Regulation of Cidea protein stability by the ubiquitin-mediated proteasomal degradation pathway

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Cidea, one of three members of the CIDE (cell-death-inducing DNA-fragmentation-factor-45-like effector) family of proteins, is highly enriched in brown adipose tissue, in which it plays a critical role in adaptive thermogenesis and fat accumulation. Cidea-null mice have increased energy expenditure with resistance to high-fat-diet-induced obesity and diabetes. However, little is known as to how the Cidea protein is regulated. In the present study we show that Cidea is a short-lived protein as measured by cycloheximide-based protein chase experiments in different cell lines or in differentiated brown adipocytes. Proteasome inhibitors specifically increased the stability of both transfected and endogenous Cidea protein. Furthermore, Cidea protein was found to be polyubiquitinated when overexpressed in different culture cells as well as in differentiated mature brown adipocytes. Extensive mutational analysis of individual lysine residues

revealed that ubiquitinated lysine residues are located in the N-terminal region of Cidea, as alteration of these lysine residues to alanine (N-5KA mutant) renders Cidea much more stable when compared with wild-type or C-terminal lysine-less mutant (C-5KA). Furthermore, K23 (Lys²³) within the N-terminus of the Cidea was identified as the major contributor to its polyubiquitination signal and the protein instability. Taken together, the results of our study demonstrated that the ubiquitin–proteasome system confers an important post-translational modification that controls the protein stability of Cidea.

Key words: brown adipose tissue, cell-death-inducing DNA-fragmentation-factor-45-like effector (CIDE), Cidea, proteasome, ubiquitin.

INTRODUCTION

Obesity represents an excess of total body fat and is the result of imbalance between energy intake and expenditure [1]. BAT (brown adipose tissue) plays a unique role in energy expenditure by uncoupling oxidative phosphorylation and dissipating energy as heat to maintain core body temperature in animals when exposed to cold [2]. The thermogenic activity of BAT is mediated by UCP1 (uncoupling protein 1), a mature brown-adipocyte-tissue-specific protein that is localized to the mitochondrial inner membrane and regulated by the sympathetic nervous system through β -adrenergic receptors [3]. CIDE (cell-death-inducing DNA-fragmentation-factor-45-like effector) proteins were originally identified by their sequence homology with the N-terminal region of DNA fragmentation, a protein complex crucial for chromosome condensation and DNA fragmentation during apoptotic induction [4]. The CIDE family consists of three members (Cidea, Cideb and Fsp27) in mice [4]. Their human counterparts have also been reported [5]. CIDE proteins contain two conserved regions: the N-terminal Cide-N domain and C-terminal Cide-C domain. Overexpression of Cidea and Cideb in several mammalian cell lines was able to induce caspase-independent cell death [4,6]. NMR structural analysis indicated that the Cide-N domain, which is conserved among all CIDE proteins and DNA fragmentation factor, comprises a novel bipolar structure with two oppositely charged regions on the surface that could serve as a potential protein–protein interaction interface [7]. The Cide-C domain was reported to be sufficient to induce cell

death when overexpressed in different cells and form homodimers or heterodimers among CIDE family members [4,6]. However, the functional role of the amino acid sequences at the extreme N-terminus and the linker region between the Cide-N and Cide-C domains within Cide family protein have not yet been defined.

Our previous study demonstrated that Cidea protein is highly enriched in BAT, but is not expressed in white adipose in mice [8]. Cidea deficiency results in increased whole-body metabolism, reduced levels of both plasma triacylglycerols and non-esterified fatty acids. Furthermore, we observed that the BAT of Cidea-null mice has increased lipolysis and less lipid accumulation during cold induction. Consequently, Cidea-deficient mice are resistant to high-fat-diet-induced obesity and diabetes [8]. A nucleotide polymorphism in the *Cidea* gene, resulting in the mutant V115F, was discovered to be associated with obesity in a Swedish population [9]. Another study on human white adipocytes suggests that Cidea could play an important role in monitoring lipolysis and other metabolic activities [10].

