Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 317 (2004) 285–293
BBRC
www.elsevier.com/locate/ybbrc

cDNA cloning and functional characterization of a novel splice variant of c-Cbl-associated protein from mouse skeletal muscle☆

Oscar Alcazar, Richard C. Ho, Nobuharu Fujii, and Laurie J. Goodyear*

The Research Division, Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02215, USA

Received 19 February 2004

Abstract

c-Cbl-associated protein (CAP) is an SH3-containing adapter protein that binds to the proto-oncogene c-Cbl. Recent work suggests that signaling through these molecules is involved in the regulation of insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Skeletal muscle is the major site of insulin-stimulated glucose disposal but there have been no reports of CAP function in this tissue. Using RT-PCR of mouse skeletal muscle RNA, we discovered a novel splice variant of CAP (CAP_{SM}; GenBank Accession No. AF521593) that is different from the adipocyte form by inclusion of a novel 168 bp fragment. This fragment encodes a peptide sequence that shows very high similarity with exon 25 of the human homologue of CAP (SORBS1). To understand the function of CAP_{SM} in glucose uptake regulation, L6 myotubes were transfected with either CAP_{SM} or a truncated CAP_{SM} devoid of all three SH3-binding domains (CAPΔSH3), which prevents CAP association with c-Cbl. Transfection with CAPΔSH3 decreased insulin-stimulated 2-deoxyglucose (2-DG) uptake and reduced c-Cbl phosphorylation. In contrast, transfection of L6 myotubes with CAPΔSH3 had no effect on dinitrophenol (DNP)- or hypoxia-stimulated glucose uptake, stimuli that work through insulin-independent mechanisms for the regulation of glucose uptake. These data demonstrate the existence of a novel CAP isoform expressed in skeletal muscle, and suggest the involvement of the CAP/Cbl pathway in the regulation of insulin-stimulated glucose uptake in L6 myotubes.
© 2004 Elsevier Inc. All rights reserved.

Keywords: CAP; c-Cbl; Insulin; Hypoxia; DNP; L6 cells

Insulin increases glucose uptake in skeletal muscle and adipose cells by a mechanism that involves insulin receptor substrate (IRS) molecules and phosphatidylinositol 3-kinase (PI 3-kinase) signaling to stimulate GLUT4 translocation [1–3]. However, there is considerable evidence that the IRS/PI 3-kinase signaling pathway is not sufficient for some of the actions of insulin, and that the generation of a separate signal may be required for normal insulin-stimulated glucose uptake [4–7]. Recently, it has been reported that the adaptor protein c-Cbl-associated protein (CAP/Ponsin/SH3P12) plays a role in PI 3-kinase independent signaling leading to glucose uptake in 3T3-L1 adipocyte cells [8]. CAP is a versatile adaptor protein that features three C-terminus SH3-binding domains and one sorbin-like region. Its functional association with the proto-oncogene product c-Cbl is mediated by the most carboxyl-terminal SH3-binding domain. c-Cbl is ubiquitously expressed and has been most extensively studied by virtue of its involvement in T cell antigen receptor-mediated signaling [9].

Evidence for CAP and c-Cbl involvement in insulin-stimulated glucose uptake comes from one study in the adipocyte cell line 3T3-L1 showing that the insulin receptor phosphorylates c-Cbl, which then associates with CAP [10]. The c-Cbl/CAP complex is translocated into specialized membrane subdomains referred to as caveolae, where the adaptor protein CrkII, along with the adenosine exchange factor C3G, is further recruited. The C3G permits the activation of the small G-protein TC10 that ultimately contributes to the translocation of

☆ *Abbreviations:* CAP, c-Cbl-associated protein; AICAR, 5-amino-imidazole-4-carboxamide ribonucleoside; DNP, dinitrophenol; AMPK, AMP-activated protein kinase.

* Corresponding author. Fax: 1-617-732-2650.
E-mail address: laurie.goodyear@joslin.harvard.edu (L.J. Goodyear).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.038

the intracellular GLUT4 storage pool into the plasma membrane [11]. Expression of a truncated CAP mutant that is devoid of SH3-binding domains (CAPΔSH3) in cultured 3T3-L1 adipocytes prevents recruitment of c-Cbl to caveolae [8]. This is associated with inhibition of insulin-stimulated GLUT4 translocation and glucose uptake, suggesting that CAP expression is crucial for normal functioning of this signaling mechanism.

