Adenylate Kinase 1 Deficiency Induces Molecular and Structural Adaptations to Support Muscle Energy Metabolism*

Edwin Janssen‡, Ad de Groof‡, Mietske Wijers‡, Jack Fransen‡, Petras P. Dzeja§, Andre Terzic§, and Bé Wieringa‡¶

From the ‡Department of Cell Biology, Nijmegen Center for Molecular Life Sciences, University Medical Center, University of Nijmegen, The Netherlands and the §Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology, and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota 55905

Genetic ablation of adenylate kinase 1 (AK1), a member of the AK family of phosphotransfer enzymes, disturbs muscle energetic economy and decreases tolerance to metabolic stress, despite rearrangements in alternative high energy phosphoryl transfer pathways. To define the mechanisms of this adaptive response, soleus and gastrocnemius muscles from $AK1^{-/-}$ mice were characterized by cDNA array profiling, Western blot and ultrastructural analysis. We demonstrate that AK1 deficiency induces fiber-type specific variation in groups of transcripts involved in glycolysis and mitochondrial metabolism and in gene products defining structural and myogenic events. This was associated with increased phosphotransfer capacities of the glycolytic enzymes pyruvate kinase and 3-phosphoglycerate kinase. Moreover, in $AK1^{-/-}$ mice, fast-twitch gastrocnemius, but not slow-twitch soleus, had an increase in adenine nucleotide translocator (ANT) and mitochondrial creatine kinase protein, along with a doubling of the intermyofibrillar mitochondrial volume. These results provide molecular evidence for wide-scale remodeling in AK1-deficient muscles aimed at preservation of efficient energetic communication between ATP producing and utilizing cellular sites.

The metabolic status of skeletal muscle is reciprocally linked to fiber-type composition and functional demand. This implies that myocytes must undergo constant reprogramming of their gene expression in response to fluctuations in intrinsic or extrinsic physiological signals such as intracellular Ca^{2+} concentrations, hormonal stimulation, or altered workload (1–3). Moreover, disturbance of cellular energetics by metabolic inhibitors or genetic mutation may also induce alterations in the muscle phenotype via changes in the gene program for fiber-type specification (4–6). The high plasticity in the responsiveness of muscle is exemplified by myosin isoform transitions, mitochondrial division, and alterations in gene expression of enzymes involved in oxidative and glycolytic pathways (3, 7–10). Although many other aspects of myocyte infrastructure may change in concert, the molecular mechanisms regulating reprogramming of muscle energetics are still unknown.

Indeed, an important unresolved issue is the relationship between muscle design and metabolic pathways maintaining cellular energy homeostasis. Adenylate kinases (AK,[1] EC 2.7.4.3) are evolutionary strongly conserved enzymes that catalyze the reaction ATP + AMP $\leftrightharpoons$ 2 ADP. This reaction is one of the principal steps in adenine nucleotide metabolism and high energy phosphoryl ($\sim$P) transfer in the cellular bioenergetic network (11–13). Among several AK isoenzymes found in mammals, skeletal muscle is particularly rich in AK1, the major cytosolic isoform (14).

Along with the AK circuit, the creatine kinase (CK, EC 2.7.3.2)/phosphocreatine (PCr), nucleoside diphosphokinase (NDPK or nm23, EC 2.7.4.6), and glycolytic phosphotransfer systems coexist in skeletal muscle and serve to balance adenylates at ATP-consuming and ATP-generating intracellular sites (4, 13, 15–17). The relative importance of the AK, CK, and glycolytic phosphotransfer system is muscle fiber-type-dependent (3, 18, 19). For example, the CK/PCr phosphotransfer circuit is most active in fast-twitch fibers as demonstrated by a high content of PCr and high levels of cytosolic muscle-type (M)-CK. Slow-twitch fibers exhibit lower amounts of PCr and M-CK, but because of the high mitochondrial content possess relatively large amounts of ScCKmit (20). Expression of glycolytic proteins is most abundant in fast fibers and the mechanisms by which glycolytic genes are collectively or individually activated have in part been identified (21). In fact, the redox cofactors NAD(P)H and NAD(P)$^+$, which couple glycolysis or the pentose phosphate cycle to the Krebs cycle and oxidative phosphorylation in mitochondria, are important regulators of glycolytic gene transcription (22). Other coupling exists with calcium calcineurin (1), calcium/calmodulin-dependent protein kinase (9, 10), and AMP-activated kinase (AMPK) signaling (6), which regulate myocyte programs for mitochondrial biogenesis and glycolytic machinery. The mechanism underlying the regulation and distribution of AK and NDPK gene products in specific fiber-types of muscle is less clear.

We have recently demonstrated that inactivation of the AK circuit induces flux redistribution in the cellular phosphotransfer network, associated with an elevated glycolytic metabolism (23). Preserved muscle function, albeit with lower efficiency, in these animals suggests metabolic and cellular adaptations induced by genetic stress associated with AK deficiency. Indeed,

¶ To whom correspondence should be addressed: Dept. of Cell Biology, NCMLS University Medical Center, University of Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel.: 31-24-3614329/3614287; Fax: 31-24-3615317; E-mail: b.wieringa@ncmls.kun.nl.

[1] The abbreviations used are: AK, adenylate kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CK, creatine kinase; M-CK, muscle-type CK; RLU, relative light unit; A.U., arbitrary units; PK, pyruvate kinase; PGK, 3-phosphoglycerate kinase; MHC, myosin heavy chain; LDH, lactate dehydrogenase; MLC, myosin light chain; GPDH, glycerol-3-phosphate dehydrogenase; NDPK, nucleoside diphosphokinase; GDH, glutamate dehydrogenase.

we here uncovered a coherent reprogramming in the genetic and molecular profile of soleus and gastrocnemius muscles from mice lacking AK1. These adaptations occur at the mRNA and/or protein level and could support energy metabolism and performance in muscles compromised by AK1 deficiency.

MATERIALS AND METHODS

AK1 Knockout Mice—Gene-targeted mice carrying a HygroB[R] replacement mutation in the exon-3–5 region of the *AK1* gene were derived as described in detail elsewhere (23). Age and sex matched (3–5 months old; born on identical dates) homozygous AK1-deficient and wild-type control animals (both with 50–50% C57BL/6 × 129/Ola mixed inbred background) were used throughout experiments. Housing conditions were kept exactly identical to exclude effects of variations in (steroid) hormone levels, or food supply. Growth rates of wild-type and AK1 knockout mice were similar, resulting in body weights varying between 26–28 and 22–24 g for 3–5-month-old males and females, respectively. The investigation conformed to the Guidelines for the Care and Use of Laboratory Animals of the Dutch Council and was approved by the Institutional Animal Care and Use Committee at the University of Nijmegen.

Macroarray Hybridization—Total RNA from freshly isolated skeletal gastrocnemius and soleus muscles was extracted using the lithium chloride-urea method (23). Macroarrays were prepared by spotting individual plasmids with cDNA insert (134 different mouse sequences for glycolytic enzymes, mitochondrial enzymes and transporters, fatty acid metabolism enzymes, muscle regulatory factors, proteins active in Ca^{2+} signaling, glucose transport, tissue oxygenation as well as cytoskeletal components and transcription factors (see www.ncmls.kun.nl/celbio/data.htm for detailed information) onto Hybond N[+] membranes using a gridding robot (24). ^{32}P-labeled single-stranded cDNA was used as probe to hybridize membranes with gridded cDNA arrays as previously described in detail elsewhere (24). Four AK1 knockout and control mice were analyzed and statistically compared.