The ubiquitin-mediated proteasomal degradation system regulates the turnover of a wide range of proteins that control many cellular events, such as cell-cycle progression, transcriptional activity and metabolic regulation [11–13]. As fat accumulation in animals is dependent on environmental conditions such as temperature, hormone stimulation and feeding conditions, we hypothesized that Cidea proteins might be highly regulated at the post-translational level. In the present paper we report that Cidea is a short-lived protein when transiently expressed in cultured cells or endogenously expressed in mature brown adipocytes

Abbreviations used: ALLN, *N*-acetyl-L-leucyl-L-norleucinal; BAT, brown adipose tissue; CHO-K1, Chinese-hamster ovary-K1; CHX, cycloheximide; CIDE, cell-death-inducing DNA-fragmentation-factor-45-like effector; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal-bovine serum; GFP, green fluorescent protein; HA, haemagglutinin; hCidea, human Cidea; HEK-293T, human embryonic kidney-293T; IBMX, 3-isobutyl-1-methylxanthine; KO, knockout; K23 (etc.), Lys²³ (etc.); UCP1, uncoupling protein 1; WT, wild-type.

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after differentiation from preadipocytes. Degradation of Cidea is dependent on ubiquitination and proteasomal activity. Extensive mutational analysis identifies a critical lysine residue, K23 (Lys²³) within the N-terminal region of Cidea that confers a switch for Cidea protein stability.

EXPERIMENTAL

Reagents and antibodies

Anti-HA (anti-haemagglutinin; F-7), anti-Myc (9E10), anti-ubiquitin (SC-8017) and control rabbit IgG antibodies were purchased from Santa Cruz Biotechnology. Anti-Flag and anti- β -tubulin antibodies were obtained from Sigma. Anti-UCP1 antibody was from Calbiochem. Rabbit anti-Cidea antibody was raised against mouse Cidea-(1–172)-peptide in rabbits, and rabbit anti-Cidea serum was purified by affinity chromatography using glutathione transferase–Cidea-(1–123)-peptide conjugated to CNBr-activated Sepharose 4B beads (Amersham). M2 beads (covalently linked to anti-Flag) were from Sigma. Protein A/G Plus beads were from Santa Cruz Biotechnology. Protein A–horseradish peroxidase was from BD Biosciences. Liposomal Dosper transfection reagent was from Roche. Cycloheximide, pepstatin, ALLN (*N*-acetyl-L-leucyl-L-norleucinal), MG132 (benzyloxycarbonyl-Leu-Leu-CHO), chloroquine, NH₄Cl, IBMX (3-isobutyl-1-methylxanthine) and dexamethasone were purchased from Sigma. Leupeptin and aprotinin were purchased from Amersham. Pioglitazone (a thiazolidinedione) was obtained from Alexis.

Plasmids

Human HA and Flag-tagged Cidea constructs were obtained from expressed-sequence-tag clones and subcloned into CMV5 (cytomegalovirus 5) vectors carrying HA and Flag as described previously [8]. pXJ-40-HA-Ub, pXJ-40-Myc-Ub and pXJ-40-HA-Bax were obtained from Dr Victor Yu (Institute of Molecular and Cell Biology, Singapore). All lysine-to-alanine point mutations of human Cidea were generated using the PCR-based QuikChange[®] site-directed-mutagenesis method with Pfu polymerase (Stratagene). All constructs created were verified by DNA sequencing.

Cell culture and transfection

HEK-293T (human embryonic kidney-293T), CHO-K1 (Chinese-hamster ovary-K1) and H1299 lung-cancer cells were obtained from the A.T.C.C. and were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco) containing 10% (v/v) FBS (fetal-bovine serum), 100 i.u. of penicillin and 100 μ g/ml streptomycin. Transfections were performed in 60-mm-diameter dishes using either Dosper reagent (Roche) or the calcium phosphate method [14]. For CHX (cycloheximide) or other drug treatments, the culture media were replaced with fresh DMEM+10% FBS 1 h prior to stimulation.

Co-immunoprecipitation and Western blotting

Cells were transfected with various plasmids as indicated. At 36 h post-transfection, cells were harvested with a Lysis Buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin and 1 mM PMSF). Cell extracts were sonicated (12 times, 1 s burst each time), then centrifuged at 16 100 *g* for 30 min at 4°C. Supernatants were subjected to immunoprecipitation with the indicated antibodies and protein

A/G Plus–agarose beads (Santa Cruz Biotechnology) at 4°C for about 3 h. Then the beads were spun down at 800 *g* for 3 min and washed three times with 700 μ l of Lysis Buffer. The proteins were eluted with 2 $\times$ SDS sample buffer [20% (v/v) glycerol, 0.48% SDS, 10% (v/v) 2-mercaptoethanol and 0.1 M Tris, pH 6.8] and the immunoprecipitates and total cell lysates were analysed by Western blotting.