Skeletal muscle accounts for the majority of blood glucose disposal in the body. Interestingly, skeletal muscle contains very high levels of CAP mRNA [10] and is also enriched in caveolae [12]. However, to our knowledge there have been no studies that have investigated the role of CAP/Cbl signaling in the regulation of glucose uptake in skeletal muscle, or any cells other than 3T3-L1 adipocytes. In the current study, we investigated the putative role of CAP/Cbl signaling in the regulation of glucose uptake in skeletal muscle. For this purpose, we determined the identity of the predominant CAP species expressed in skeletal muscle tissue, and investigated the potential involvement of CAP in both insulin- and hypoxia-stimulated glucose uptake in L6 myotubes. Our data show that a splice variant of CAP (CAP_{SM}) is predominantly expressed in mouse skeletal muscle. We also demonstrate that in L6 myotubes CAP_{SM} is involved in the regulation of insulin-stimulated glucose uptake in a c-Cbl-dependent manner. While previous studies exclusively focused on 3T3-L1 adipocytes, this is the first study to report that CAP regulates glucose uptake in a muscle cell line.

Methods

Materials. Cell culture materials were purchased from Gibco-BRL (Gaithersburg, MD). The following antibodies were purchased: anti-phospho-Cbl (Upstate Biotechnology, VA), anti-FLAG (Sigma, St. Louis, MO), anti-GLUT1 (Chemicon, CA), anti-GLUT4 (gift from Robert Smith), anti-phospho-Akt (Cell Signaling Technology, Beverly, MA), anti-phospho-ERK (Promega, WI), and anti-phospho-tyrosine pY99 (Santa Cruz, CA). LipofectAMINE 2000 was obtained from Life Technologies, (Invitrogen, CA), and anti-mouse and anti-rabbit IgG-HRP were from Transduction Laboratories (San Diego, CA). The ECL-improved Western blotting detection kit and 2-deoxy-D-[^{3}H]glucose were purchased from NEN (Perkin–Elmer, Boston, MA). All other reagents were of analytical grade and were from Merck (Darmstadt, Germany). The L6 cell line was kindly provided by Dr. A. Klip (The Hospital for Sick Children, Toronto, Canada).

cDNA cloning of CAP and construct generation. A FLAG epitope-tagged CAP cDNA in the pCAGGS vector was constructed as follows. Total cellular RNA was isolated using the GTC-acidic phenol method [13]. The cDNA of CAP was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from mouse gastrocnemius muscle as template. Primers (Forward: 5′-AGTTCTGAA TGTGATGTTGGAAGC-3′; Reverse: 5′-TTATAGATATAAAGGT TTTACATAGTTGCCTG-3′) were constructed based on the published coding region sequence of the mRNA for CAP (GenBank Accession No. U58883). The forward primer was designed to have a *Eco*RI restriction site, followed by the coding sequence for a FLAG epitope in frame. The single cDNA fragment obtained was TA-cloned into a pCR2.1-TOPO vector (Invitrogen, CA) using standard molecular biology techniques [14] and subsequently sequenced by the Sanger method [15]. The expression vector used was pCAGGS (gift from Dr. J. Miyazaki, Osaka University) [16]. The FLAG-CAP construct was excised with *Eco*RI and transferred into the *Eco*RI site between the CAG promoter and a 3′-flanking region of a rabbit β-globin gene. Further restriction digestion with *Bgl*II allowed for selection of the construct harboring the insert with the correct orientation. This expression vector drives a target gene under the CAG (cytomegalovirus immediate-early enhancer-chicken β-actin hybrid) promoter. The CAG promoter has extremely high activity in muscle, as demonstrated in both transgenic mice [17] and intramuscular naked DNA injection [18]. The FLAG-tagged truncated mutant CAPSH3, devoid of all three SH3-binding domains of the CAP molecule, was generated using the same strategy described above, but utilizing a different reverse primer (5′-GAATTCGCGGCCGCTTAGTCTTGGTGACTCTGAATCTTT AG-3′).

Tissue distribution of CAP_{SM} mRNA in mouse was assessed by RT-PCR using primers (forward: 5′-ATGAGTTCTGAATGTGATG TTGGAAGC-3′; reverse: 5′-GCAATATCGGCCTCTTCTTGCTCG -3′) that spans from the 5′-end of the coding region until the 168 bp novel fragment characteristic of CAP_{SM}.

The on-line internet tools BLAST, ScanProsite, and Coils (EMBL) were used in combination with the software package VectorNTI for analysis of the sequence.

Cell culture. L6 myoblasts (passage 5) were seeded into 12-well plates and maintained in growth medium consisting of α-minimum essential medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Myoblasts were grown in monolayers until they reached a confluency of 50–60%. Culture medium was then switched from a 10% FBS into a 2% FBS containing α-MEM allowing differentiation of myoblast into myotubes. After six days of differentiation, around 60% of the L6 cells fused to form multinucleated myotubes and fibers. Standard lipotransfection protocols were then applied and protein synthesis was allowed for 48 h after transfection. For insulin stimulation experiments, L6 myotubes were serum-starved 5 h prior to challenge with 100 nM insulin at the indicated times. Hypoxia conditions were generated placing the cell culture plates at 37 °C inside an anaerobic jar with a controlled atmosphere of nitrogen for the time period indicated.

