

The death effector domain-containing DEDD forms a complex with Akt and Hsp90, and supports their stability

Nobuya Kurabe¹, Mayumi Mori, Jun Kurokawa, Kaori Taniguchi, Hisatoshi Aoyama, Kazuhiro Atsuda, Akemi Nishijima, Nariaki Odawara, Saori Harada, Katsuhiko Nakashima, Satoko Arai, Toru Miyazaki*

Division of Molecular Biomedicine for Pathogenesis, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

Insulin secretion and glucose transport are the major mechanisms to balance glucose homeostasis. Recently, we found that the death effector domain-containing DEDD inhibits cyclin-dependent kinase-1 (Cdk1) function, thereby preventing Cdk1-dependent inhibitory phosphorylation of S6 kinase-1 (S6K1), downstream of phosphatidylinositol 3-kinase (PI3K), which overall results in maintenance of S6K1 activity. Here we newly show that DEDD forms a complex with Akt and heat-shock protein 90 (Hsp90), and supports the stability of both proteins. Hence, in DEDD^{-/-} mice, Akt protein levels are diminished in skeletal muscles and adipose tissues, which interferes with the translocation of glucose-transporter 4 (GLUT4) upon insulin stimulation, leading to inefficient incorporation of glucose in these organs. Interestingly, as for the activation of S6K1, suppression of Cdk1 is involved in the stabilization of Akt protein by DEDD, since diminishment of Cdk1 in DEDD^{-/-} cells via siRNA expression or treatment with a Cdk1-inhibitor, increases both Akt and Hsp90 protein levels. Such multifaceted involvement of DEDD in glucose homeostasis by supporting both insulin secretion (via maintenance of S6K1 activity) and glucose uptake (via stabilizing Akt protein), may suggest an association of DEDD-deficiency with the pathogenesis of type 2 diabetes mellitus.

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Introduction

The signalling cascade involving mitogen-related phosphatidylinositol 3-kinase (PI3K), Akt and their downstream TOR (target of rapamycin) is the central pathway that maintains glucose homeostasis in the body [1–4]. In mammals, upon stimulation by growth factors including insulin, the mammalian TOR (mTOR) cooperates with PI3K-dependent effectors to activate p70 ribosomal protein S6 kinase-1 (S6K1), thereby phosphorylating the 40S-ribosomal protein S6, and subsequently enhances translation of the 5'-terminal oligopyrimidine (5'-TOP) sequences that encode components of the translational machinery. This reaction increases the number of ribosomes and the efficacy of protein synthesis, thus critically promoting growth of types of cells including insulin-producing β cells

within the pancreatic Langerhans islet [5–8]. The insulin mass was diminished in S6K1-deficient (S6K1^{-/-}) mice, resulting in ineffective secretion of insulin upon glucose administration [9]. Thus, S6K1 is involved in the machinery controlling glucose tolerance by supporting the size of β cells [10,11]. On the other hand, activation of Akt (in particular Akt2, the primary isoform in insulin-responsive tissues) induces translocation of glucose-transporter 4 (GLUT4) to the plasma membrane [12–15]. This response is responsible for glucose transport into cells. Thus, dysfunction of these elements provokes a phenotype similar to type 2 diabetes mellitus, which is a multifactorial disease with a variety of pathological defects in glucose homeostasis [16–18].

Recently, we defined the DEDD molecule as a critical element that maintains the activity of S6K1, thereby supporting the size of β cells and insulin mass in mice [19]. DEDD was initially described as a member of the death effector domain (DED)-containing protein family [20]. We previously found that DEDD is associated with the Cdk1/cyclin B1 complex, and that it decreases the kinase activity of Cdk1 [21]. This response impedes the Cdk1-dependent mitotic program to shut off synthesis of ribosomal RNA (rRNA) and protein, and is consequently useful in gaining sufficient cell growth [21,22]. Interestingly, DEDD also associates with S6K1, and interferes with the Cdk1-dependent inhibitory phosphorylation of S6K1 at several serine/threonine (Ser/Thr)

Abbreviations: DEDD, death effector domain-containing DNA binding protein; Cdk1, cyclin-dependent kinase-1; S6K1, S6 kinase-1; PI3K, phosphatidylinositol 3-kinase; rRNA, ribosomal RNA; TOR, target of rapamycin; mTOR, mammalian TOR; 5'-TOP, 5'-terminal oligopyrimidine; Thr, threonine; Ser, serine; MEF, mouse embryonic fibroblast; PDK1, phosphoinositide-dependent protein kinase-1; Hsp90, heat-shock protein 90; GLUT4, glucose-transporter 4.