For Western blotting, the boiled protein samples were separated on SDS/8–14% (w/v) polyacrylamide gels and transferred on to a PVDF membrane (Roche Applied Science). After blocking with 5% (w/v) dried skimmed milk (or 4% BSA when necessary) in PBST (PBS with 0.1% Tween 20) for at least 1 h, the membranes were probed with antibodies as indicated. Bound antibodies were visualized using an enhanced-chemiluminescence kit (Pierce) using horseradish-peroxidase-conjugated antibodies.

CHX-based protein chase experiment

Approx. 70% confluent HEK-293T cells were co-transfected with plasmids (1 μ g of pCMV-HA-Cidea and 0.5 μ g of pEGFP-N1) by the calcium phosphate method. Dosper liposomal transfection was used for CHO-K1 and H1299 cells. At 24 h post-transfection, and 1 h prior to the addition of CHX, the medium was replaced with fresh DMEM plus 10% FBS. Cells were harvested at fixed time points (0, 30, 60 and 120 min), after addition of CHX to a final concentration of 100 μ g/ml to stop protein synthesis, and lysed in a 0.5 ml of Lysis Buffer. Immunoprecipitates or total cell lysates were analysed by Western blotting as described above.

In vivo ubiquitination assay

Briefly, and as described above, HEK-293T cells were transfected with 1 μ g of CMV-tagged hCidea (human Cidea) and 0.5 μ g of pEGFP-N1 with or without 0.5 μ g of pXJ40-HA-Ub or pXJ40-Myc-Ub using the calcium phosphate method. At 24 h post-transfection, cells were treated with 10 μ M MG132 for 2 h, harvested, sonicated in Lysis Buffer with 0.5% SDS and 5 mM dithiothreitol, then heated at 90°C for 5 min [15,16]. Heated lysates were then cooled, centrifuged at 16 100 *g* for 30 min, and 0.25 ml of the supernatant was diluted with Lysis Buffer until the concentration of SDS was 0.1% for immunoprecipitation by the corresponding Cidea-tagged antibodies. Immunoprecipitates or total cell lysates were analysed by Western blotting as described previously.

Differentiation of primary brown preadipocytes from BAT or the brown preadipocyte cell line HB2

Brown preadipocytes were isolated from fresh BAT of 4-week-old Balb/c mice, or HB2 cells were grown to confluency in 100-mm-diameter tissue-culture dishes maintained in DMEM plus 10% FBS [17,18]. Subsequently brown preadipocytes were differentiated into mature brown adipocytes in the presence 0.5 mM IBMX and 10 μ M dexamethasone at day 0; then, at day 2, the differentiation medium was replaced with DMEM plus 10% FBS, 1 μ g/ml insulin and 10 μ M pioglitazone in the differentiation medium. Then the differentiated medium was changed daily until day 8. Before treatment with drugs, the medium of differentiated mature brown adipocytes was replaced with fresh medium without the differentiation agents.

Apoptosis assay

CHO-K1 cells were plated in 35-mm-diameter culture dishes and transfected with 1.5 μ g of the expression plasmids and 0.5 μ g of pEGFP-N1 using Dosper reagent according to the manufacturer's instructions. At 24 h post-transfection, 1 μ g/ml Hoechst 33 342

Figure 1 Cidea is a short-lived protein and its degradation is proteasome-dependent

(A, B and C) A 1 μ g portion of CMV5-HA-hCidea was co-transfected with 0.5 μ g of pEGFP-N1 into HEK-293T, CHO-K1 and H1299 cells in 60-mm-diameter dishes using Dosper reagent. CHX-based protein chase experiments were performed as described below. At 24 h post-transfection, and 1 h prior to the addition of CHX, the medium was replaced with fresh medium, and then CHX was added to a final concentration of 100 μ g/ml. Cells were harvested in 0.5 ml of lysis buffer at different time points: 0, 30, 60 and 120 min. Total cell lysates were prepared and analysed by Western blotting (IB (immunoblotting)) using anti-HA and anti-GFP as primary antibodies. The amount of GFP transiently co-expressed by pEGFP-N1 present in total cell lysates was used for the normalization of transfection protein. (D) Graph showing band intensities corresponding to HA-Cidea and GFP scanned and quantified using ImageQuant-TL software (Amersham). Results are expressed as the percentage of the signal of HA-Cidea compared with that of GFP. The ratio was set to 100 % at zero time. The results shown are representative of three independent experiments. (E) HEK-293T cells in 60-mm-diameter dishes were co-transfected with 1 μ g of CMV-HA vector (lane 1) or 1 μ g of CMV5-HA-hCidea (lane 2–11) and 0.5 μ g of pEGFP-N1 using the calcium phosphate method. After 24 h, CHX was added to 100 μ g/ml (lanes 3–11) along with different reagents [DMSO, ethanol, pepstatin (0.5 μ g/ml), leupeptin (5 μ g/ml), aprotinin (2 μ g/ml), ALLN (50 μ M), MG132 (10 μ M), chloroquine (50 μ g/ml) and NH₄Cl (2.5 mM)] for 2 h. Cells were harvested in 0.5 ml of lysis buffer after the 2 h treatment. Total cell lysates were subjected to Western blotting and detected with anti-HA and anti-GFP antibodies.

