochim Biophys Acta 2000;1478:102–12.

[7] Hershko A, Heller H, Eytan E, Kaklij G, Rose IA. Role of the α-amino group of protein in ubiquitin-mediated protein breakdown. Proc Natl Acad Sci USA 1984;81:7021–5.

[8] Tsunasawa S, Sakiyama F. Amino-terminal acetylation of proteins: an overview. Meth Enzymol 1984;106:165–70.

[9] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[10] Dimmeler S, Lottspeich F, Brune B. Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. J Biol Chem 1992;267:16771–4.

[11] Tso JY, Sun X-H, Kao T, Reece KS, Wu R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucl Acids Res 1985;13:2485–502.

[12] Scaloni A, Jones WM, Barra D, Pospischil S, Sassa S, Popowics A, Manning LR, Schneewind O, Manning JM. Acylpeptide hydrolase: inhibitors and some active site residues of the human enzyme. J Biol Chem 1992;267:3811–8.

[13] Ercolani L, Florence B, Denaro M, Alexander M. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. J Biol Chem 1988;263:15335–41.

[14] Roche E, Knecht E, Grisolia S. 2,3-Bisphosphoglycerate protects mitochondrial and cytosolic proteins from proteolytic inactivation. Biochem Biophys Res Commun 1987;142:680–7.

[15] Aniento F, Roche E, Cuervo AM, Knecht E. Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes. J Biol Chem 1993;268:10463–70.

[16] Cuervo AM, Dice JF, Knecht E. A population of rat liver lysosomes responsible for the selective uptake and degradation of cytosolic proteins. J Biol Chem 1997;272:5606–15.

[17] Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1999;1432:159–84.

[18] Ishitani R, Sunaga K, Hirano A, Saunders P, Katsube N, Chuang D-M. Evidence that glyceraldehyde-3-phosphate dehydrogenase is involved in age-induced apoptosis in mature cerebeller neurons in culture. J Neurochem 1996;66:928–35.

[19] Ishitani R, Kimura M, Sunaga K, Katsube N, Tanaka M, Chuang D-M. An antisense oligodeoxynucleotide to glyceraldehyde-3-phosphate dehydrogenase blocks age-induced apoptosis of mature cerebrocortical neurons in culture. J Pharmacol Exp Ther 1996;278:447–54.

[20] Nakazawa M, Uehara T, Nomura Y. Koningic acid (a potent glyceraldehyde-3-phosphate dehydrogenase inhibitor)-induced fragmentation and condensation of DNA in NG108-15 cells. J Neurochem 1997;68:2493–9.

[21] Kim JW, Kim TE, Kim YK, Kim YW, Kim SJ, Lee JM, Kim I-K, Namkoong SE. Antisense oligonucleotide of glyceraldehyde-3-phosphate dehydrogenase gene inhibits cell proliferation and induces apoptosis in human cervical carcinoma cell lines. Antisense Nucl Acid Drug Dev 1999;9:507–13.