Data Analysis—Acquisition of radioactive signals from the gridded arrays was performed on a Bio-Rad GS 363 phosphorimager using the Molecular Analyst software from Bio-Rad. Because the signal intensity varied widely, membranes were exposed for 30 min (detection of abundant RNA) and 16 h (non-abundant RNA) and hybridization levels for individual cDNAs expressed as the mean Arbitrary Units (A.U.) of duplicate spots. Signals were divided by the value for the sum of detectable signals, resulting in the identification of an unaffected subset of genes that were used for final normalization.

Western Blot Analysis—Gastrocnemius and soleus muscles excised from the hind legs of 3 mice were pooled. Protein extracts were prepared and analyzed as described (25). For Western blot analysis, extracted proteins were separated on 10% SDS-polyacrylamide gels and proteins electrophoretically transferred onto nitrocellulose membranes. Specific proteins were detected using antibodies raised against species-specific protein or synthetic peptides of corresponding proteins as described elsewhere (25). After image acquisition with a densitometer (Bio-Rad GS-690), proteins were quantified using the Molecular Analyst software from Bio-Rad. For correlation, intensities of signals were compared with intensities of signals in the C57BL/6 calibration series. To confirm equal loading of protein a representative gel was stained with Coomassie Brilliant Blue (R-250) and, after immunodetection, blots were stained with Ponceau S.

Northern Blot Analysis—RNA blots were prepared as described (23) and hybridized with ^{32}P-labeled cDNAs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), muscle pyruvate kinase (PK-M), β-enolase, MLC-2s, ScCKmit, GLUT4, and α-actin as probe (probe length, 0.5–2 kbp). Hybridization was carried out overnight at 68 °C in 0.5 M $NaPO_4$ buffer, pH 7.2, 7% (w/v) SDS, 1 mM EDTA, and blots were washed to a final stringency of 0.1× SSC/1% (w/v) SDS at 68 °C and exposed to Kodak X-Omat films. Signals were quantified by phosphorimager analysis as described above. Per genotype, four animals were analyzed (independently from macroarray experiments) and statistically compared.

Enzyme Activity—Protein extracts were prepared from frozen powdered muscles as described in detail elsewhere (26). AK activity in supernatant, expressed as relative light units (RLU)/min/mg protein, was measured by a luciferase-based ATP bioluminescence assay (Sigma) in the presence of 2 mM ADP using a BioOrbit 1253 luminometer. NDPK activity, expressed as RLU/min/mg protein, was determined with the same bioluminescence assay in the presence of the AK inhibitor, diadenosine pentaphosphate (Ap_5A; 200 μM), and the reaction started with dGTP (2 mM). Three animals per genotype were analyzed and statistically compared. The activity of GAPDH, PK, and 3-phospho-

TABLE I
Differential mRNA expression in gastrocnemius and soleus skeletal muscle

Data are expressed in arbitrary units as mean ± S.E. ($n = 4$): $p < 0.01$.

| EST/cDNA	Gastrocnemius	Soleus
GDH	0.23 ± 0.02	0.07 ± 0.02
Tfam	0.31 ± 0.04	0.08 ± 0.04
Glutamine synthetase	0.54 ± 0.05	0.17 ± 0.03
GPDH	0.97 ± 0.07	0.21 ± 0.03
B-CK	1.0 ± 0.2	0.36 ± 0.08
PGK	1.08 ± 0.09	0.40 ± 0.07
GAPDH	1.3 ± 0.2	0.4 ± 0.1
LDH-A	2.4 ± 0.4	0.91 ± 0.06
AK1	3.0 ± 0.3	1.4 ± 0.2
Glycogen phosphorylase B	3.3 ± 0.2	1.06 ± 0.09
Aldolase A	14.1 ± 0.5	4.5 ± 0.4
β-Enolase	13.4 ± 1.1	4.7 ± 0.2
M-CK	73 ± 8	20 ± 1
Serca2	0.4 ± 0.1	2.3 ± 0.1
MLC-2s; partial cDNA	0.55 ± 0.04	3.7 ± 0.7
MLC-1s/v	0.8 ± 0.2	6.6 ± 1.0
MLC-2s	1.1 ± 0.1	6.9 ± 1.0
Myoglobin	0.9 ± 0.1	3.5 ± 1.0
PiC	1.06 ± 0.06	2.1 ± 0.3
ScCKmit	2.1 ± 0.2	4.5 ± 0.5

glycerate kinase (PGK) were measured using coupled enzyme assays (27). Reactions were started by the addition of 10 μl (GAPDH and PGK) or 20 μl (PK) of muscle extract and activities spectrophotometrically recorded at 340 nm with a Beckman DU 7400 spectrophotometer. Per genotype, five animals were analyzed and statistically compared.

Electron Microscopy and Morphometric Analysis—Mice were anesthetized with 2,2,2,-tribromorthanol (350 mg/kg intraperitoneal) and the GPS muscle complex was fixed using a clamp and dissected during immersion fixation with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Subsequently, muscles were prepared and examined on a JEOL JEM1010 electron microscope (4, 15). Intermyofibrillar mitochondrial volumes in superficial gastrocnemius muscle were estimated from electron micrographs at a magnification of ×8000 by point counting. For each individual muscle, at least three randomly taken micrographs were analyzed. Per genotype, six animals were measured and statistically compared.

Statistics—Data are presented as mean ± S.E. Student's *t* test for unpaired samples was used for statistical analysis, and $p < 0.05$ was considered significant.

RESULTS

mRNA Profiles of Fast-twitch and Slow-twitch Fibers—Prior to typing the cellular and molecular adaptations that are evoked as a response to AK1 deficiency we established the normal gene expression characteristics of two distinctly different skeletal muscle types, gastrocnemius and soleus muscles. These muscles can be considered the archetypes of fast and slow skeletal muscle in the mouse hind leg (19), for which differences in contractile performance and metabolic design are mirrored in their transcription profiles (1, 3). Using a customized cDNA macroarray assay and experimental conditions that avoid mRNA amplification (24) a selective subset of mouse-muscle cDNAs was probed for their potential to discriminate between gastrocnemius and soleus. Ultimately, 86 candidate cDNAs were chosen that belonged to different functional clusters, gave good signal to noise ratio, and were considered suitable indicators for inter- and intramuscular comparison. About 65% of the signals (56 signals) represented mRNAs with similar expression levels in soleus and gastrocnemius muscles. The remaining signals (30 signals) represented mRNAs with expression levels that were significantly different ($p < 0.05$; $n = 4$). Transcripts with expression levels that differed by a magnitude of 2 or more ($p < 0.01$; $n = 4$) between these two muscles are shown in Table I and Fig. 1. Quantitative data for the remaining 10 mRNAs, which differed by a magnitude less than 2 can be found at www.ncmls.kun.nl/celbio/data.htm.