* Corresponding author. Fax: +81 3 5841 1438.

E-mail address: tm@m.u-tokyo.ac.jp (T. Miyazaki).

¹ Present address: Department of Molecular Anatomy, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192, Japan.

residues, including Ser411 and Ser424 sites within the auto-inhibitory tail [19,23,24]. This response maintains the activity of S6K1 preserving a high level of phosphorylation at Thr389, a hallmark of active S6K1 [19]. Hence in DEDD^{-/-} mice, the activity of S6K1 was reduced in various cell types, and as observed in S6K1^{-/-} mice, the insulin mass within pancreatic islets is reduced, resulting in overt glucose intolerance [19].

Having discovered the functional association of DEDD with S6K1, we here address a possible interaction of DEDD with Akt, and investigate a novel involvement of DEDD in the regulation of the insulin signaling cascade.

Material and methods

Mice. DEDD^{-/-} mice [21] had been backcrossed to C57BL/6 (B6) for 17 generations before used for experiments. Mice are maintained under a SPF condition.

Antibodies. Antibodies used are: anti-total Akt (clone 11E7), anti-Akt phosphorylated at Thr308 (clone 244F9) (all are from Cell Signaling Technology, Beverly, MA); anti-Hsp90 (clone SPA-830) and anti-Cdk1 (clone A17) (from Stressgen, Victoria, BC, Canada, and Zymed Laboratories Inc., South San Francisco, CA).

Glucose incorporation. This assay was performed as described previously [25] with some modifications. Pieces from epididymal white fat pads and the soleus muscles of the mice were used. To determine 2-DG uptake, the muscles and fat pads were transferred to buffer A containing 1 mM 2-DG (0.5 μ Ci/ml 2-deoxy-D-[1-¹⁴C]glucose) and 1 mM L-glucose (5 μ Ci/ml L-[1-³H]glucose) with or without 10 nM insulin and incubated at 30 °C for 10 min. After the reaction is terminated, the samples were neutralized with 5 N HCl and dissolved in ACSII (Amersham Biosciences). ¹⁴C and ³H specific activities were counted by a liquid scintillation counter (Packard Instrument Co.).

GLUT4 translocation. Primary MEF cells prepared from DEDD^{+/+} or DEDD^{-/-} embryos were differentiated to adipocytes according to established protocols [26]. GLUT4 translocation assay was performed as previously described [26,27], using a GFP-fused GLUT4 expression vector [27]. Confluent primary MEF cells prepared from DEDD^{+/+} or DEDD^{-/-} mice were induced to differentiate by incubating the cells with DMEM containing 10 μ g/ml of insulin (Sigma), 1 μ M of dexamethasone (Sigma), and 500 μ M of isobutylmethylxanthine (Sigma). After 48 h, the cells were fed with DMEM containing 10 μ g/ml of insulin every 2 days prior to use. On 8–10 days after induction of differentiation, cells were transfected with the construction coding GFP-GLUT4-myc [27]. Cells were serum-starved for 4 hours, and then incubated with or without 200 nM of insulin for 30 min. GFP-GLUT4 trafficking was followed by a fluorescent microscope. A ratio for GFP-GLUT4 distribution within peri-membrane areas to peri-nuclear areas was measured using NIH-Image.

Na₃VO₄ treatment. DEDD^{+/+} or DEDD^{-/-} MEF cells were enriched in metaphase by a treatment with 500 nM nocodazole (Sigma) for 24 h. Cells were further incubated with 10 mM Na₃VO₄ for an additional 6 h, and lysed in SDS sample buffer. The cell extracts were subjected to Western blotting using anti-Akt or anti-Hsp90 antibodies.