2-Deoxyglucose uptake. L6 myotubes were serum-starved for 5 h in α-MEM prior to any treatment. Cells were washed twice with ice-cold phosphate-buffered saline and pre-incubated in insulin (100 nM; 20 min), DNP (500 μM; 10 min) or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; 1 mM; 1 h). Following stimulation, 2-deoxy-D-[^{3}H]glucose uptake was measured by incubating cells at room temperature for 5 min in transport solution (140 mM NaCl, 20 mM Hepes–Na, pH 7.4, 10 μM 2-deoxy-D-glucose, 5 mM KCl, 0.5 μCi/mL 2-deoxy-D-[^{3}H]glucose, 2.5 mM $MgSO_4$, and 1 mM $CaCl_2$). Non-specific glucose transport was determined in the presence of 10 μM cytochalasin B. Accumulation of 2-deoxy-D-[^{3}H]glucose in the cells was determined and rates of uptake were calculated.

Immunoprecipitation. L6 cells ($\approx 0.5 \times 10^7$) were washed twice with ice-cold phosphate-buffered saline and lysed with buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM $NaVO_4$, 1% NP-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 3 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. After centrifugation at 10,000*g* for 15 min at 4 °C, protein content in the supernatants was determined by the Bradford method. 500 μg of total protein was incubated with anti-Cbl antibody (Upstate Biolabs) overnight at 4 °C. The immune complexes were precipitated with protein G-plus protein A–agarose beads (50% each, w/v) for 2 h and were washed extensively with lysis buffer before solubilization in Laemmli sample buffer. Bound proteins were subjected to immunoblotting.

Immunoblotting. Following the experimental protocols, L6 myotubes were lysed in buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM $NaVO_4$, 1% NP-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 3 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. After centrifugation at 10,000*g* for 10 min at 4 °C, total protein concentration was determined by the Bradford method (BioRad, CA) in the clarified supernatants. Samples were resolved in 10% SDS–PAGE, transferred to nitrocellulose membranes, and blocked in 5% BSA. Individual proteins were detected with the specified antibodies and visualized by blotting with horseradish peroxidase-linked secondary antibodies. Immunocomplexes were detected using chemioluminescence. Bands were scanned and quantitated by densitometry. To reprobe immunoblots, the nitrocellulose membranes were incubated for 30 min at 60 °C with 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 0.7% of 2-mercaptoethanol, and then washed extensively with 10 mM Tris–HCl (pH 8), 150 mM NaCl.

Statistical analysis. Data are expressed as means ± SEM. Statistical comparisons were performed using one-way ANOVA and Student's *t* test. Data were considered statistically significant if $p < 0.05$.

Results

cDNA cloning of CAP_{SM}

There is some evidence that there may be a number of splice variants of CAP in different tissues [22]. Therefore, our first goal was to investigate the molecular identity of the CAP isoform predominantly expressed in skeletal muscle. Total cellular RNA was purified from mouse gastrocnemius muscle, and RT-PCR was performed following standard protocols using primers based on the published mouse CAP cDNA sequence (GenBank Accession No. U58883), as described in Methods. This yielded a single 2.2 kb PCR product that was subsequently TA-cloned into a pCR2.1-TOPO vector. Three different clones were sequenced. The resulting cDNA sequence (Fig. 1A) is 2223 bp in length and is predicted to encode a protein of 80.5 kDa. A pairwise alignment against the published mouse CAP cDNA sequence (GenBank Accession No. U58883) revealed complete identity of the nucleotide sequence, with the exception of a fragment that is 168 bp in length and lies at position 1333 (data not shown). BLAST analysis of the 168 bp fragment showed a very high degree of similarity (*E* value: 1e − 42) with exon 25 of the human CAP gene homologue, termed SORBS1, obtained from human skeletal muscle (GenBank Accession No. AF136381). Preservation of this sequence fragment in the same tissue type in different species suggests its physiological relevancy. The 56 amino acids encoded by the novel fragment match those of the SORBS1 exon 25 with the exception of two single amino acid residues (Fig. 1A). A similarity plot of the published mouse CAP cDNA sequence versus SORBS1 shows the absence of a matching homologue sequence for SORBS1 exon 25 in mouse CAP that is predominant in adipose tissue (Fig. 1B). This result opens the possibility that CAP_{SM} in muscle tissue has different functions and/or regulatory mechanisms compared with the CAP variant expressed in adipose cell types. The expression pattern of CAP_{SM} was investigated using total RNA from diverse mouse tissues by RT-PCR using a primer specific for the novel fragment. CAP_{SM} showed a tissue distribution consistent with expression in muscle type tissues, i.e., skeletal muscle and heart (Fig. 1C).

