Energy metabolism is the most fundamental capacity for mammals, impairment of which causes a variety of diseases such as type 2 diabetes and insulin resistance. Here, we identified a novel gene, termed diabetes-related ankyrin repeat protein (DARP) that is up-regulated in the heart of KKA\(^\sharp\) mouse, a type 2 diabetes and insulin resistance model animal. DARP contains putative nuclear localization signals and four tandem ankyrin-like repeats. Its expression is restricted in heart, skeletal muscle, and brown adipose. Western blot analysis and immunocytochemistry of DARP-transfected Chinese hamster ovary (CHO) and COS-7 cells reveal that DARP is a nuclear protein. When DARP is expressed in CHO cells, \([1-14C]\)palmitate uptake is significantly decreased, whereas the palmitate oxidation does not show significant change. Furthermore, DARP expression is altered by the change of energy supply induced by excess fatty acid treatment of skeletal myotube \textit{in vitro} and fasting treatment of C57 mouse \textit{in vivo}. We confirmed that DARP expression is also altered in Zucker fatty rat, another insulin resistance model animal. Taken together, these data suggest that DARP is a novel nuclear protein potentially involved in the energy metabolism. Detailed analysis of DARP may provide new insights in the energy metabolism.

Metabolic disorders cause wide variety of diseases including hyperlipidemia, hyperuricaemia, diabetes, and insulin resistance. Among these diseases, diabetes and insulin resistance are epidemic worldwide and are expected to affect 300 million people by 2025 (1). Recently, abnormalities of fatty-acid metabolism are recognized as key components of the pathogenesis of type 2 diabetes and insulin resistance (2–5). High fat diet and raised levels of circulating free fatty acids are sufficient to induce insulin resistance that is related to the fat content of skeletal muscle in rats (6). Accumulation of lipids inside muscle and, specifically, an increase in muscle long chain fatty acyl-CoA content are reported to cause insulin resistance. This suggests that abnormal fatty acid metabolism and the accumulation of lipid in skeletal muscle play crucial roles in the pathogenesis of insulin resistance (7, 8). Moreover, the relation between insulin resistance and muscle triglyceride content is independent of total adiposity. Although the details of the mechanisms connecting lipid accumulation and insulin resistance are still unclear, studies of insulin receptor signaling reveal that the accumulation of lipid products causes the phosphorylation of insulin receptor as well as insulin receptor substrate (IRS)-1 through protein kinase C activation. This results in the inhibition of insulin receptor signaling (4).

Recently identified molecules involved in the pathogenesis of insulin resistance act at least partially through the alteration of fatty acid metabolism. Adiponectin, whose secretion from white adipose tissue is reduced in insulin-resistant animal models, induces tissue fatty acid oxidation, leading to a reduction of tissue steatosis and reduced plasma glucose, triglycerides, and free fatty acids concentrations (9). The new class of insulin-sensitizing agents, thiazolidinediones, affects a wide variety of metabolic genes in insulin-sensitive tissues and has direct effects on mitochondrial fuel oxidation (10–12).

Here, we report a novel nuclear protein, termed diabetes-related ankyrin repeat protein (DARP),\(^1\) that is up-regulated in the heart of KKA\(^\sharp\) mouse, a type 2 diabetes and insulin resistance model animal. DARP-expressing CHO cells demonstrate significantly decreased \([1-14C]\)palmitate uptake. Furthermore, DARP expression in skeletal muscle is altered by a change of energy supply both \textit{in vitro} and \textit{in vivo}. Also, DARP expression is altered in Zucker fatty rat, another insulin resistance model animal. These results suggest that DARP is a novel nuclear protein that is potentially involved in energy metabolism.