A

B
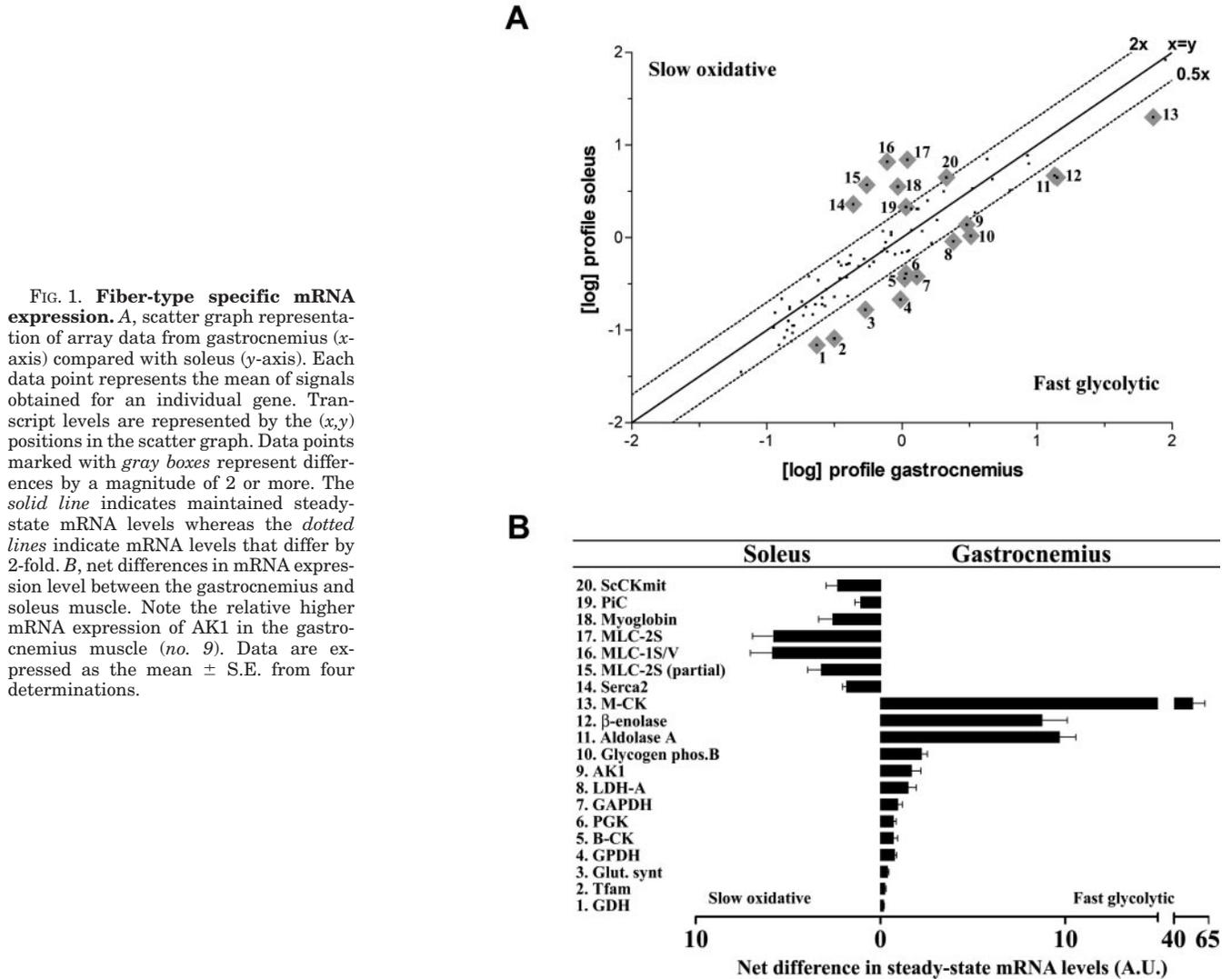
FIG. 1. **Fiber-type specific mRNA expression.** *A*, scatter graph representation of array data from gastrocnemius (*x*-axis) compared with soleus (*y*-axis). Each data point represents the mean of signals obtained for an individual gene. Transcript levels are represented by the (*x,y*) positions in the scatter graph. Data points marked with *gray boxes* represent differences by a magnitude of 2 or more. The *solid line* indicates maintained steady-state mRNA levels whereas the *dotted lines* indicate mRNA levels that differ by 2-fold. *B*, net differences in mRNA expression level between the gastrocnemius and soleus muscle. Note the relative higher mRNA expression of AK1 in the gastrocnemius muscle (*no. 9*). Data are expressed as the mean ± S.E. from four determinations.

Differential regulation of energy generation, consumption and distribution pathways is central to the determination of the nature and function of the muscle fiber (1–3). We therefore assessed the expression level of mRNAs for several key enzymes in these pathways. The relative abundance of AK1 mRNA was 2.2-fold higher in the gastrocnemius muscle compared with the soleus muscle (Fig. 2*A*). This result was confirmed by the observation that AK-catalyzed phosphotransfer activity was ~3-fold higher in gastrocnemius muscle than in soleus muscle (Fig. 2*A*). Also the composition and function of the CK phosphotransfer system depends on the specific muscle type (20). Our macroarray analysis showed that M-CK mRNA is 3.7-fold higher expressed in gastrocnemius compared with the soleus muscle. In contrast, the ScCKmit gene expression level is 2.1-fold higher in soleus muscle (Fig. 2*B*). Still, the ScCKmit mRNA level was only 3 and 23% of that of M-CK mRNA in gastrocnemius and soleus muscle, respectively. Subsequent comparison of M-CK and ScCKmit protein levels in soleus and gastrocnemius muscle by means of quantitative blot analysis, using specific antibodies directed against ScCKmit and CK-MM (Fig. 2*B*, *inset*), indicated that mRNA and protein product levels were well correlated. When normalized to total protein content, the M-CK protein level appeared 1.8-fold higher in gastrocnemius than in soleus, while the ScCKmit protein level was 2.7-fold higher in soleus than in gastrocnemius muscle.

Along with the AK- and CK-catalyzed high-energy phospho-

ryl transfer, the NDPK phosphotransfer pathway warrants transport and distribution of ~P over nucleotide di- and triphosphates (13, 16, 17). The expression level of different NDPK isoforms present on the gridded membranes (nm23-M1, nm23-M2, nm23-M4 and the human homologue of mouse nm23-M3, DR-nm23), did not differ more than 1.2-fold between gastrocnemius and soleus muscles. Taken into account that the expression of the nm23-M2 isoform was the highest in skeletal muscle (data not shown), this finding was corroborated by the observation that there was a good correlation between the transcript level for nm23-M2, and the accompanying total NDPK activity (Fig. 2*C*). The total NDPK activity was not significantly different between soleus and gastrocnemius. Thus, whereas NDPK gene product and activity levels are equally maintained in soleus and gastrocnemius muscle, AK- and CK-mediated phosphoryl transfer activity is clearly dependent on muscle phenotype.