Software-based sequence predictions

We next analyzed the sequence of the novel fragment in search of potential regulatory motifs susceptible to being phosphorylated or covalently modified. Prosite analysis of CAP_{SM} for the distinct segment of 56 amino acids predicts a N-myristoylation site and several potential phosphorylation sites including cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and casein kinase II (CK2) (Fig. 2A). This result suggests that this sequence fragment might confer potentially new regulatory properties to CAP_{SM} in comparison to CAP. An additional structural feature is revealed using the analysis software Coils (EMBL) for predicting coiled coil regions in proteins. The novel segment of 56 amino acid residues introduces a high likelihood for an additional coiled coil conformation within the CAP_{SM} molecule (Fig. 2B). This feature of the tertiary structure introduced by the novel fragment argues again in favor of a functional role of this sequence, which might be of relevance for normal muscle physiology.

Time-course of insulin-stimulated c-Cbl phosphorylation in L6 myotubes

To determine whether L6 myotubes show the ability to alter the phosphorylation status of c-Cbl when challenged with insulin, we performed a time-course study. Changes in Cbl phosphorylation were assessed by immunoprecipitation of cell lysates with anti-c-Cbl antibody followed by immunoblotting with anti-phosphotyrosine antibody, after stimulation with 100 nM insulin at 0.5, 1, 5, and 10 min. c-Cbl phosphorylation was increased up to twofold and reached statistical significance with 5 min of insulin stimulation ($p < 0.05$, $n = 4$) (Fig. 3). The increases in c-Cbl phosphorylation were transient, declining towards baseline by 10 min. Based on these results, we used the 5 min time point for subsequent studies of insulin stimulation.

Expression of a dominant negative mutant of CAP_{SM} ($CAP\Delta SH3$) diminishes insulin-stimulated glucose uptake in L6 myotubes

Because signaling through CAP has been reported to be essential for insulin-stimulated glucose uptake in 3T3-L1 adipocytes, we investigated whether CAP_{SM}