**EXPERIMENTAL PROCEDURES**

Cloning of DARP—Total RNA was extracted from the heart of 10-week-old KKA\(^\sharp\) and C57BL/6J mice (Nihon Crea) using ISOGEN (Wako Pure Chemical Industries). Suppression-subtractive hybridization was performed using PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech) with 2 \(\mu\)g of poly(A)+ RNA purified from total RNA using the FastTrack 2.0 Kit (Invitrogen) as recommended by the manufacturers. Subtracted cDNAs were subcloned into pET vector (Novagen) and sequenced. The 5’ end of DARP cDNA was cloned by 5’-rapid amplification of cDNA ends (5’-RACE) (Invitrogen) against C57BL/6J mouse heart. The primers for 5’-RACE were designed according to the sequences obtained by the search of GenBank\(^\#\) (5’-TTCACCACGCT-GTCGGTGCCCTCAGACA-3’ for synthesis of first strand cDNA, 5’-CAGTGACTTGCTGAATCCAGGGCTCTGAGTG-3’ for first PCR, and

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\(^\sharp\) The abbreviations used are: DARP, diabetes-related ankyrin repeat protein; CHO, Chinese hamster ovary; RACE, rapid amplification of cDNA ends; RT, reverse transcription; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; NGS, normal goat serum; BSA, bovine serum albumin; ANK, ankyrin; CARP, cardiac ankyrin-repeat protein.
palmitate oxidation, cells were cultured in the sealed flask containing a suspended filter paper. The 14CO2 in the medium was liberated by addition of 1 ml of 6% hydrochloric acid. The 14CO2, collected overnight on the filter paper, was alkalinized with 2% sodium hydroxide and quantified by scintillation counting.

In Vivo Experiments—Animal care and procedures were in accordance with guidelines and regulations of the institutional animal care committee. To examine DARP expression, 8-week-old Zucker fatty and lean rats (Charles River, Japan) were used. Female 8-week-old C57BL/6J mice were divided into three groups. One group was maintained on chow, a second was fasted for 48 h, and a third was fasted for 48 h followed by unrestricted access to chow for 48 h.

Statistical Analysis—All data are presented as mean ± S.E. as indicated. Statistical analyses of the characteristics of Zucker rats and Northern blot analyses were performed with a Mann-Whitney’s U test. Differences between mean values obtained for palmitate metabolism studies were determined by a Student’s t test. p < 0.05 was considered significant.

RESULTS

Identification of DARP—In the heart, energy metabolism is appreciably active and dynamic. Alteration of heart energy metabolism is reported in diabetes and insulin resistance model animals (17, 18). To isolate genes that are involved in energy metabolism, we have performed suppression-subtractive hybridization using the heart of the KKA mouse, a model mouse of type 2 diabetes and insulin resistance. Since the KKA mouse shows obesity and insulin resistance due to polygene impairment, there is no authentic normal control mouse with the same genetic background. Although the KK mouse is used as a control for the KKA mouse in several studies, the KK mouse shows mild diabetic phenotype. Thus, KK mouse is not an appropriate control mouse for our experiments, and we used the C57 mouse as a control.

We then successfully identified a novel gene, termed DARP, whose expression is up-regulated in KKA mouse heart as compared with C57 mouse heart (Fig. 1). Nucleotide homology search of the GenBank and 5'-RACE using mouse heart total RNA allowed us to isolate a full-length DARP cDNA. DARP encodes 306 amino acids containing putative nuclear localization signals and four tandem ankyrin (ANK)-like repeats. The amino acid sequence of DARP showed high similarity to cardiac ankyrin-repeat protein (CARP) and ankyrin-repeat domain 2 (Ankrd2) with 45 and 36% identities, respectively (Fig. 2A). We also isolated human DARP cDNA by RT-PCR using human skeletal muscle total RNA (Fig. 2B). Searching the GenBank of human DARP gene revealed that it is located at chromosome 2p11.1-2q11.1 where no genetic diseases are reported.

Expression of DARP in Eukaryotic Cells—Since DARP contains putative nuclear localization signals, we investigated
whether DARP is indeed a nuclear protein. We prepared CHO cells that stably express DARP with FLAG tag attached at its C terminus (CHO/DARP-FLAG). Western blot analysis of the cell lysate of CHO/DARP-FLAG demonstrated appropriate DARP expression in the size of 34.3 kDa, although nonspecific signals were observed in both CHO/DARP-FLAG and vector-transfected CHO cells (CHO/MOCK) (Fig. 4A). Immunocytochemistry of CHO/MOCK demonstrated relatively strong immunoreactivities dispersed diffusely, presumably due to nonspecific cross-reaction with anti-FLAG antibody (data not shown). We then performed Western blot analysis of nuclear and cytoplasmic fractions of CHO/DARP-FLAG as described. Appreciable amount of DARP expression was observed in the nuclear fraction, although a significant amount of protein still remained in the cytoplasmic fraction, presumably due to ongoing protein synthesis (Fig. 4B). To further confirm its nuclear localization, we performed Western blot analysis of nuclear and cytoplasmic fractions of COS-7 cells in which DARP-FLAG was transiently transfected (COS/DARP-FLAG). In COS/DARP-FLAG cells, we also detected the appreciable amount of DARP expression in the nuclear fraction (Fig. 4B). Immunocytochemistry of COS/DARP-FLAG cells demonstrated strong immunoreactivities in the nucleus, whereas no significant immunoreactivities were observed in vector-transfected COS-7 cells (COS/MOCK) (Fig. 4C). These findings indicate that DARP is a nuclear protein.