Protein degradation and phosphorylation assay. Subconfluent DEDD^{+/+} or DEDD^{-/-} MEF cells were cultured accordingly and harvested at indicated time points after treatment with 100 μ g/ml cyclohexamide with or without 10 μ M MG-132 and 0.4 mM chloroquine, lysed with Brij 97 lysis buffer supplemented with 5 mM iodoacetamide, 5 μ g/ml leupeptin, 0.2 mM AEBSF, 1 mM Na₃VO₄, and 10 mM NaF. Lysates were resolved onto SDS-PAGE, and immunoblotted anti-Akt antibody.

siRNA transfection. Double strands siRNA targeting DEDD or Cdk1 were purchased from Applied Biosystems or SIGMA, respectively. Wild-type MEF cells at 50% confluency were transfected with 10 μ M siRNA using Lipofectamine 2000 (Invitrogen Inc.). Forty-eight hours after the transfection, the cells were harvested and analyzed by Western blotting or RT-PCR. Sequences of the oligonucleotides were as follows: DEDD siRNA#1: 5'-GCCCTGATC TTGTAGACAATT-3', DEDD siRNA#2: 5'-AAATGGACGTGACTTCTTA TT-3', Cdk1 siRNA#1: 5'-CTATGATCCTGCCAAACGATT-3', Cdk1 siRNA#2: 5'-GTTGTTTACCGTTGGCTCTTT-3', Cdk1 siRNA#3: 5'-CAATC AAAGTGGCTGATTTTT-3'. For a control, an oligonucleotide targeting GFP sequence (Sigma) was used.

Primers for RT-PCR. Primers used are as follows: for, forward primer; rev, reverse primer. Hsp90 α for: 5'-CGCGCAAAGACAAGAAA AAG-3'; Hsp90 α rev: 5'-CAAGTGGTCTCCAGTCAT-3'; Hsp90 β for: 5'-CTGGGTCAAGCAGAAAGGAG-3'; Hsp90 β rev: 5'-TCTCTGTTG CTTCCCGACTT-3'; Akt1 for: 5'-CCACGCTACTTCTCTCTC-3'; Akt1 rev: 5'-TGCCCTTGCCAAACAGTCTGAAGCA-3'; Akt2 for: 5'-GTCCGC AACAGTCTGAAGCA-3'; Akt2 rev: 5'-GAGAGAGGTGAAAAACAG C-3'; G3PDH for: 5'-ACCACAGTCCATGCCATCAC-3'; G3PDH rev: 5'-TCCACCACCTGTTGCTGTA-3'; β -actin for: 5'-GTGGCTACAGCTTC ACCACCAG-3'; β -actin rev: 5'-GCATCCTGTACGAATGCCTGGG T-3'; DEDD for: 5'-GCGGGATCCGCGGGCCATAAGAGGC-3'; DEDD rev: 5'-GCGTCTAGAGTCTACAGATCAGGGC-3'.

Quantification of immuno-blots. Quantification of the immuno-blots was performed using the NIH-Image. Relative phosphorylation levels to those in control (shown as 1.0 \pm SEM) are presented. For all immuno-blotting experiments, at least three independent blotting were performed.

Statistical analysis. A two-tailed Mann-Whitney test was used to calculate P-values. (**): P < 0.01, (*): P < 0.05. Error bars: SEM.

Results

Lack of DEDD decreases the amount of Akt protein

Having observed reduced activity of S6K1 in the absence of DEDD [19], we wondered whether upstream of S6K1 in the insulin signalling pathway might also be influenced by the lack of DEDD, and thus assessed the situation of Akt protein in DEDD^{-/-} mouse embryonic fibroblast (MEF) cells by Western blotting. To our surprise, the amount of Akt was also greatly decreased in DEDD^{-/-} MEF cells compared with DEDD^{+/+} MEF cells, when tested by using an antibody that detects all isoforms of Akt (Fig. 1A, total Akt). Signals for activated Akt phosphorylated at Thr308 residue were also reduced, along with the diminished amounts of total Akt protein (Fig. 1A, p-Akt). A reduction in the amount of Akt as well as in the Thr308 phosphorylation level, was also clear in DEDD^{-/-} skeletal muscles and adipose tissues, where Akt plays an important role in the regulation of glucose homeostasis [12,13] (Fig. 1B). The effect of an acute loss of DEDD on Akt was also assessed by knocking down DEDD in cells. As presented in Fig. 1C, downregulation of DEDD expression by introducing a double-stranded siRNA for DEDD into wild-type MEF cells significantly reduced the amount of Akt. Consistent with these observations, the activating phosphorylation (at Ser2448) of mTOR, downstream of Akt, was decreased in DEDD^{-/-} compared with DEDD^{+/+} cells (Fig. 1D, left). In contrast, phosphorylation levels of 3-phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates Akt, were comparable in the presence or absence of DEDD, suggesting that the less phosphorylation of Akt in the absence of DEDD is mainly caused by a reduction in the total amount of Akt protein (Fig. 1E). Together, the lack of DEDD decreases the amount of all types of Akt protein both in MEF cells and in tissues from DEDD^{-/-} mice, which is accompanied with lower Akt activity. This