The sarcomeric myosin molecule is a hexamer consisting of two myosin heavy chains (MHCs), two essential myosin light chains (ELCs), and two regulatory myosin light chains (RLCs). Because myofibrillar protein isoforms generally show tissue-specific distribution these proteins may serve as useful markers for skeletal muscle fiber typing. As anticipated on the basis of a slow or fast-twitch muscle fiber (3), the expression level of genes encoding the MLC-1s/v and MLC-2s mRNAs differed 6–8-fold between gastrocnemius and soleus muscle (Table I and Fig. 1). Also the signals for the mRNAs for mitochondrial
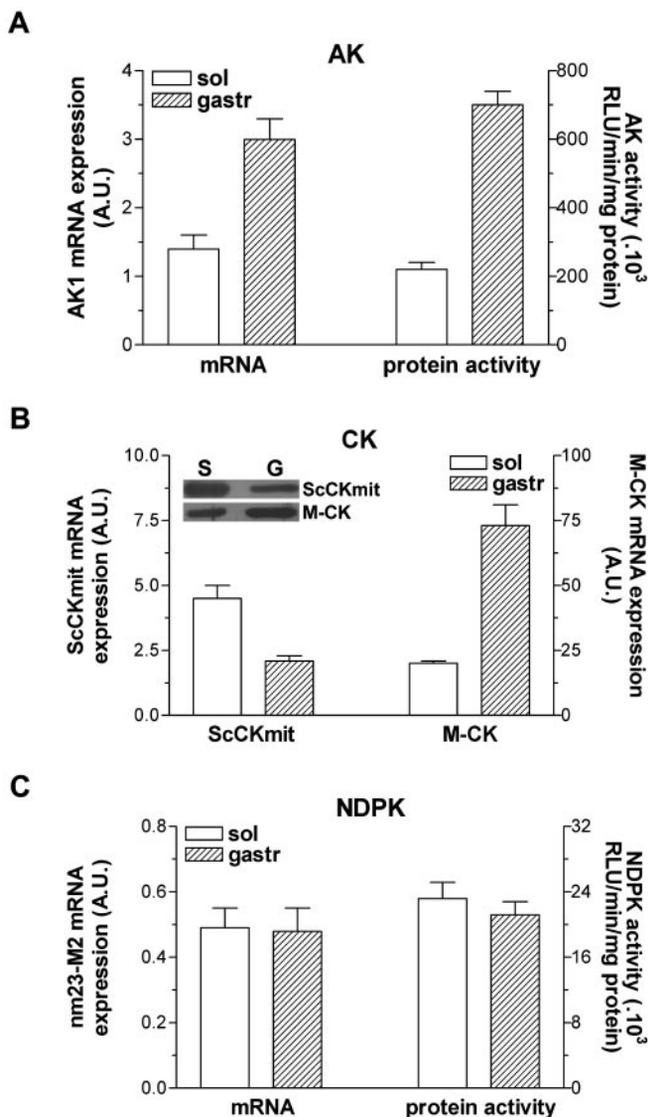
FIG. 2. **Fiber-type distribution of AK, CK, and NDPK.** *A*, relative abundance of AK1 transcript and total AK enzymatic activity in soleus and gastrocnemius muscles. *B*, relative abundance of ScCKmit and M-CK in soleus and gastrocnemius muscles. The *inset* shows a Western blot of immunoreactive ScCKmit and M-CK from soleus (*S*) and gastrocnemius (*G*) muscles. *C*, relative abundance of NDPK-M2 transcript and total NDPK enzymatic activity in soleus and gastrocnemius muscles. Data are expressed as the mean ± S.E. from three or four determinations.

TABLE II
Transcript profiling in wild-type and $AK1^{-/-}$ gastrocnemius muscle

EST/cDNA	Wild type[a]	AK1KO[a]
Gastrocnemius		
Glycolytic metabolism		
β-Enolase[b]	100 ± 11	143 ± 5
Pyruvate kinase M[b]	100 ± 2	134 ± 7
GPDH[b]	100 ± 6	147 ± 4
GAPDH[b]	100 ± 11	187 ± 7
Mitochondrial metabolism		
Glutamate dehydrogenase	100 ± 6	46 ± 19
Acyl-CoA dehydrogenase	100 ± 6	65 ± 11
Mono- or diphosphate kinases		
Adenylate kinase 1	100 ± 9	4 ± 1
DR-NM23 (NDPK-M3)	100 ± 17	214 ± 32
Structural		
Myosin light chain 2s	100 ± 10	63 ± 5
Myosin light chain 2s (partial)	100 ± 11	61 ± 8
Myogenesis		
MYF5	100 ± 35	327 ± 56
Soleus		
Glycolytic metabolism		
α-Enolase	100 ± 18	181 ± 13
Lactate dehydrogenase A	100 ± 6	77 ± 2
Monophosphate kinase		
Adenylate kinase 1	100 ± 12	5 ± 1

[a] Data are expressed as mean ± S.E. ($n = 4$); $p < 0.05$. Values for wild type were set to 100%.
[b] Data acquisition after 30-min exposure in phosphorimager.

inorganic phosphate carrier (PiC), Ca^{2+}-ATPase isoform Serca2, and myoglobin, of which the translation products are associated with oxidative metabolism, were 2.0-, 3.8-, and 5.2-fold higher in the soleus muscle, respectively (Table I and Fig. 1). Strikingly, the steady-state transcript level for the nuclear-encoded mitochondrial transcription factor Tfam (or mtTFA) was 3.9-fold higher in the glycolytic gastrocnemius muscle compared with the oxidative soleus muscle. This observation was surprising because the gastrocnemius is a mitochondria-poor muscle, but may be explained by the fact that cooperation of more than one factor is needed to regulate mitochondrial biogenesis (9, 10). Unexpectedly, relatively high steady-state B-CK transcript levels were detected in soleus and gastrocnemius muscle. This observation is most easily explained by the fact that immature muscle cells (*i.e.* satellite cells) actively transcribe the *B-CK* gene (15). As expected, signal intensities for mRNAs related to glycolysis (β-enolase, aldolase A, GAPDH, and PGK) or coupled pathways (glycogen phosphoryl-ase B, LDH-A, and GPDH) were higher in gastrocnemius than in soleus muscle (Table I and Fig. 1). Thus, we may conclude that the cDNA macroarray has sufficient discriminating power to distinguish mRNA profiles of fast and slow type muscles.

Differences in Gene Products for High Energy Phosphoryl Transfer in the Absence and Presence of AK1—Changes at the molecular level may occur when the muscle genotype or the integrity of various intrinsic or extrinsic physiological control mechanisms are disturbed (6, 9, 25, 28). To determine the spectrum of molecular changes related to the absence of AK1 we compared mRNA profiles of gastrocnemius and soleus, between wild-type and $AK1^{-/-}$ animals. Altogether, fourteen genes were identified for which the expression levels differed significantly between AK1-proficient and -deficient muscles (Table II and Fig. 3).

Knockout of AK1 produced a dramatic 96% decrease in the AK1 mRNA signal (Table II and Fig. 3, *A–B*) for gastrocnemius muscle. Likewise, soleus muscle of $AK1^{-/-}$ animals showed a 95% reduction in AK1 mRNA content (Table II and Fig. 3*C*). We surmised that transcript profiles of other enzymes involved in energy transfer pathways might be among the principal targets for adaptation to AK1 deficiency. Yet, M-CK transcript levels on the arrays of AK1 knockout gastrocnemius and soleus were not significantly different from wild type. The steady-state M-CK transcript level was respectively 88 ± 8 A.U. and 85 ± 12 A.U. in the wild type and AK1 knockout gastrocnemius muscle ($n = 4, p > 0.05$) and 16 ± 1 A.U and 16 ± 2 A.U. in the wild-type and AK1 knockout soleus muscle ($n = 4, p > 0.05$), respectively. Also the level of the mRNA for the mitochondrial CK isoform, ScCKmit, was unaffected (2.6 ± 0.1 *versus* 2.3 ± 0.1 A.U. in the gastrocnemius and 3.5 ± 0.2 *versus* 3.5 ± 0.6 A.U. in the soleus of wild-type and AK1-deficient mice, respectively; $n = 4, p > 0.05$). Thus, in response to *AK1* gene deletion, the levels of transcripts encoding the ScCKmit and M-CK isoforms are not changed. We observed, however, a 2-fold increase in the signal level for DR-nm23 mRNA in the absence of AK1 in gastrocnemius muscle (Table II and Fig. 3*B*). DR-nm23 mRNA level in soleus muscle did not differ by genotype. Total NDPK-catalyzed phosphoryl transfer capacity in the gastrocnemius and soleus muscles was not affected by *AK1* gene deletion (21 ±