Fig. 2. Amino acid sequence of human and mouse DARP. A, amino acid sequence of mouse DARP aligned with mouse CARP and Ankrd2. Conserved residues are marked by closed boxes. Underlined residues are the four tandem ankyrin-like repeats. Putative nuclear localization signals are marked by asterisks. B, amino acid sequence of human DARP aligned with mouse DARP. Dots represent identical amino acids to those in human DARP. The sequences are available under GenBank accession number AF492400 and AF492401.

Fig. 3. Tissue distribution of mouse DARP. 15 μg of total RNA isolated from a wide variety of mouse tissues were subjected to Northern blot analysis. 28S rRNA is shown at the bottom.

Effects of DARP on Fatty Acid Metabolism—Recently, evi-
dence that abnormalities of fatty acid metabolism in skeletal muscle play crucial roles in the pathogenesis of insulin resistance is increasing. Abundant expression of DARP in skeletal muscle and its altered expression in the KKAy mouse led us to investigate its function in fatty acid metabolism. We prepared three individual stable transfectants of CHO cells that stably express DARP-FLAG (CHO/DARP-1, 2 and 3) and compared their [1-14C]palmitate metabolism to that of parental CHO cells (CHO/control). The expression of DARP-FLAG mRNA and protein were confirmed by Northern blot and Western blot analysis (Fig. 5, A and B). After 6 h of incubation in medium containing 40 μM [1-14C]palmitate, palmitate uptake, measured as the amount of radioactivity in the cells, slightly but significantly decreased in all three stable transfectants that express DARP-FLAG compared with control (Fig. 5C). On the other hand, palmitate oxidation in DARP-FLAG transfectants did not show significant differences from that of control cells (Fig. 5D).

DARP Expression Is Regulated by Energy Supply—We then examined the effect of exogenous energy supply on DARP expression. In skeletal myotube, addition of 250 μM oleate in differentiation medium containing glucose significantly increased DARP expression after 24 h of incubation, indicating that DARP expression is altered by exogenous energy supply in vitro (Fig. 6A). There was no significant morphological change after addition of oleate, and the expression of acetylcholine receptor α subunit (AchRα), a marker of differentiated myo-

![Fig. 4. Western blot analysis and immunocytochemistry of CHO/DARP-FLAG and COS/DARP-FLAG cells. A, CHO/DARP-FLAG cell lysate was subjected to Western blot analysis using anti-FLAG M2 antibody. B, nuclear (N) and cytoplasmic (C) fractions of CHO/DARP-FLAG and COS/DARP-FLAG cells were prepared as described under “Experimental Procedures,” and 30 μg of each fraction were subjected to Western blot analysis using anti-FLAG M2 antibody. C, immunocytochemistry of COS/DARP-FLAG and COS/MOCK using anti-FLAG M2 antibody.

![Fig. 5. Effect of DARP expression on [1-14C]palmitate metabolism. A, total RNAs were extracted from each stable transfectant and subjected to Northern blot analysis. All three transfectants express DARP-FLAG mRNA, whereas no expression of DARP was detected in parental CHO cell. B, cell lysate of each stable transfectant was subjected to Western blot analysis using anti-FLAG M2 antibody. All three transfectants express DARP-FLAG protein. C, palmitate uptake measured as the amount of radioactivity in CHO/DARP and CHO/control cells incubated with [1-14C]palmitate as described under “Experimental Procedures.” Counts per minute was determined by scintillation counting and was normalized to the value of CHO/control cells (n = 10). Mean ± S.E. are shown; *, p < 0.005 versus CHO/control; and **, p < 0.005 versus CHO/control. D, total 14CO2 produced from [1-14C]palmitate in CHO/DARP and CHO/control cells (n = 9 for control group and n = 5 for each CHO/DARP group). Palmitate oxidation did not show any significant difference between groups.