was added directly into the 35-mm-diameter culture dish. Approx. 300 GFP (green fluorescent protein)-positive cells were counted for each transfection ($n=3$) from random fields under a fluorescence microscope. The percentage of apoptotic cells that were positive was calculated as the ratio of cells positive for both GFP fluorescence and chromatin condensation and cells positive for total GFP.

RESULTS

Cidea protein degradation is dependent on proteasomal activity

To determine whether proteolysis has a role in regulating the steady-state levels of the Cidea protein in different cells, Cidea tagged with HA at its N-terminus was co-transfected into HEK-293T, CHO-K1 and H1299 cells with pEGFP-N1 that served as an internal control for protein transfection efficiency. At 24 h post-transfection, cells were treated with 100 μ g/ml CHX to block protein synthesis. Cidea was found to be very unstable compared with the GFP control in all HEK-293T, CHO-K1 and H1299 cells (Figures 1A–1C). Its half-life in these different cells was determined from the protein degradation curves at the time when the percentage of protein remaining is 50 % of that at zero time of CHX treatment (Figure 1D). The half-life was very similar in each cell line, being shorter than 30 min. Similar results were obtained by immunoprecipitation and subsequent Western-blot analysis of HA-Cidea expressed in different cells (results not shown). These

data demonstrate that Cidea undergoes rapid proteolysis in the cell.

We then questioned whether Cidea is degraded by the ubiquitin–proteasome pathway. The rate of Cidea protein degradation in HEK-293T cells in the presence of various protease inhibitors was examined. As shown in Figure 1(E), in the presence of general protease inhibitors (pepstatin, leupeptin and aprotinin) together with CHX, compared with the control, similar levels of Cidea were detected, suggesting that these protease inhibitors did not block Cidea degradation. Similarly, treatment of cells with the lysosomal inhibitors chloroquine and NH₄Cl had little effect on the accumulation of Cidea proteins. By contrast, when cells were treated with the cysteine protease/proteasome inhibitor ALLN or the specific proteasome inhibitor MG132, large amounts of the Cidea protein accumulated. Similar results were obtained when Cidea was overexpressed in CHO-K1 and H1299 cells (results not shown). These findings strongly suggest that Cidea protein degradation is dependent on proteasomal activity.

Endogenous Cidea protein is unstable and its degradation can be inhibited by proteasome inhibitors

To investigate the stability of endogenous Cidea in mature brown adipocytes, preadipocytes were isolated from the BAT of 4-week-old mice, which was then induced via differentiation to become mature brown adipocytes [18]. As shown in Figure 2(A), no Cidea or UCP1 protein was detected in undifferentiated preadipocytes,

Figure 2 Endogenous Cidea in differentiated brown adipocytes is also unstable and proteasome inhibitor MG132 can prevent its degradation

Brown preadipocytes, obtained by isolation of fresh BAT from 4-week-old Balb/c mice, were grown to confluency in 100-mm-diameter tissue-culture dishes and maintained in DMEM plus 10% FBS. After that, brown preadipocytes were allowed to differentiate into mature adipocytes in the presence of 0.5 mM IBMX and 10 μ M dexamethasone at day 0. Then, at day 2, 48 h later, the differentiation medium was replaced with DMEM plus 10% FBS, 1 μ g/ml insulin and 10 μ M pioglitazone. The differentiation medium was continually changed each day until day 8. At 2 h before treatment with drugs, the medium of differentiated brown mature adipocytes was replaced with fresh medium without the differentiation agents. DMSO, 100 μ g/ml CHX and 10 μ M MG132 were added for 2 h, and 0.5 ml portions of cell extract were harvested in lysis buffer for Western-blot analysis using anti-Cidea, anti-UCP1 and anti- β -tubulin antibodies. (A) Lane 1, undifferentiated cells from primary preadipocytes; lanes 2 and 3, differentiated mature brown adipocytes treated with control DMSO and CHX for 2 h. (B) Lane 1, undifferentiated cells from primary preadipocytes; lanes 2 and 3, differentiated mature brown adipocytes treated with control DMSO and MG132 for 2 h. IB, immunoblotting.

but they were detected in mature adipocytes upon differentiation. Levels of Cidea protein in the mature adipocytes decreased dramatically in the presence of CHX (Figure 2A). However, when

mature brown adipocytes were treated with specific proteasome inhibitor MG132, Cidea protein was accumulated to a much higher level than in untreated cells (Figure 2B). In summary, these data suggest that endogenous Cidea protein in mature brown adipocytes is also unstable and can undergo proteasome-dependent degradation.