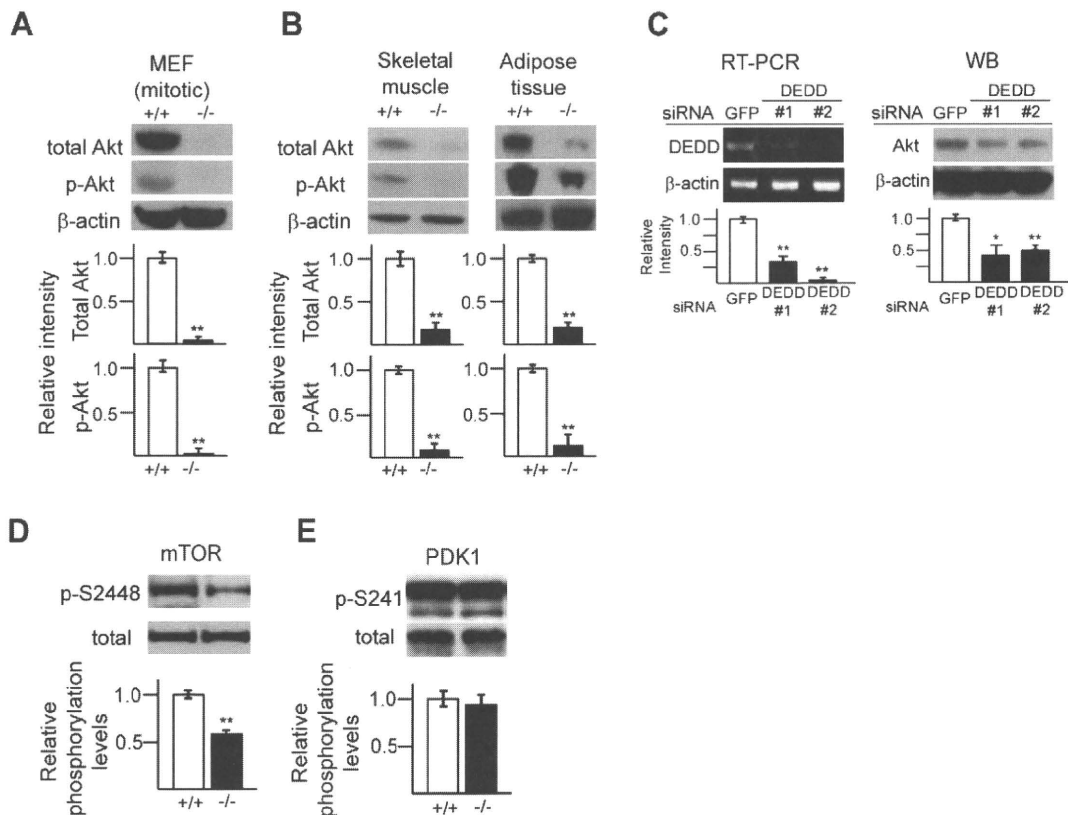


Fig. 1. Reduced Akt protein amounts in the absence of DEDD. (A) Total Akt or phosphorylated (activated) Akt analyzed in DEDD^{+/+} and DEDD^{-/-} MEF cells, and (B) in tissues from DEDD^{+/+} and DEDD^{-/-} mice. The representative immuno-blots and the averages of relative intensities (+/+ as 1.0) from all experiments are presented. Error bar: SEM. (C) Increase of Akt levels by diminishment of DEDD using siRNA in DEDD^{-/-} MEF cells. DEDD mRNA and Akt protein levels relative to those in control (shown as 1.0) are also presented. (D) Activative phosphorylation of mTOR (at Ser2448 site) and (E) PDK1 (at Ser241 site) in DEDD^{+/+} or DEDD^{-/-} MEF cells was analyzed by Western blotting. For PDK1, two bands appear when this polyclonal antibody (Cell signalling; #3061) is used as described in its data sheet provided from the company. The upper band is the phosphorylated PDK1. Three independent experiments (for both D and E) were performed.

result also indicates that in the absence of DEDD, the reduction in Akt activity may partly be responsible for the decreased S6K1 activity, in addition to the increased phosphorylation levels at the inhibitory residues of S6K1 brought about by the hyper activity of Cdk1 [19].