![Fig. 6. Western blot analysis and immunocytochemistry of CHO/DARP-FLAG and COS/DARP-FLAG cells. A, CHO/DARP-FLAG cell lysate was subjected to Western blot analysis using anti-FLAG M2 antibody. B, nuclear (N) and cytoplasmic (C) fractions of CHO/DARP-FLAG and COS/DARP-FLAG cells were prepared as described under “Experimental Procedures,” and 30 μg of each fraction were subjected to Western blot analysis using anti-FLAG M2 antibody. C, immunocytochemistry of COS/DARP-FLAG and COS/MOCK using anti-FLAG M2 antibody.](image)
increased DARP expression in skeletal muscle to an even higher level than that of control mice without fasting (Fig. 6B). However, fasting showed no significant effects on DARP expression in either the heart or brown adipose tissue (data not shown). These observations indicate that DARP expression is, at least partially, regulated by the energy supply in skeletal muscle both in vitro and in vivo.

**DARP Expression Is Altered in Insulin Resistance Model Animals**—We cannot exclude the possibility that enhanced DARP expression in KKAy mouse heart is due to the difference of genetic background between KKAy and C57 mice. Therefore, we examined DARP expression in another type 2 diabetes and insulin resistance model animal, Zucker fatty rats. Zucker fatty rats showed significantly higher body weight and plasma insulin level than those of Zucker lean control rats, whereas the blood glucose of both groups was not significantly different.

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**Fig. 6.** DARP expression is regulated by energy supply. A, 30 μg of total RNA isolated from skeletal myotube incubated with or without 250 μM oleate for 24 h were subjected to Northern blot analysis of DARP and AchRα. The same blot was stripped and reprobed for β-actin mRNA. B, 10 μg of total RNA extracted from skeletal muscle of mice with unrestricted feeding, fasted, or fasted and then refed were subjected to Northern blot analysis. Radioactivities of DARP mRNA signals were normalized with actin signal (n = 5 for control and fasted group, and n = 6 for fasted and then refed group). Values (mean ± S.E.) are presented as a percent of control (100%). *p < 0.01 versus control; **p < 0.05 versus fasted group; and ***p < 0.05 versus control.

**Fig. 7.** DARP expression is altered in insulin resistance model animals. 20 μg of total RNA from the heart (A) and brown adipose (C) and 5 μg of total RNA from skeletal muscle (B) of Zucker fatty rats and Zucker lean rats were subjected to Northern blot analysis of DARP. The same blots were stripped and reprobed with β-actin probe. Radioactivities of DARP mRNA signals were normalized with actin signal. Values (mean ± S.E.) are presented as a percent of lean control (100%) (n = 5 for each group); *p < 0.05 versus lean rats; and **p < 0.01 versus lean rats.

**Table I**

| Characteristics of Zucker lean and fatty rats |
|----------------------------------------------|
| Body weight | Blood glucose | Plasma insulin |
|-------------|---------------|----------------|
| g           | mg/dl         | ng/ml          |
| Zucker lean rat | 212.40 ± 2.68 | 179.20 ± 34.42 | 2.00 ± 0.52 |
| Zucker fatty rat | 283.40 ± 2.11* | 185.60 ± 18.91 | 9.66 ± 2.03* |

* Data are mean ± S.E. (n = five for each group); *p < 0.01 versus lean rat.
indicating that they are appropriate model animal for insulin resistance (Table I). DARP expression in heart and skeletal muscle was significantly higher in Zucker fatty rats than Zucker lean control rats (Fig. 7, A and B). In contrast, DARP expression in brown adipose was significantly lower in Zucker fatty rats as compared with that of control rats (Fig. 7C). These results indicate that DARP expression is indeed altered in insulin resistance animals.