Cidea is ubiquitinated

To assess whether Cidea is ubiquitinated prior to its degradation, *in vivo* ubiquitination assays were performed. Flag-tagged Cidea was co-transfected with HA-tagged ubiquitin in 293T cells; at 24 h post-transfection, cells were treated with MG132 and incubated for additional 2 h. Cidea proteins were then immunoprecipitated with anti-Flag antibody. As shown in Figure 3(A) (top panel), when the proteins were blotted with anti-ubiquitin antibody, a typical high-molecular-mass protein ladder was observed, suggesting that Cidea protein was polyubiquitinated. A similar protein ladder was also detected using anti-Flag antibody (Figure 3A, middle panel), consistent with Cidea being polyubiquitinated. Since ubiquitin is a quite abundant molecule in the cell [11], we tested whether Cidea can be polyubiquitinated by endogenous ubiquitin by transiently expressing HA-Cidea alone in HEK-293T cells in the presence or absence of MG132. After immunoprecipitation with anti-HA antibody, a stronger polyubiquitination signal was detected from cells treated with MG132 as compared with DMSO-treated cells, suggesting that exogenous Cidea can be polyubiquitinated by endogenous ubiquitin for proteasomal degradation (Figure 3B). To evaluate

Figure 3 Cidea can be ubiquitinated by exogenous or endogenous ubiquitin

(A) HEK-293T cells in 60-mm-diameter dishes were co-transfected with 0.5 μ g of pXJ-40-HA-Ub, pEGFP-N1 and 1 μ g of CMV5-Flag control vector or CMV5-Flag-hCidea using the calcium phosphate method. *In vivo* ubiquitination was determined as follows. At 1 h before treatment with MG132, the medium was replaced with fresh medium and then MG132 was added to a final concentration of 10 μ M for 2 h. Then cells were harvested in 0.5 ml of lysis buffer containing 0.5% SDS, 5 mM dithiothreitol and heated at 90 $^{\circ}$ C for 5 min, 0.25 ml of sonicated and centrifuged lysate was then subjected to IP (immunoprecipitation) by diluting SDS to 0.1% in lysis buffer using M2 beads, and Western-blotting analysis was conducted using anti-ubiquitin, anti-Flag and anti-GFP antibodies. (B) HEK-293T cells in 60-mm-diameter dishes were co-transfected with 1 μ g of CMV5-HA control vector or CMV5-HA-Cidea and 0.5 μ g of pEGFP-N1 using the calcium phosphate method. At 24 h post-transfection, MG132 was added to a final concentration of 10 μ M for 2 h (lanes 2 and 3) as indicated. Then cells were harvested using the same procedure as for the *in vivo* ubiquitination assay as described above and were subjected to IP using anti-HA antibody and Protein A/G Plus-agarose beads. The immunoprecipitates and total cell lysates were analysed by Western blotting using anti-ubiquitin, anti-HA and anti-GFP antibodies. (C) One 100-mm-diameter dish of differentiated mature adipocytes from the HB2 cell line at day 8, 2 h prior to treatment with MG132, the medium was replaced with fresh medium and then MG132 (10 μ M) was added for 2 h. After MG132 treatment, cells were harvested in lysis buffer and lysates prepared were subjected to IP with anti-Cidea antibody (lanes 1 and 2) and control IgG (lane 3) for 4 h as indicated. The IP product was washed in lysis buffer four times and finally dissolved in 30 μ l of lysis buffer. The freshly boiled samples were detected by Western blotting using anti-ubiquitin, anti-Cidea and anti- β -tubulin antibodies. IB, immunoblotting.