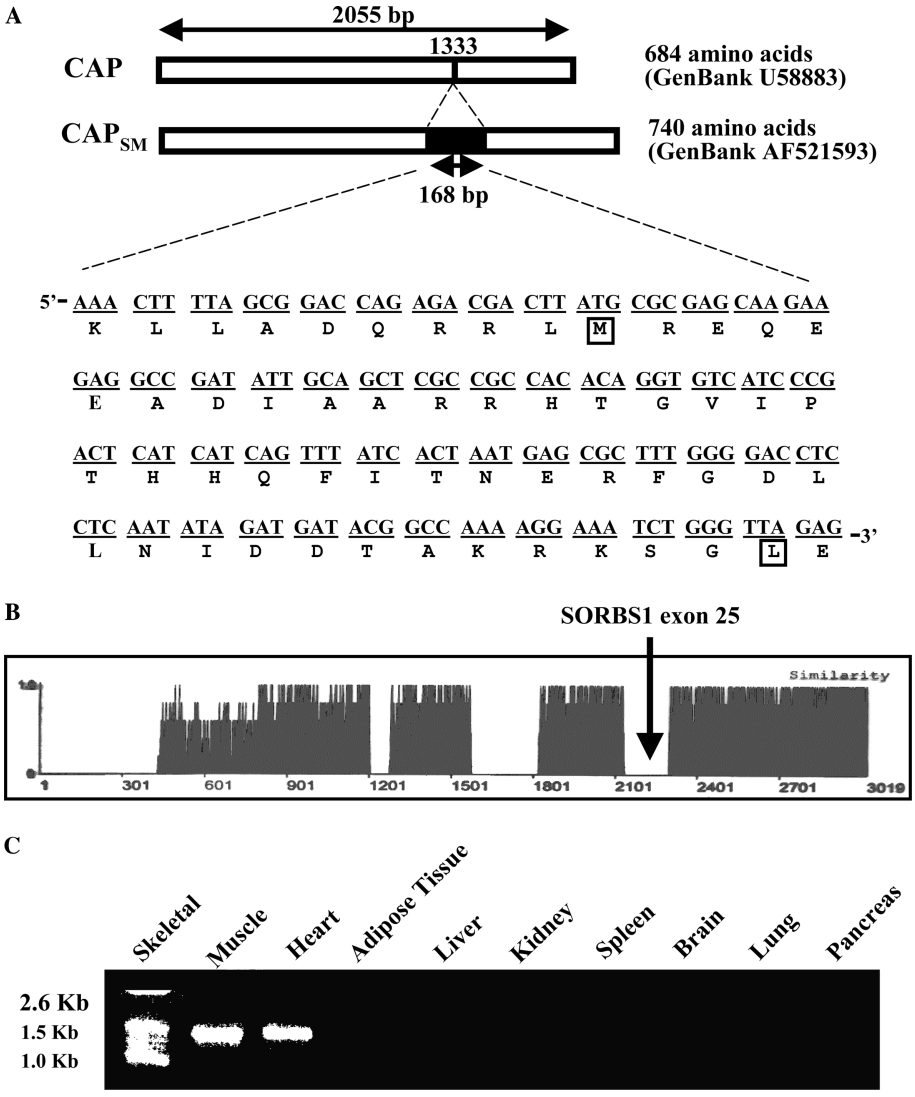

Fig. 1. Sequence analysis and tissue distribution of CAP_{SM}. (A) Schematic representation comparing CAP and CAP_{SM} cDNA sequences including GenBank accession numbers. Nucleotide sequence and predicted amino acid sequence of the novel fragment characteristic of CAP_{SM} that spans 168 bp are shown. Amino acid residues that differ between CAP and CAP_{SM} sequences are indicated. (B) Similarity plot between CAP and its human homologue termed SORBS1. The software VectorNTI package was utilized to generate the similarity plot. SORBS1 exon 25 sequence is nearly identical to the novel fragment present in CAP_{SM} but is absent in CAP as shown. (C) Tissue distribution of CAP_{SM} mRNA by RT-PCR in various mouse tissues. A forward primer annealing at the 5′-end of the nucleotide sequence was used in combination with a reverse primer that anneals within the 168 bp novel fragment characteristic of CAP_{SM}. Expression pattern reveals muscle tissue type distribution.

plays a similar role in L6 myotubes. L6 cells were transfected with empty pCAGGS vector (Mock), wild type CAP_{SM} (CAP_{SM}), or the truncated mutant of CAP_{SM} devoid of all three SH3-binding domains (CAPΔSH3). Efficiency of transfection was shown to be approximately 40–45% as assessed by β-galactosidase staining after transfection with the reporter gene *LacZ* (data not shown). L6 myotubes were transfected on day 6 of differentiation and experiments were performed 48 h later, as detailed in Methods. L6 myotubes were challenged with 100 nM insulin for 5 min and glucose uptake was determined using 2-DG. Rates of 2-DG uptake increased twofold in mock-transfected cells with insulin treatment. Basal rates of 2-DG uptake were unchanged after transfection with either CAP_{SM} or CAPΔSH3. On the other hand, transfection with CAPSH3 resulted in a significant decrease ($18 \pm 5\%$, $n = 4$, $p < 0.05$) in insulin-stimulated glucose uptake (Fig. 4A). Consistent with previous findings in 3T3-L1 adipocytes [4,12], overexpression of wild type CAP did not further increase 2-DG uptake in the cells. These data demonstrate that in L6 myotubes CAP_{SM} is involved in insulin-stimulated glucose uptake, and that the SH3-binding domains are necessary for this effect.

Immunoblotting of L6 cell lysates shows that insulin increased c-Cbl phosphorylation by 2.3 ± 0.3-fold over basal in the mock transfected cells (Fig. 4B). This increase was significantly blunted in L6 myotubes