**DISCUSSION**

We have described the cloning and characterization of DARP, a novel nuclear protein, whose mRNA expression is altered in type 2 diabetes and insulin resistance model animals. From the data presented in this manuscript, we are unable to determine whether DARP has a clear function in free fatty acid metabolism. However, its restricted expression in heart, skeletal muscle, and brown adipose and relevance to fatty acid metabolism suggest that analysis of DARP may reveal new insights in the energy metabolism.

Amino acid sequencing of DARP revealed that it contains putative nuclear localization signals and four tandem ANK-like repeats, sharing high homology with CARP and Ankrd2. CARP was initially identified as a cytokine-inducible nuclear protein from human endothelial cells (19). Later, CARP was reported to be a downstream molecule in the Nkx2–5 homeobox gene pathway in cardiomyocyte (20) and to be a downstream target of TGF-β/Smad signaling in vascular smooth muscle cell (21). However, its physiological function is still unclear. Ankrd2 was identified from mouse skeletal muscle as a gene putatively responsible for stretch-induced muscle hypertrophy (22, 23). Their identical structural features are nuclear localization signals and the ANK repeat motif. Although ANK repeats were initially reported to mediate protein-protein interactions, their function is more diverse. ANK repeat proteins carry out a wide variety of biological activities, and this motif has been recognized in more than 400 proteins including cyclin-dependent kinase inhibitors, transcriptional regulators, cytoskeletal organizers, developmental regulators, and toxins (24). Thus, the ANK repeat motif does not determine the specific function of DARP, although it may play key roles in DARP function.

Immunocytochemistry of COS/DARP-FLAG cells and Western blot analysis of nuclear and cytoplasmic fractions of COS/DARP-FLAG and CHO/DARP-FLAG demonstrated that DARP is a nuclear protein. Its nuclear localization suggests that DARP may play a role in the regulation of gene expression. It was reported that subcellular localization of CARP is altered by a change of circumstance of the cell, such as serum depletion in vitro (20). Since the function of protein is sometimes regulated by its subcellular localization (25, 26), detailed analysis of DARP localization may provide important clues to clarify the physiological function of DARP.

DARP expression in CHO cells caused a slight but significant decrease of palmitate uptake, suggesting that DARP may be involved in fatty acid metabolism. However, in CHO cells, it appeared that the effect of DARP on fatty acid metabolism was not enhanced proportionately to its expression level. Because DARP possesses ANK-like repeats that may mediate protein-protein interaction, DARP may functionally require partner molecule(s). Therefore, over-expression of DARP in CHO cells may not be sufficient. However, our results strongly suggest that DARP is potentially involved in fatty acid metabolism. Further experiments are required to elucidate the detailed mechanism of DARP effect on fatty acid metabolism.

Because skeletal muscle is the principal tissue for insulin-mediated glucose disposal and a major site of peripheral insulin resistance, the correlation between skeletal muscle fuel handling and insulin resistance has been extensively investigated. Recent studies revealed that skeletal muscle in insulin resistance shows increased glucose oxidation and decreased fatty acid oxidation under basal conditions and decreased glucose oxidation and increased fatty acid oxidation under insulin-stimulated conditions. This is referred to as a state of "metabolic inflexibility" (4). Decreased fatty acid oxidation under basal conditions could lead to lipid accumulation within skeletal muscle that is strongly associated with insulin resistance. Since DARP-expressing CHO cells demonstrated a significant decrease of palmitate uptake, DARP expression may be up-regulated in insulin-resistant animals to partially compensate for abnormalities in fatty acid accumulation in skeletal muscle. However, further analyses are required to address this point.

DARP expression is altered by a change of energy supply and energy metabolic condition, induced by excess fatty acid treatment in vitro and fasting in vivo. Initially, we expected that fasting would enhance the expression of DARP as well as excess fatty acid treatment in vitro since fasting was shown to increase plasma fatty acid level (27). Unexpectedly, fasting resulted in a decrease in DARP expression. This could be due to a significantly reduced glucose supply under fasted conditions. Although detailed mechanisms are unknown, our observations suggest that DARP expression is, at least partially, regulated by energy supply. Since energy supply appreciably affects energy metabolism (27, 28), these findings further suggest that DARP is implicated in energy metabolism. To clarify the physiological function of DARP, gene targeting and transgenic animal studies will likely be required. Detailed analysis of DARP will provide new insights of energy metabolism and crucial information as to the molecular regulatory mechanisms of energy metabolism.

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