DEDD forms a complex with Akt and Hsp90, and stabilizes these proteins

Although the amount of Akt protein is markedly reduced, mRNA for both Akt1 and Akt2 were expressed at similar levels in DEDD^{-/-} and DEDD^{+/+} tissues and cells (Fig. 2A). This result suggests that DEDD may be necessary for the maintenance of Akt protein. To test this possibility, we measured the half-life of Akt protein in DEDD^{-/-} and DEDD^{+/+} MEF cells. Importantly, the amount of Akt protein was decreased in 10 hours in DEDD^{-/-} cells, but not in DEDD^{+/+} cells (Fig. 2B). The presence of MG-132, a proteasome inhibitor, tempered the reduction observed in DEDD^{-/-} cells (Fig. 2B). Thus, the lack of DEDD results in instability of Akt protein.

Several groups reported that heat-shock protein 90 (Hsp90), a chaperone required for the conformational maturation of certain signalling proteins, forms a complex with Akt and is involved in its stabilization [28,29]. Thus, we assessed the protein levels of Hsp90 in DEDD^{+/+} and DEDD^{-/-} MEF and tissues. As depicted in Fig. 2C, the amount of Hsp90 protein also decreased in skeletal muscle, adipose tissue, as well as in MEF cells from DEDD^{-/-} mice compared with those from DEDD^{+/+} mice, whereas the transcripts of *Hsp90* (both α and β) genes were at an equivalent level in both types of mice (Fig. 2D). Furthermore, as depicted in Fig. 2E, immunoprecipitation assays revealed that DEDD associates with Akt

(both 1 and 2) and Hsp90. Together, DEDD appears to facilitate a stable complex with Akt and Hsp90, supporting the levels of these proteins.

Suppression of Cdk1 increases Akt protein levels in DEDD^{-/-} cells

As we demonstrated in a previous report, DEDD modulates the activity of S6K1 partly via suppressing Cdk1 activity [19]. To assess whether the inhibitory effect of DEDD on Cdk1 is also involved in stabilizing Akt protein, we knocked down Cdk1 in DEDD^{-/-} MEF cells by introducing double-stranded siRNA for Cdk1, and analyzed the Akt and Hsp90 protein levels. As demonstrated in Fig. 3A, the levels of both proteins increased in DEDD^{-/-} cells when Cdk1 protein was diminished. In addition, treatment of DEDD^{-/-} MEF cells with sodium orthovanadate (VO₄), which is commonly used to inactivate Cdk1 [24], significantly increased the levels of both Akt and Hsp90 (Fig. 3B). These data suggest that in DEDD^{-/-} cells, the increase in Cdk1 activity appeared to be responsible to the instability of Akt protein.

Attenuated glucose incorporation in DEDD^{-/-} skeletal muscles and adipose tissues

One of a variety of functions for Akt is the regulation of incorporation of glucose into cells in response to insulin [30–32]. It is well known that translocation of GLUT4 to the plasma membrane upon insulin stimulation is a key mechanism of glucose transport into cells [12,13], and that this translocation of GLUT4 is dependent on activation of Akt, in particular Akt2 [33–35]. Therefore, we assessed how the reduction of the amount of Akt caused by the ab-