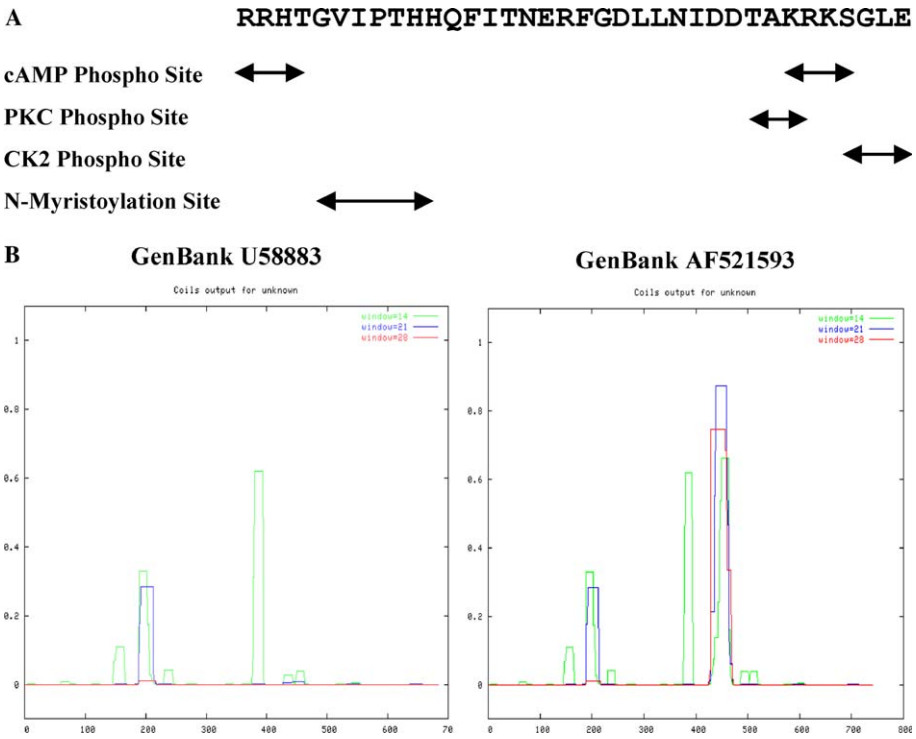

Fig. 2. In silico amino acid sequence analysis of the novel fragment of CAP_{SM}. (A) Schematic representation of the predicted phosphorylation sites after a PROSITE search using the amino acid sequence derived from the novel fragment of CAP_{SM}. Putative phosphorylation sites for PKA (2 sites), PKC (1 site), and CK2 (1 site) are revealed. An additional feature consists of the presence of a N-myristoylation site. (B) Prediction for coiled coil tertiary structure in CAP (GenBank U58883) and CAP_{SM} (GenBank AF521593) proteins. Translation of the 168 bp novel fragment characteristic of CAP_{SM} introduces an additional coiled coil region in the protein tertiary structure, as shown by using the analysis software coils (EMBL).

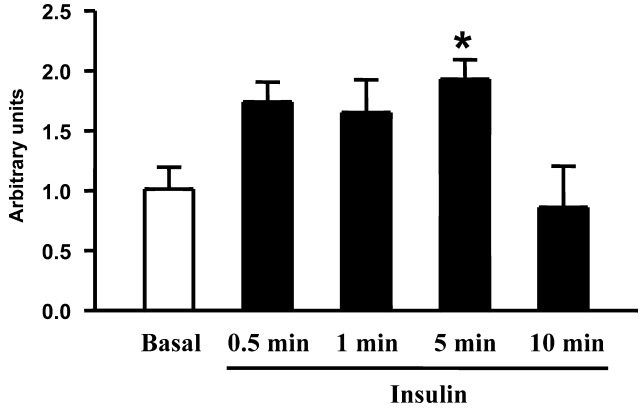

Fig. 3. Time-dependent effects of insulin on c-Cbl phosphorylation. L6 cells were maintained in culture and differentiated as described in Methods, and further challenged with 100 nM insulin at the indicated times. Cell lysates were immunoprecipitated using anti-c-Cbl antibody and probed against anti-phosphotyrosine antibody. Thereafter, membranes were stripped and reprobed with anti-c-Cbl antibody. Quantitative values were normalized and corrected. Results represent means ± SEM of four independent experiments. Basal, open bar; insulin treatment, shaded bars. $*p < 0.05$, compared with basal non-insulin stimulation.

transfected with the truncated CAPΔSH3 (reduction of $41 \pm 17\%$, $n = 4$, $p < 0.05$) (Fig. 4B). Insulin increased ERK and Akt phosphorylation, an effect that was not altered by overexpression of CAP_{SM} or CAPΔSH3, suggesting that insulin signaling via the classical PI 3-K pathway is not regulated by CAP_{SM}. Phosphorylation of the AMP-activated protein kinase (AMPK), an insulin-independent signaling mechanism that regulates glucose uptake, was also not affected by insulin stimulation or alterations in CAP_{SM} expression (not shown). Taken together, these data demonstrate that insulin-induced phosphorylation of c-Cbl is dependent on CAP_{SM} and suggest that the CAP/Cbl pathway is important for glucose uptake in L6 myotubes.

Effects of CAP_{SM} on dinitrophenol and hypoxia-stimulated glucose uptake in L6 myotubes

There are numerous insulin-independent stimuli that increase GLUT4 translocation and glucose uptake in muscle, presumably through a mechanism that primarily involves activation of AMPK.

5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), DNP, and hypoxia are extensively used treatments that result in large increases in AMPK Thr^{172} phosphorylation and activity in skeletal muscle tissue [19,20]. Interestingly, we found that AICAR had minimal effects on AMPK phosphorylation in L6 myotubes (data not shown) and also failed to significantly increase 2-DG uptake (Fig. 5A). The inability of AICAR to stimulate glucose uptake in L6 myotubes is intriguing