FIG. 3. **Expression profiling of skeletal muscles from AK1$^{-/-}$ and wild-type mice.** *A*, autoradiograms of cDNA array profiles from wild-type and AK1 knockout gastrocnemius muscle. Total RNA was isolated, and first strand cDNA product was ^{32}P-labeled and used as hybridization probe. Signal intensities of individual spots were quantified by phosphorimager analysis and normalized to the sum of detectable signals. Note the absence of AK1 mRNA in AK1 knockout muscle (position B15). *B*, scatter graph for the comparison of transcript profiles between AK1 mutant and wild-type gastrocnemius muscles. Each data point represents the mean ± S.E. of signals obtained for an individual gene from four determinations. Transcript levels are represented by the (x,y) positions in the scatter graph. X-axis and y-axis represent wild-type and AK1 knockout RNA profiles, respectively. The *solid line* indicates maintained steady-state mRNA levels. Significant different mRNA levels are marked with *gray boxes*. *C*, scatter graph for comparison of transcript profiles between AK1 mutant and wild-type soleus muscles. *D*, Northern blots of total RNAs isolated from wild type ($n = 4$) and AK1 knockout ($n = 4$) gastrocnemius muscles. Specific probes for ScCKmit, GLUT4, GAPDH, PK-M, β-enolase, and α-actin were used for hybridization. RNA levels were quantified by phosphorimager analysis and normalized to the α-actin signal (see text).
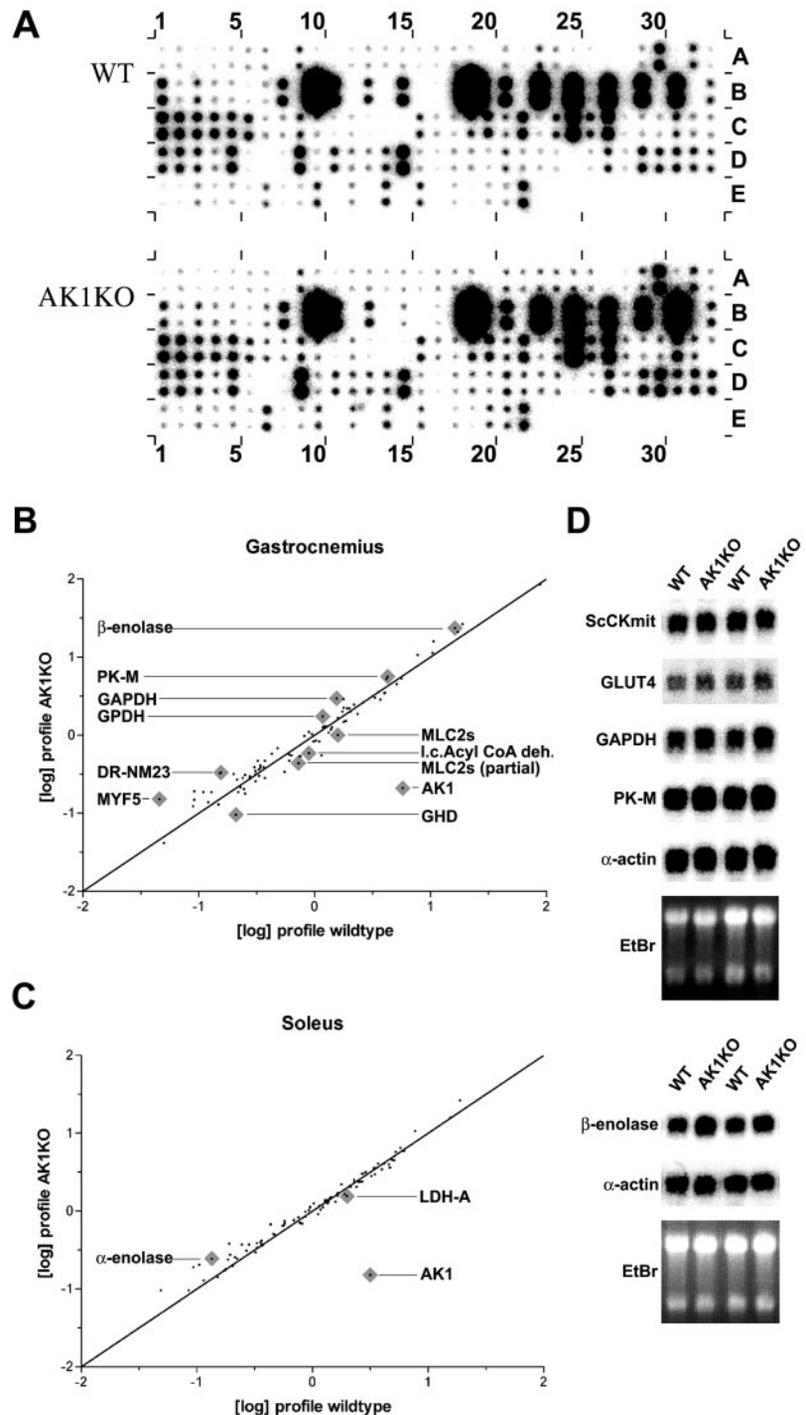
2 *versus* 20 ± 2 .10^3 RLU/min/mg protein and 23 ± 2 *versus* 27 ± 2 .10^3 RLU/min/mg protein in wild-type and AK1 knockout, respectively; $p > 0.05$, $n = 3$).

Adaptation in Glycolytic, Mitochondrial, and Structural Gene Product Levels—Several studies support the functional interaction of the AK circuit with the glycolytic machinery for energy production (13, 29–32). Previous studies showed that AK1$^{-/-}$ muscles have up-regulated glycolytic flux. In keeping with this finding, we observed that four of the affected signals in gastrocnemius represented mRNAs that encode enzymes acting in the glycolytic pathway. For muscle enolase, consisting of two subunits encoded by two distinct genes (enolase α and β), there was a significant increase in the steady-state level of the β-enolase mRNA in the gastrocnemius knockout muscle (Table II and Fig. 3*B*). In soleus muscle a similar increase, but now for α-enolase mRNA, was seen (Table II and Fig. 3*C*). As the β-enolase level is dependent on muscle energetic demand (33, 34), this result suggests a similar role for the α-enolase subunit in skeletal muscle. Similarly, the levels of mRNAs for PK-M, GAPDH, and GPDH were ~1.5-fold increased in mutant gastrocnemius compared with wild type (Table II and Fig. 3*B*). Also the PGK mRNA level tended to be greater (30%) in the AK1-deficient gastrocnemius muscle ($p < 0.08$). Conversely, there was a 23% decrease in the level of the mRNA for the tetrameric glycolytic enzyme lactate dehydrogenase (LDH), LDH-A, in soleus muscle in AK1$^{-/-}$ compared with wild type (Table II and Fig. 3*C*). Levels of mRNAs for PK-M, GAPDH, and GPDH in soleus and LDH-A in gastrocnemius did not

significantly differ between knockout and wild type muscles. Because the flux through glycolysis was increased in gastrocnemius-plantaris-soleus (GPS) muscle complex of AK1 knockout mice (23) we next raised the question whether the mRNA level for the insulin and AMPK-dependent glucose importer, GLUT4, was possibly altered. The steady-state GLUT4 transcript level appeared unaffected by AK1 absence in both gastrocnemius (1.8 ± 0.4 and 2.9 ± 0.7 A.U. in wild type and AK1 knockout, respectively; $p > 0.05$, $n = 4$) and soleus (1.4 ± 0.3 and 1.4 ± 0.4 A.U. in wild type and AK1 knockout, $p > 0.05$; $n = 4$) muscle.