Figure 4 Cidea N-terminal lysine residues play a predominant role in controlling protein stability

(A) Schematic drawing of human Cidea, WT, N-5KA, C-5KA and KO mutants. (B) A 1.0 μ g portion of CMV5-HA-Cidea, CMV5-Cidea-N-5KA, CMV5-Cidea-C-5KA and CMV5-Cidea-KO were co-transfected with 0.5 μ g of pEGFP-N1 in HEK-293T cells in 60-mm-diameter dishes using the calcium phosphate method. The CHX-based protein chase experiment was performed as described above. Total cell lysates were detected by Western blotting using anti-HA and anti-GFP antibodies. The blot shown is representative of three separate experiments. (C) Quantification of scanned gel bands shown in (B) was performed using ImageQuant-TL software (Amersham). Results are expressed as the percentage of the signal of HA-Cidea compared with that of GFP. The value was set to 100% at zero time. The results shown are representative of the three independent experiments. (D) HEK-293T cells in 60-mm-diameter dishes were co-transfected with 1.0 μ g of CMV5-HA control vector, CMV5-HA-Cidea (WT), CMV5-HA-Cidea-N-5KA and CMV5-HA-Cidea-C-5KA or CMV5-HA-Cidea-KO control vector with 0.5 μ g of pXJ40-Myc-Ub and pEGFP-N1. Then *in vivo* ubiquitination assays were performed as described above and cell lysates were subjected to IP using anti-HA in protein A/G beads. The immunoprecipitates were analysed by Western blotting using anti-Myc, anti-HA and anti-GFP antibodies. (E) Apoptosis induced by Cidea and its lysine-less mutants. A 1.5 μ g portion of the indicated plasmids and 0.5 μ g of pEGFP-N1 were co-transfected into CHO-K1 cells. Apoptotic cells were quantified, and the percentage of cell death was calculated relative to the GFP-positive cells. Results are means $\pm$ S.D. for at least three independent experiments. IB, immunoblotting.

whether endogenous Cidea proteins can be ubiquitinated, Cidea protein was immunoprecipitated from mature brown adipocytes after complete differentiation from the brown preadipocyte cell line HB2 using anti-Cidea antibody or control IgG. As shown in Figure 3(C), polyubiquitination signals were detected on Cidea protein in the presence of MG132, but not in the absence of MG132. No ubiquitin signal was detected in the immunoprecipitate using IgG as a control. Our data thus indicate that both exogenous and endogenous Cidea proteins can be ubiquitinated and degraded via a proteasome-dependent pathway.

Cidea N-terminal lysine residues control protein stability

To identify (a) crucial lysine residue(s) that could serve as the predominant acceptor site(s) for covalent ubiquitin attachment in regulating Cidea stability, we used site-directed mutagenesis to replace all lysine residues with alanine individually or in

combination. Human Cidea contains ten lysine residues: one in the extreme N-terminal region (K23), three in the Cide-N domain, one in the linker region (K122), three in the Cide-C domain and two in the extreme C-terminal region (Figure 4A). As expected, Cidea protein in which all lysine residues were replaced with alanine [KO (knockout)] was very stable (Figures 4B and 4C, and Table 1), as similar levels of Cidea proteins were observed before and after 2 h of CHX treatment. Interestingly, the Cidea N-terminal lysine-less mutant (Cidea-N-5KA) was also very stable, with a half-life similar to that of Cidea lysine-less mutant (Cidea-KO). By contrast, Cidea proteins containing amino acid replacements for C-terminal lysine residues by alanine (Cidea-C-5KA) can still turn over rapidly, with a half-life similar to that of WT (wild-type) Cidea protein (Figures 4B and 4C, and Table 1). When examined for ubiquitination, Cidea-KO protein was not ubiquitinated, as no ubiquitination signal was detected in immunoprecipitated Cidea-KO protein (Figure 4D).

Figure 5 Cidea K23 is the major contributor to protein instability

(A) Amino acid sequence alignment of Cidea sequence in mammals using ClustalX (version 1.83) software. The shaded regions indicate identical amino acids and conserved lysine residues. The proposed Cidea Cide-N domains are overlined. (B) A 1.0 μ g portion of CMV5-HA vector, CMV5-HA-Cidea and CMV5-HA-Cidea-K23A, CMV5-HA-Cidea-K23E, CMV5-HA-Cidea-K23G or CMV5-HA-Cidea-K23R was co-transfected with 0.5 μ g of pEGFP-N1 in HEK-293T cells in 60-mm-diameter dishes using the calcium phosphate method. The CHX-based protein chase experiment was performed as described above. Total cell lysates were detected by Western blotting using anti-HA and anti-GFP antibodies. The blot shown is representative of three separate experiments. (C) Quantification of scanned gel bands shown in (B) was performed using ImageQuant-TL software (Amersham) and results are expressed as the percentage of the signal of HA-Cidea compared with that of GFP. The ratio was set to 100% at zero time. The results shown are representative of three independent experiments. (D) HEK-293T cells were co-transfected with 1.0 μ g of CMV5-HA control vector, CMV5-HA-Cidea, CMV5-HA-Cidea-K23A, CMV5-HA-Cidea-K23E, or CMV5-HA-Cidea-K23G or CMV5-HA-Cidea-K23R with 0.5 μ g of pXJ-40-Myc-Ub and pEGFP-N1. Then an *in vivo* ubiquitination assay was performed as described above and cell lysates were subjected to IP (immunoprecipitation) using anti-HA and Protein A/G Plus-agarose beads. The immunoprecipitates were analysed by Western blotting using anti-Myc, anti-HA and anti-GFP antibodies. IB, immunoblotting.