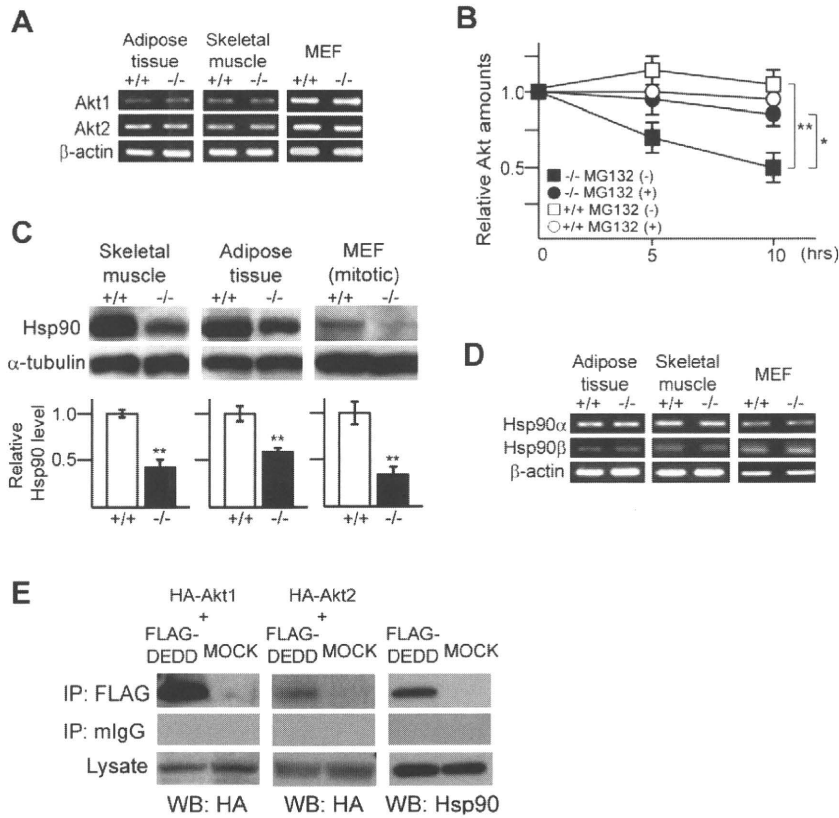


Fig. 2. DEDD forms a complex with Akt and Hsp90 and supports the stability of these proteins. (A) RT-PCR for Akt1, Akt2, and β-actin as a control (performed within the linear range). (B) Protein degradation assay for Akt. Representative data out of comparable results obtained by two independent experiments is presented. (C) Hsp90 protein levels in DEDD^{+/+} or DEDD^{-/-} skeletal muscle, adipose tissue or mitotic MEF cells. (D) RT-PCR assay for Hsp90 mRNA. (E) Association of DEDD with Akt1, Akt2, and Hsp90. DEDD was precipitated from 293T cells expressing HA-tagged AKT1 or Akt2 with or without FLAG-DEDD, and the precipitates were analyzed for HA-Akt1 or 2 using an anti-HA antibody (left and middle). Likewise, endogenous Hsp90 was co-precipitated with FLAG-DEDD from 293T cells expressing FLAG-DEDD (right). Results from the control IP using a mouse-IgG (mlgG) are also demonstrated.

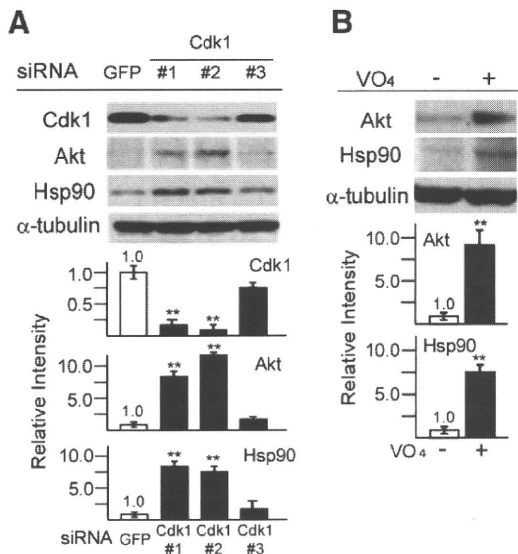


Fig. 3. Involvement of Cdk1 in stabilization of Akt by DEDD. (A) Akt and Hsp90 levels in mitotic DEDD^{-/-} MEF cells after treatment with siRNA targeting Cdk1 (three distinct sequences), or (B) with Na₃VO₄ (VO₄) for 6 h. The levels of Akt and Hsp90 relative to those in control (shown as 1.0) are also presented.

sence of DEDD affects glucose uptake in mice. As shown in Fig. 4A, the uptake of glucose by skeletal muscle (soleus muscles) and adipose tissue in response to insulin was significantly damaged in

DEDD^{-/-} mice. We also tested GLUT4 translocation in response to insulin, using DEDD^{-/-} and DEDD^{+/+} adipocytes differentiated from MEF cells. The increase of GLUT4 on the cell membrane after an insulin challenge was significantly less in DEDD^{-/-} compared to DEDD^{+/+} cells (Fig. 4B). Hence, diminished levels of Akt correlated with inefficient induction of GLUT4 translocation, resulting in deficient glucose transport into cells in DEDD^{-/-} skeletal muscle and adipose tissue.