In addition to glycolytic mRNAs also transcripts encoding enzymes in other related catabolic pathways were analyzed. In the glycolytic gastrocnemius muscle of AK1 knockouts we observed lower mRNA levels for long chain acyl-CoA dehydrogenase and glutamate dehydrogenase (35 and 54% reduction, respectively; Table II and Fig. 3B). Both messengers encode proteins that reside in the mitochondrial matrix and are involved in the oxidation of glutamate and fatty acids. In the oxidative soleus muscle these messenger signals were unaffected by the absence of AK1 (data not shown).

Based on the intramuscular comparison (Table I and Fig. 1) the regulatory cardiac/slow-twitch myosin light chain 2 (MLC-2s) may be considered a marker for oxidative fiber-types. In AK1 animals, signals representing the MLC-2s mRNA (full-length and partial) were downregulated by ~40% in the gastrocnemius muscle, but not affected in soleus muscles (Table II and Fig. 3B). This observation suggests that down-regulation of *MLC-2s* gene transcription is essential in evoking changes in isoenzyme composition for the regulatory MLCs. Interestingly, also the essential cardiac/slow-twitch MLC-1s/v transcript in gastrocnemius muscle inclined to a decrease in concentration (50% reduction; $p < 0.07$; $n = 4$).

Among the key factors involved in transcriptional control of myofibers are the myogenic basic helix-loop-helix (bHLH) transcription factors. Expression levels of MyoD and Myf5 strongly correlate with the fast muscle phenotype (35, 36), while myogenin has been shown to be associated with the slow muscle phenotype (37). In fast-twitch gastrocnemius muscles lacking AK1 the MYF5 transcript level was increased by more than 200% (Table II and Fig. 3B). Whereas signals of myogenin transcripts could not be reliably detected, the MyoD mRNA level tended to be up-regulated (73% with low significance $p < 0.09$; $n = 4$). In the soleus muscle no differences were observed for the bHLH transcription factors.

Northern blot analysis of newly isolated mRNA samples from gastrocnemius muscles independently confirmed the results from our macroarray experiments (Fig. 3D). Signals obtained with probes specific for ScCKmit and GLUT4 were at similar strength for wild-type and AK1-deficient muscles (0.68 ± 0.05 *versus* 0.68 ± 0.04 and 0.37 ± 0.02 *versus* 0.36 ± 0.02 A.U. in AK1KO and wild type, respectively; $p > 0.05$; $n = 4$). The intensities of mRNA bands for β-enolase and PK-M were 1.3- and 1.4-fold up-regulated in mutant gastrocnemius muscle (2.1 ± 0.1 *versus* 1.6 ± 0.1 and 1.01 ± 0.02 *versus* 0.71 ± 0.04 A.U. in AK1KO and wild type, respectively; $p < 0.03$; $n = 4$). Also the GAPDH mRNA signal was increased 1.4-fold in mutant gastrocnemius (0.46 ± 0.03 *versus* 0.34 ± 0.02 A.U. in AK1KO and wild type, respectively; $p < 0.02$; $n = 4$), but less pronounced than in the macroarray data set (1.8-fold increase). Use of the MLC-2s cDNA probe confirmed the earlier observed decrease in MLC-2s transcript level (signal intensity 0.8 ± 0.2 *versus* 1.5 ± 0.2 A.U. in AK1KO and wild type, respectively; $p = 0.05$; $n = 4$). Independent confirmation of the mRNA array data for soleus muscle was not achieved because the RNA yield from this muscle was too low for use in the Northern blot assay.

Increased Glycolytic Phosphotransfer Capacity in AK1 Knockout Skeletal Muscle—We next used protein-chemical and functional assays to establish the relationship between mRNA and protein levels and enzymatic activities, focusing on key phosphoryl transfer enzymes in the glycolytic pathway, PK, GAPDH, and PGK. Semiquantitative blot and enzymatic analysis of GAPDH showed maintained protein levels and activities for GAPDH in mutant skeletal muscle (Fig. 4A). The protein level for PK was increased 2-fold in the absence of AK1 and was paralleled by 2-fold increase in PK-catalyzed phosphotransfer capacity (Fig. 4, B–D). Thus, the increase in GAPDH mRNA level is not followed by an increase in GAPDH protein quantity and enzymatic activity. For the adaptive PK up-regulation in AK1$^{-/-}$ gastrocnemius muscle mRNA level, protein content as well as enzymatic activity appeared linearly coupled.

In addition to PK, PGK is the other principal phosphoryl transfer enzyme that produces ATP in the glycolytic pathway. PGK catalyzed phosphotransfer activity was increased 2-fold (Fig. 4E). Together, the increased steady-state levels for several glycolytic mRNA species and the more direct observation of increased enzymatic activity of PK and PGK, indicate that glycolysis-driven phosphoryl exchange between ADP and ATP may be one of the principal targets for adaptation in fast-twitch muscles of AK1$^{-/-}$ mice.

AK1-deficient Muscle Show Increased Mitochondrial Content—Based on theoretical considerations and experimental evidence it is now commonly accepted that the AK, CK, and glycolytic circuits may be intertwined with mitochondrial activity and together form integrated networks for high energy phosphoryl transfer at different subcellular locales (13, 16, 23, 32). As shown in Fig. 5A and *inset 4C*, the level of the cytosolic M-CK protein was not affected in skeletal muscle. We did, however, observe a 40% increase in the level of mitochondrial ScCKmit in the gastrocnemius muscle of AK1-deficient mice. Also, the signal for the mitochondrial adenine nucleotide transporter (ANT) was similarly increased. Similar results were obtained for another fast-twitch muscle, the psoas major muscle (data not shown). Intriguingly, the increase in steady-state protein levels of ANT and ScCKmit in AK1 knockout gastrocnemius muscle was not accompanied by a corresponding increase in mRNA level. The level of two other mitochondrial proteins, the inorganic phosphate carrier (PiC) and voltage-dependent anion channel (VDAC; porin), were not changed in AK1-deficient gastrocnemius muscle (data not shown). In mutant soleus muscle no significant alterations in the level of ANT and ScCKmit protein were detected.

To analyze whether the changes in mitochondrial marker proteins were also reflected in variation of mitochondrial density or appearance we applied EM-morphometric analysis. Indeed, a 2-fold increase in mitochondrial volume was detected in AK1-deficient gastrocnemius muscles (Fig. 5, B and C). Taken together, these findings indicate that in response to *AK1* gene deletion cell type-dependent adaptations occur to maintain cellular energetic homeostasis.

DISCUSSION

Previously we had demonstrated that inactivation of the *AK1* gene compromises economic efficiency of the cellular energetic network despite the fact that there is rewiring of fluxes through other pathways for metabolic energy transfer. These adaptive responses apparently ameliorate, but do not entirely obscure, the effects of loss of the AK1 phosphotransfer activity (23, 26, 38). Here we provide a detailed analysis of the molecular events underlying compensatory responses to muscle AK1 deficiency, demonstrating that regulation at the level of gene transcript abundance, enzymatic activity, as well as (re)orga-

A

GAPDH

FIG. 4. **Increased glycolytic phosphotransfer capacity in AK1$^{-/-}$ gastrocnemius skeletal muscle.** *A*, GAPDH activity in wild-type and AK1 knockout gastrocnemius muscles (*n* = 5 each). The *inset* illustrates a Western blot image of immunoreactive GAPDH in wild-type and AK1 knockout gastrocnemius muscle. *B*, densitometric analysis of Western blot image (*inset*) for PK-M. Protein extracts were pooled from three mice, electrophoretically separated on 10% SDS-polyacrylamide gels, and subjected to Western blot analysis. WT (○) and AK1KO (●) PK-M protein levels are indicated on the linear calibration plot. This plot serves to correlate signal intensities with known quantities of immunoreactive PK-M protein. Note the 2-fold-increased PK-M protein level in AK1KO (1.6) compared with wild type (0.8) gastrocnemius protein extracts. *C*, densitometric analysis of Western blot image for M-CK (*inset*). The blot shown in *B* was reprobed with the M-CK antibody. Note equal M-CK protein levels in wild-type (1.2) and AK1KO (1.2) gastrocnemius protein extracts. *D*, PK activity in wild-type and AK1 knockout gastrocnemius muscles (*n* = 5). *E*, PGK activity in wild-type and AK1 knockout gastrocnemius muscles (*n* = 5). *Asterisk* indicates significant difference between groups.