Intriguingly, both the Cidea N-5KA and C-5KA mutants can still be polyubiquitinated, albeit to a much lesser extent than that of WT protein (Figure 4D). Our data suggest that both N- and C-terminal lysine residues can be ubiquitinated and that no particular lysine residue serves as a specific acceptor site for Cidea ubiquitination. However, as shown above (Figure 4B), lysine residues located at the N-terminus of Cidea contribute predominantly to the regulation of its protein stability.

To determine whether the molecular structure and cellular function of lysine substitutions of Cidea remain functionally intact, an apoptosis assay was conducted in CHO-K1 cells. As shown in Figure 4(E), Cidea WT can induce cell death in CHO-K1 cells to the extent of approx. 66%, whereas Cidea N-5KA caused even a higher apoptotic function (82%). The difference between WT Cidea and N-5KA may be due to the increased protein stability of Cidea N-5KA protein [4,6]. Replacement of Cidea C-terminal lysine residues with alanine (C-5KA) slightly decreased its cell-death-inducing activity (56%). Surprisingly, Cidea protein in which all lysine residues were replaced with

alanine remains active, albeit with a lower associated death rate (46%). The lower cell death efficiency for Cidea-KO may be due to minor conformational changes resulted from the substitution of all ten lysine residues. Nevertheless, these data suggest that replacement of lysine residues in Cidea does not result in drastic alteration of Cidea protein structure and does not constitute a main factor in controlling Cidea stability at the structural level.

Cidea protein instability is mainly conferred by K23

The N-terminal region of Cidea contains five lysine residues, three of them (K23, K61 and K107) being conserved among the Cidea proteins in all mammals (Figure 5A). To identify any specific lysine residue(s) in this region that is (are) responsible for controlling Cidea protein stability, we individually replaced these five lysine residues with alanine and measured the extent of ubiquitination and rates of protein degradation. As shown in Table 1, replacement of lysine residues K61, K104, K107 or K122 in Cidea with alanine did not affect its stability. They all have a

Table 1 Protein half-lives of the Cidea lysine (K)-to-alanine (A) mutants determined in HEK-293T cells by CHX-based protein chase experiments

Western blots from three different independent experiments of transiently expressed HA-Cidea and its lysine-to-alanine mutants in HEK-293T cells assayed by a CHX-based protein chase experiment were scanned and analysed using ImageQuant-TL software (Amersham). The ratio of HA-Cidea to the GFP band signal was set at 100 % at zero time. The half-life of the corresponding protein was determined from the degradation curve when the protein remaining reached 50 %. The data presented are the averages of the half-lives obtained from three separate experiments.

Cidea lysine-to-alanine mutant	Protein half-life (min) (mean $\pm$ S.D.)
Cidea lysine-less mutants	
WT	29.6 $\pm$ 6.3
N-5KA	>120
C-5KA	41 $\pm$ 11.1
KO	>120
N-terminal single mutants	
K23A	>120
K61A	49.3 $\pm$ 2.9
K104A	42.9 $\pm$ 15.4
K107A	26.4 $\pm$ 2.6
K122A	30.1 $\pm$ 12.0
N-terminal double mutants	
K23,61A	>120
K23,104A	>120
K23,107A	>120
K23,122A	>120
K61,104A	41.6 $\pm$ 16.0
K61,107A	64.2 $\pm$ 17.1
K61,122A	31.6 $\pm$ 10.6
K104,107A	28.6 $\pm$ 6.1
K104,122A	30.7 $\pm$ 5.3
K107,122A	37.6 $\pm$ 12.1