Interestingly, however, Akt levels were almost comparable in the liver in DEDD^{-/-} and DEDD^{+/+} mice, in contrast to levels in skeletal muscle and adipose tissue (Fig. 4C). As the endogenous DEDD expression level was lower in the liver compared with that in the skeletal muscle (Fig. 4D), the loss of DEDD might not strongly influence Akt stability in the liver as it did in the skeletal muscle or adipose tissue.

Discussion

In addition to our previous report in which DEDD maintains the activity of S6K1 supporting the insulin mass within pancreatic islets, our current study has demonstrated that DEDD stabilizes Akt protein, leading to efficient glucose transport into skeletal muscles and adipose tissues. Thus, DEDD is involved in the insulin signalling pathway at diverse levels (summarized in Fig. 4E). As type 2 diabetes mellitus is a multifactorial disease [17], our findings suggest that DEDD-deficiency might play a certain role in the pathology of type 2 diabetes mellitus. Indeed, the defect in glucose transport observed in DEDD^{-/-} mice is one of the essential pathogenic features in type 2 diabetes mellitus. Evidence has also

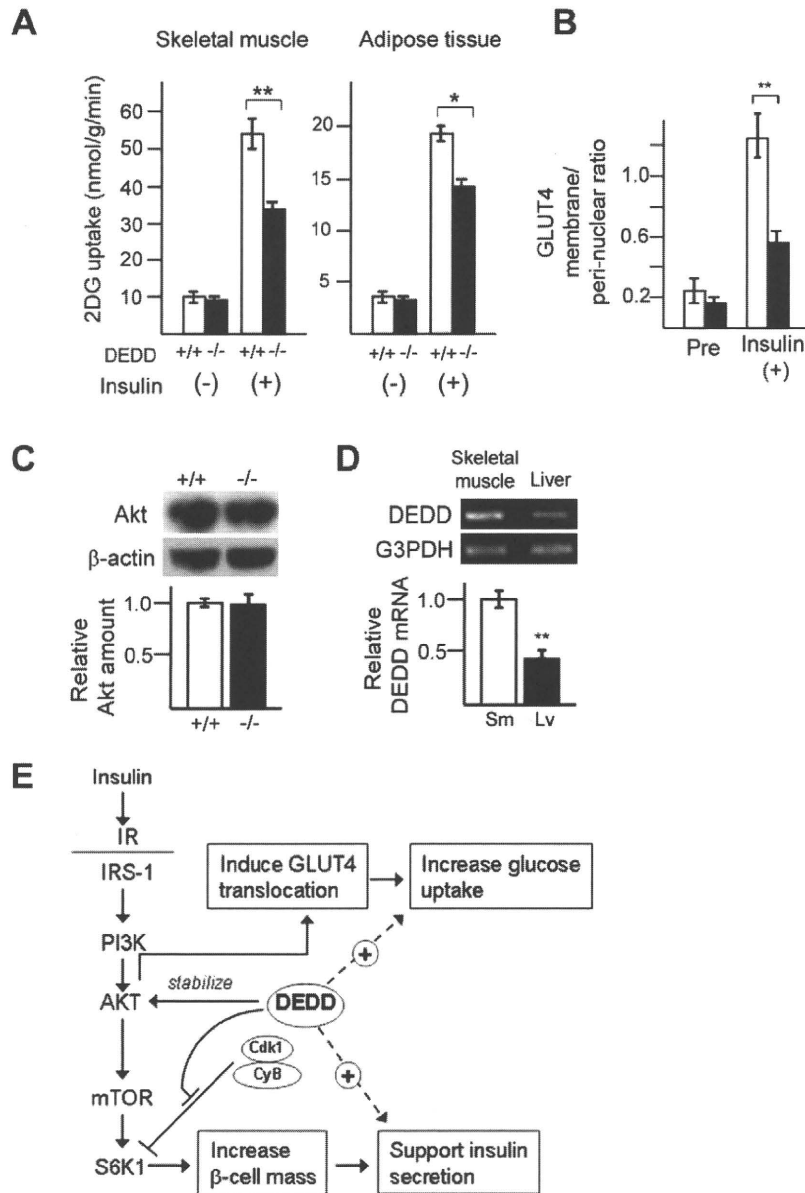


Fig. 4. Defects in glucose incorporation in DEDD^{-/-} tissues. (A) Glucose incorporation by skeletal muscle (left) or adipose tissue (right) (n = 5 each). (B) Translocation of GLUT4 in DEDD^{+/+} (white bars) or DEDD^{-/-} (black bars) adipocytes. Data represent the relative amount of GFP-GLUT4 at the membrane locus to that at the peri-nuclear area. Data are the averages of results from 30 cells. (C) Akt protein levels in DEDD^{-/-} and DEDD^{+/+} liver. (D) DEDD mRNA level expressed in the liver (Lv) relative to that in the skeletal muscle (Sm) is presented (lower panel). (E) A scheme for the plausible involvement of DEDD in glucose homeostasis. IR, insulin receptor; CyB, cyclin B1. The + with a dashed line (that starts from DEDD) means positive effect.

shown that a decrease in insulin secretion and reduced β cell mass do contribute to development of the disease [16,18].

It is interesting that the decrease in Akt protein levels was variable in different DEDD^{-/-} tissues important for glucose homeostasis; i.e. it was prominent in the skeletal muscles and adipose tissues, but was not very significant in the liver. This might cause a variable aberrancy in glucose transport in different organs in DEDD^{-/-} mice. Additional experiments such as to test the insulin sensitivity in each tissue, will test this possibility. It may be noteworthy that such a variation in insulin sensitivity in different tissues is often seen in human patients [16–18]. It will also be important to address whether any dysfunction of DEDD is present, either in the whole body or in specific tissues, in a subset of type 2 diabetes patients.