B PK-M

C M-CK

D PK-M

E PGK
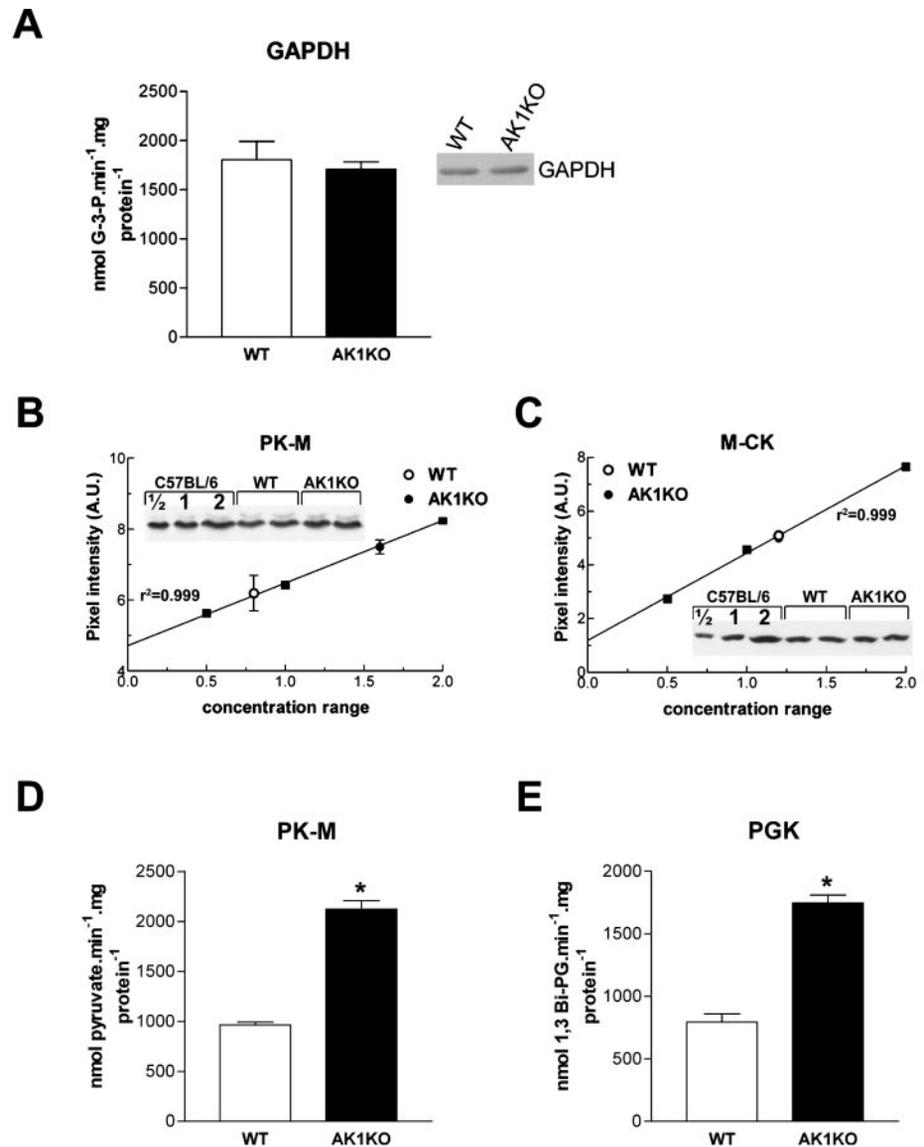
nization of the cellular ultrastructure is involved, with a signature to sustain cellular high energy phosphoryl generation and transfer capacity.

Although regulation of the *AK1* gene and its products has been described in several reports (23, 39), not much attention has been paid to the relevance for metabolic context or cell type requirements of AK-mediated phosphoryl exchange. We demonstrate here by mRNA profiling and biochemical activity measurement that AK1 transcript level is well correlated to enzymatic capacity (when normalized to total RNA or protein content) and is 2–3-fold higher in fast-twitch gastrocnemius compared with slow-twitch soleus muscle. This underscores the relative importance of AK catalysis for muscle that is relatively poor in mitochondria and highly dependent on glycolytic ATP production. Similar fiber-type specificity was noted for mRNA and protein products of the gene for muscle-type cytosolic creatine kinase, M-CK. Although we still do not fully understand how cytosolic AK and CK enzymes are integrated in the cellular energy network (13, 32), predominance in fast-twitch myocytes would fit to their role in protecting the cell from threshold effects of abnormal ATP/ADP/AMP ratios during transient periods of sudden and profound energy demand. No correlation to fiber-type was found for expression of members of the NDPK family of genes (nm23-M1, nm23-M2, nm23-M3/DR-nm23, and nm23-M4). Based on this finding it is tempting to speculate that NDPK-mediated phosphotransfer may feed high-energy phosphoryls into other metabolic pathways, not directly involved in energy homeostasis associated with muscle contraction.

Our array profiling demonstrated that AK1 absence caused a parallel, 1.5–2-fold, increase in the level of PK-M, β-enolase, GAPDH, PGK, and GPDH mRNAs in gastrocnemius. Northern blot quantification supported this finding for the first three mRNAs mentioned. This strongly points to concerted transcriptional regulation of these glycolytic genes. In concordance with our earlier findings (23) we surmise that up-regulation of mRNAs encoding glycolytic enzymes in gastrocnemius serves a general need for larger capacity of the glycolytic pathway in AK1$^{-/-}$ mutants. It is of note, therefore, that the contents of mRNAs for long chain acyl-CoA dehydrogenase and glutamate dehydrogenase were decreased. This could suggest that the production of Krebs cycle intermediates from metabolic pathways other than glycolysis may be down-regulated. Again this could be viewed as a direct adaptational effect that fits the general drift toward a more glycolytic profile.