similar half-life compared with WT Cidea. However, replacement of K23 with alanine (K23A) significantly prolonged its half-life (with a half-life longer than 120 min; Figure 5B and Table 1). These data suggest that K23, in the N-terminal region, plays a predominant role controlling Cidea protein stability. To eliminate a possible charge effect of the K23 residue, we replaced K23 with a negatively charged glutamic acid residue (K23E) or a positively charged arginine residue (K23R). All mutants variants were very stable and showed no significant decrease in protein level in the presence of CHX (Figures 5B and 5C), although all K23 mutants could be significantly ubiquitinated (Figure 5D). To confirm the importance of the K23 residue, double mutants of the five lysine residues in the N-terminal region were created and their protein stability was determined. All double mutants harbouring the K23A mutation were relatively stable, with a half-life longer than 2 h, whereas other double mutants without the K23 mutation were still relatively unstable (Table 1). Taken together, our findings indicate that K23 is the main lysine residue contributing to Cidea protein instability.

DISCUSSION

Our previous studies using Cidea KO mice as a model system suggested that Cidea plays an important role in controlling energy expenditure and the development of obesity [8]. In the present study we observed that both transiently expressed Cidea protein in various cell lines and endogenous protein in mature brown adipocytes are all unstable. We provide solid evidence to show that Cidea protein stability is regulated by the ubiquitin-proteasome pathway, since Cidea proteins can only be accumulated in the presence of the specific proteasome inhibitors ALLN and MG132.

Other general proteolysis inhibitors, such as pepstatin, leupeptin and aprotinin, or lysosomal inhibitors, such as chloroquine and NH_4Cl , cannot inhibit its degradation in CHX-based protein chase experiments.

Control of protein stability via the ubiquitin-mediated proteasomal degradation pathway plays an important role in regulating many cellular processes, since many regulatory proteins in these processes, such as p21, p53, MyoD, β -catenin and acetyl-CoA carboxylase 1, a rate-limiting enzyme in fatty acid synthesis, are all regulated via this pathway [13,19–22]. Ubiquitination generally occurs on lysine residues or the N-terminal free amino group [11,23]. However, no specific lysine residue responsible for protein ubiquitination has been identified in many short-lived proteins [12,22,24]. When the preferred lysine residue is mutated, cryptic lysine residues within a molecule will be used for conjugation to ubiquitin during the ubiquitin conjugation process [16,24–26]. This is also the case for Cidea ubiquitination. We found that replacement of all lysine residues within Cidea individually did not significantly affect its overall ubiquitination (results not shown). Moreover, mutation of all five N-terminal lysine residues or all of the five lysine residues in the C-terminal region of the Cidea reduces, but does not abolish, Cidea polyubiquitination. Ubiquitination of Cidea protein can only be abolished when all ten lysine residues were replaced by alanine. Nevertheless, it is noteworthy that levels of ubiquitination of Cidea proteins harbouring replacements of all lysine residues with alanine in N-terminal region (N-5KA) and C-terminal all-lysine replacements (C-5KA) were similar. However, their stability is dramatically different, with N-5KA being much more stable than the WT and C-5KA Cidea proteins. The disparity between their respective stabilities may be due to the less quantitative measurement of the amount of ubiquitination for overexpressed proteins. Alternatively, proteins that have similar levels of ubiquitination may have differing abilities to target to proteasome for degradation. This extra step of regulation may be mediated by N-terminal lysine residues in Cidea. We then demonstrated that K23, located in the variable region at the N-terminus of Cide-N domain, is the main contributor to Cidea protein instability, since replacement of K23 by alanine or differently charged amino acid residues significantly prolonged its half-life. It is not clear how K23 plays such a critical role in governing Cidea protein stability. One possibility is that ubiquitination is, *a priori*, a condition for protein instability, but it takes additional neighbouring sequence around K23 for targeting to 26S proteasome. Implied here is also that ubiquitination occurring at different lysine residues within a single molecule may have different functions besides the ubiquitin-mediated proteasomal degradation [27–30].

As the specificity of ubiquitin-mediated proteasomal degradation is tightly controlled by various E3 ligases, which targets its substrate protein for degradation, it is of great importance to find out the precise mechanism of Cidea protein stability by identifying the specific E3 ligase that controls Cidea protein ubiquitination and degradation [11,12,23]. Our demonstration that Cidea is a strongly ubiquitinated protein and that K23 is the main residue controlling the ubiquitination-dependent proteasomal degradation of Cidea will provide an important foundation for the ultimate understanding of how Cidea is regulated by extracellular signals such as environmental or nutritional changes, and of its *in vivo* biological function in BAT.

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