The precise mechanism of how the association of DEDD with Akt and Hsp90 supports the stability of these proteins is still unclear. The DEDD's effect on Akt stability appears to be achieved

through Cdk1. As we previously demonstrated, increased Cdk1 activity in the absence of DEDD accelerates the phosphorylation levels at the inhibitory residues of S6K1, resulting in a reduction of S6K1 activity [19]. A similar scenario might also be true for Akt (and/or Hsp90), although so far, inhibitory phosphorylation sites are not known either in Akt or Hsp90. Otherwise, Cdk1 might phosphorylate and activate some ubiquitin ligase(s) that degenerate Akt. As reviewed by Hunter [36], multiple crosstalks between phosphorylation and ubiquitination occur differentially during the protein degradation. Phosphorylation can regulate ubiquitination of a protein in different manners. Firstly, phosphorylation positively or negatively regulates the activity of the E3 ligase responsible for ubiquitine transfer. It is possible that Cdk1 may phosphorylate some E3 ligase(s) responsible for ubiquitination and degradation of Akt. Indeed, some E3 ligases involved in degradation of Akt, including recently identified TTC3 [37], require phosphorylation for their activation. Whilst, activity of CHIP, a major E3

ligase responsible for ubiquitination of Akt [38,39], might be modified directly or indirectly by Cdk1-dependent phosphorylation events, though the direct phosphorylation of CHIP has not been reported. On the other hand, it is also known that phosphorylation also promotes recognition of substrates by an E3 ligase [40]. However, phosphorylation events of Akt promoted by Cdk1 have not yet demonstrated. Alternatively, the possibility that formation of DEDD/Akt/Hsp90 might structurally stabilize these participant proteins is not mutually excluded. Thus, the molecular linkage among DEDD, Cdk1 and Akt-degradation still remains to be further addressed.

Conclusions

In summary, we newly demonstrated that DEDD plays an important role in maintenance of the Akt protein level, which in consequence supports the efficient incorporation of glucose into skeletal muscles and adipose tissues. Further investigations might find an unknown relevance of DEDD to the insulin signalling pathway, and thus, with a novel pathogenesis of type 2 diabetes mellitus.

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