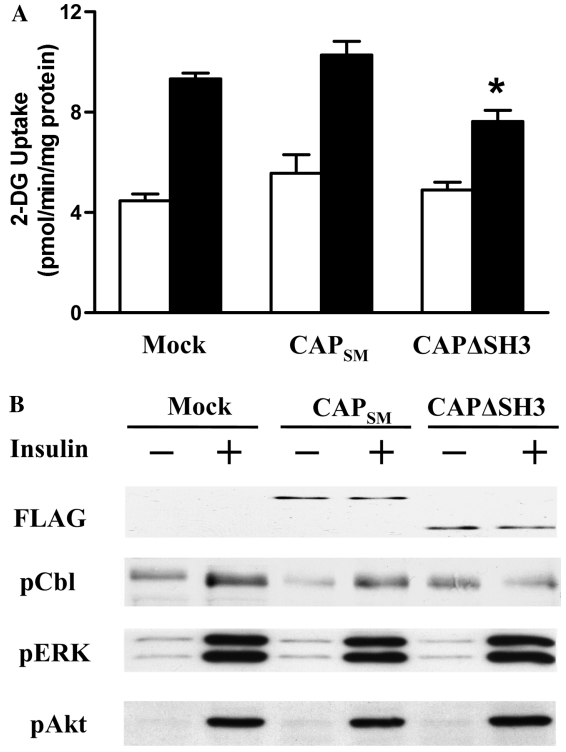

Fig. 4. Effect of the truncated mutant CAPΔSH3 on insulin action in L6 myotubes. (A) 2-DG uptake rates in L6 myotubes transfected with empty vector (mock), CAP_{SM} or CAPΔSH3. Cells were stimulated with 100 nM insulin for 5 min and glucose transport determined as described under Methods. Results represent means ± SEM of four independent experiments in triplicate. Basal, open bars; insulin treatment, shaded bars. $*p = 0.015$, compared with insulin-stimulated mock control. (B) Effects of CAP_{SM} and CAPΔSH3 overexpression on insulin signaling in L6 myotubes. Cells were challenged with 100 nM insulin for 10 min, lysed, and subjected to immunodetection with the antibodies indicated. Representative blots of four independent experiments are shown. FLAG expression was used to monitor the presence of the recombinant FLAG-tagged molecules utilized in the transfection experiments. The differences in molecular weight between CAP_{SM} and CAPΔSH3 species account for the difference in location of the FLAG-band.

and might be related to the expression pattern of the catalytic subunit isoforms of AMPK. On the other hand, DNP, an uncoupler of the mitochondrial respiratory chain, increased 2-DG uptake to levels comparable to that attained with insulin stimulation in L6 myotubes (Fig. 5A). DNP-stimulated 2-DG uptake tended to be higher in wild type CAP_{SM} overexpressing cells, but the difference was not significant (Fig. 5B). In contrast to insulin stimulation, expression of CAPΔSH3 did not induce any change in DNP-stimulated 2-DG glucose. Overexpression of CAP_{SM} and CAPΔSH3 was not associated with any detectable modification in DNP-stimulated AMPK phosphorylation (data not shown). Thus, CAP_{SM} does not appear to be a major regulator of DNP-stimulated glucose uptake in L6 myotubes.

One hour of acute hypoxia treatment increased 2-DG uptake in the L6 myotubes by approximately twofold