Various regulatory circuits like the myogenic bHLH transcription factor family, calcineurin/CaMK/PGC-1/NFAT/MEF2, myogenic regulatory factor (MRF) activity, or NPAS2/BMAL pathways have now been identified that, together, may be implemented in the transcriptional regulation of cellular
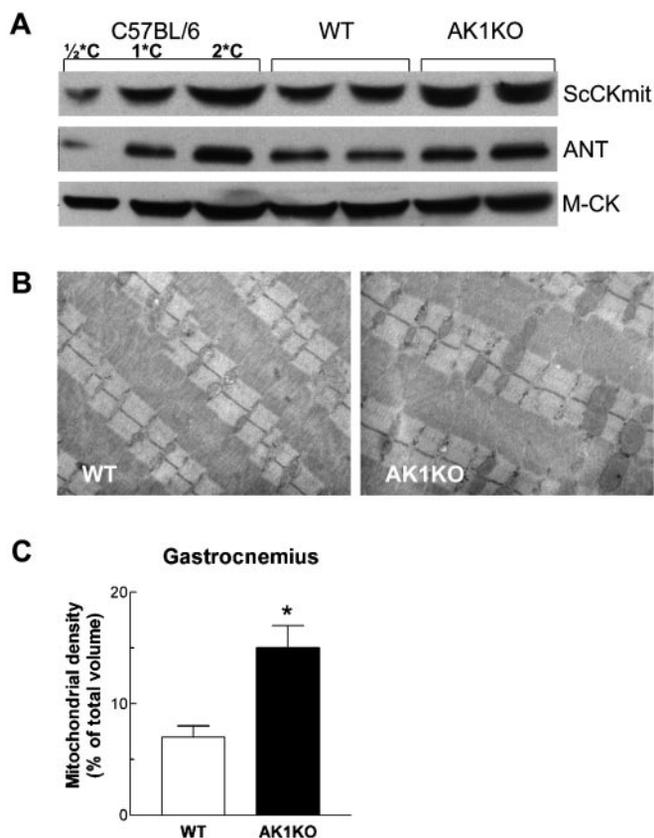
FIG. 5. **Increased mitochondrial content in AK1-deficient skeletal muscle.** *A*, Western blot images for ScCKmit, ANT, and M-CK. Protein extracts were pooled from three mice, electrophoretically separated on 10% SDS-polyacrylamide gels and subjected to Western blot analysis. Images were analyzed as described under "Materials and Methods." *B*, ultrastructural changes in gastrocnemius skeletal muscle from AK1-deficient mice. Electron micrographs of longitudinal sections through myofibers of the gastrocnemius muscle. Fast-twitch fibers of AK1 deficient mice display an increased intermyofibrillar mitochondrial volume. *C*, increased mitochondrial density in AK1-deficient gastrocnemius muscle. Mitochondrial volume density was assessed by point counting for six $AK1^{-/-}$ and control animals. *Asterisk* indicates significant difference between groups.

carbohydrate metabolism and ultrastructural design of myofibers (and other cell types as well) (1, 9, 10, 22, 35–37, 40). We found no change in abundance of mRNA for calcineurin in $AK1^{-/-}$ gastrocnemius and soleus, but this does not exclude a regulatory role for this enzyme, as the calcineurin pathway is mainly regulated at the level of factor relocation. We did observe, however, that the concentration of MYF5, a member of the bHLH family of myogenic transcription factors (TFs), was increased 3-fold. Also the level of another member, MyoD, was increased. Allen *et al.* (36) reported that MYF5 and MyoD preferentially activate the skeletal *MHCIIb* gene, specifying the myosin isoform that is highest expressed in the glycolytic fast-type fiber IIB. In parallel, a role for MyoD has been proposed in the maintenance of fast-fiber characteristics (35). Against this background, these TFs might be considered good candidates for being involved in the transition in glycolytic *versus* oxidative phenotype and the accompanying contractile properties of muscle design in our $AK1^{-/-}$ mice.

It is of note that different pathways for matching muscle infrastructure to conditions of metabolic stress might be effective in slow and fast type myofibers. In the highly oxidative soleus muscle, lack of AK1 produced an 80% increase in the steady-state level of α-enolase mRNA and down-regulated LDH-A mRNA levels with no changes in PK-M, GAPDH, and GPDH mRNAs levels. Intriguingly, recent findings indicate

that the α-enolase mRNA encodes two distinct proteins, α-enolase and Myc-binding protein (MBP)-1 protein, due to alternative usage of translation initiation sites (41). β-Enolase is involved in glycolysis, whereas the MBP-1 protein down-regulates c-Myc oncoprotein expression. Activation by c-Myc, in turn, can promote *LDH-A* gene transcription (42, 43). Increased production of the MBP-1 protein from the α-enolase mRNA would therefore be expected to result in down-regulation of c-Myc, and subsequent reduction in *LDH-A* gene activity. Although this is exactly what was observed in our array measurements, clearly more detailed study is necessary to see whether this explains our findings. Another hypothetical possibility would be that the redox state of NAD cofactors and its effect on the NPAS2/BMAL1 transcription machinery (22) is involved in the metabolic signaling and suppression of the LDH-A transcript level. We put this possibility forward because gastrocnemius muscles of $AK1^{-/-}$ mice show significant higher NAD^+ levels (20% increase).[2]

In validating our array quantification data with Northern and Western blot analyses we noticed that there was discordance between mRNA and protein levels for some genes. Most striking was that the almost 2-fold increase in GAPDH mRNA was not paralleled in GAPDH activity in muscle extract. Conversely, a relatively modest increase in PK-M mRNA level was accompanied by a 2-fold increase in PK-M content and activity. This suggests that the mechanism(s) of adaptive response may also include regulation at the rate of translation or protein turnover (44). Ultimately, shifts in isoenzyme composition, phosphorylation or protein complexation should therefore be taken into account. For example, skeletal muscle PK-catalyzed phosphotransfer activity can be increased upon binding with MM-CK resulting in an increased flux through PK, independently of its substrate concentrations (45). Interestingly, an up-regulation of GAPDH and/or PK mRNA, as we found here, has also been reported for heart and skeletal muscle in response to muscle disuse and ischemic stress (46, 47).

The adaptations in the glycolytic pathway, especially the phosphotransfer enzymes PK and PGK, should also be discussed in the context of a possible direct structural and functional association between AK1 and glycolytic enzymes. Indeed, AK1 can physically interact with phosphofructokinase and participate in the formation of a larger glycolytic enzyme complex or cluster (30, 31, 48). Whether this complex only serves to provide localized glycolytic ADP:ATP phosphotransfer capacity, presumably important for sustaining actomyosin sliding and force production, or also has any other structural-organizational function is currently unknown. Immuno-staining experiments have indicated that AK1 is present in distinct subcellular locales of skeletal muscle that coincide with localization of enzymes of the glycolytic apparatus (49). Adaptations, instead of serving a general role in cellular energetics, may therefore also have a role in guarding structural integrity of the glycolytic infrastructure of muscle.

Finally, it is important to note that AK1 is involved in the communication between myofibrillar ATPases and mitochondria (13, 27, 32, 50), thereby maintaining efficient intracellular energy flow (23). Translocation of ADP into mitochondria or release of ATP in the cytosol is achieved via translocator complexes composed of ANT, porin (VDAC) and octameric mitochondrial creatine kinase (51). Because diffusion of ADP is very limited in muscle cells (52) and the AK pathway for ADP (re)phosphorylation is depleted in $AK1^{-/-}$ gastrocnemius muscle, the observed 2-fold increase in mitochondrial volume, and concomitant increase in ANT and ScCKmit protein may serve

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[2] E. Janssen, unpublished observation.

to match the mitochondrial ADP import capacity with the increase in glycolytic flux. Apparently, post-translational regulation may be involved in this phenomenon, as the mRNA levels for ANT and ScCKmit were not accordingly adapted. Intriguingly, other mitochondrial import proteins like the PiC and VDAC were not increased. In this regard muscles of M-CK knockout mice have a similar pattern in molecular and cytoarchitectural adaptations (24, 25). In these mutants, ScCKmit and ANT1 mRNA levels were maintained whereas ScCKmit and ANT protein levels were dramatically increased. This was also paralleled by an increase in pyruvate kinase protein level and intermyofibrillar mitochondrial volume (24, 25). When combined, this suggests that the AK1- and M-CK-catalyzed phosphotransfer circuit may have functional redundancy as the deficiency for both circuits is sensed and counteracted in a highly similar manner.

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