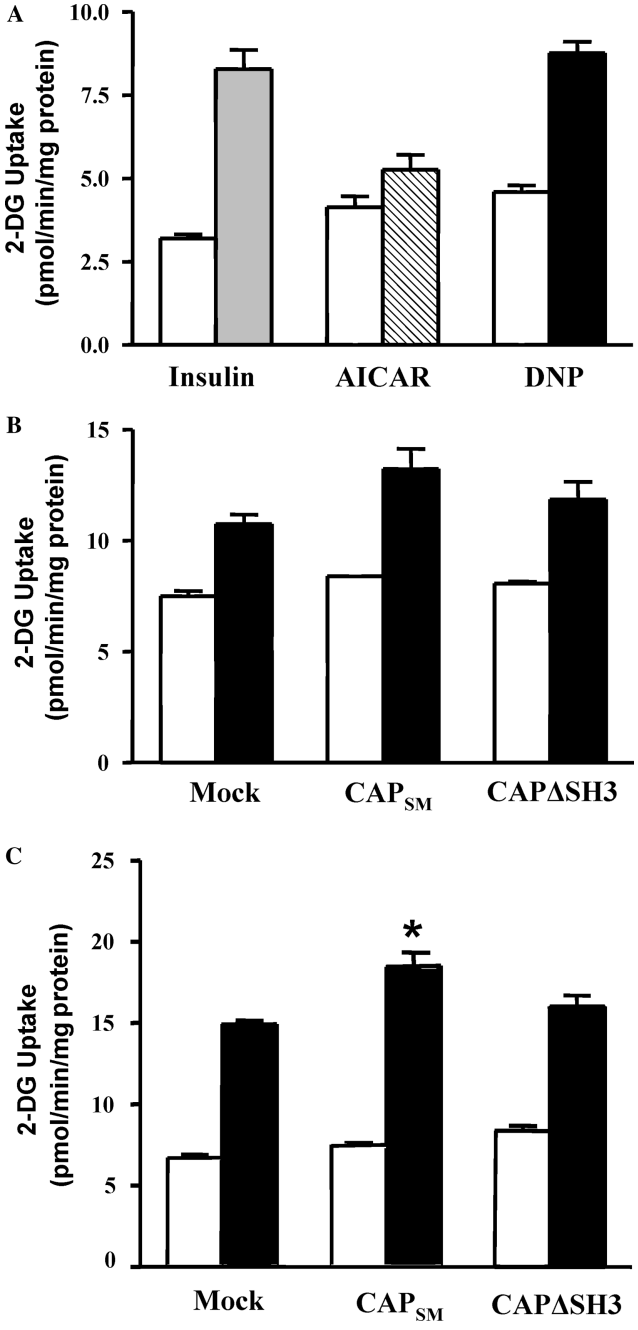

Fig. 5. Effect of the truncated mutant CAPΔSH3 on glucose uptake after treatment with AMPK activating agents. (A) 2-DG uptake rates of L6 myotubes treated with 100 nM insulin (10 min), 1 mM AICAR (1 h), or 500 μM DNP (10 min). (B) 2-DG uptake rates of L6 myotubes challenged with 500 μM DNP for 10 min, transfected with empty vector (mock), CAP_{SM} or CAPΔSH3. Basal, open bars; DNP treatment, shaded bars. (C) 2-DG uptake rates after acute (1 h) hypoxia treatment in L6 myotubes transfected with empty vector (mock), CAP_{SM} or CAPΔSH3. Basal, open bars; acute hypoxia, shaded bars. $*p < 0.05$, compared with hypoxia-treated mock control. Results (A, B, and C) represent each means ± SEM values of four independent experiments.

(Fig. 5C). Hypoxia-stimulated 2-DG uptake was 23% higher in L6 myotubes transfected with wild type CAP_{SM}. Similar to DNP, hypoxia-stimulated 2-DG

uptake was not significantly reduced in the CAPΔSH3 overexpressing cells. Changes in glucose transport were not due to changes in GLUT1 and GLUT4 since protein levels were unchanged with acute hypoxia, and there was no difference in GLUT1 protein among mock, CAP_{SM}, and CAPΔSH3 transfected cells (data not shown). Similar to DNP, hypoxia also had no effect on c-Cbl phosphorylation, and AMPK phosphorylation state was not changed by expression of CAP_{SM} or CAPΔSH3.

Discussion

Over the past few years there has been accumulating evidence in support of the idea that PI 3-kinase is necessary, but may not be sufficient for insulin-stimulated glucose uptake in insulin sensitive tissues, such as skeletal muscle and adipose. Examples include findings showing that overexpression of constitutively active mutants of PI 3-kinase only partially stimulate GLUT4 translocation induced by insulin [7], platelet-derived growth factor (PDGF), and interleukin-4 stimulate PI 3-kinase to a similar extent as insulin but has little effect on glucose uptake [4,5], and cell-permeable derivatives of PI-3,4,5-P3 (product of PI 3-kinase) cannot mimic insulin-stimulated glucose uptake [21]. These findings have raised the hypothesis that there is a segregation of the insulin signal into PI 3-kinase-dependent and -independent pathways to fully account for the effect of insulin on GLUT4 translocation and glucose uptake.

In humans, the CAP homologue termed SORBS1 has a number of splice variants [22], making it important to determine the identity of CAP expressed in skeletal muscle. Here we reveal the expression of a unique isoform of c-Cbl-associated protein in mouse skeletal muscle (CAP_{SM}; GenBank AF521593) that is different from adipocyte CAP (GenBank U58883) in that it includes a novel 168 bp fragment. Within this fragment, there are a number of potential motifs prone to be regulated by protein kinases that have been determined in silico and require further functional characterization to be confirmed. The fact that this fragment has been evolutionarily preserved in the same tissue type in different species (mouse and human) strongly argues in favor of physiological relevancy. In addition, it is not surprising that tissues metabolically different, e.g., fat and muscle, might require specific features in common proteins that could be met by the presence/absence of particular amino acid sequences conferring differential regulatory mechanisms in each tissue. For instance, we can speculate that some proteins of muscle, which is a tissue more enriched in membranes than adipose tissue, might require additional membrane anchoring capabilities by harboring a palmitoylation site that helps for this purpose. Therefore, understanding the function of CAP_{SM} in glucose uptake regulation in skeletal muscle was another goal in this study. Functional studies were performed on L6 cells, as they are widely utilized as an in vitro model of skeletal muscle by virtue of the well-preserved morphological and metabolic properties of skeletal muscle [23].

Given that the association of CAP with c-Cbl requires the carboxyl-terminal SH3 domain of CAP, we generated a truncated CAP_{SM} (CAPΔSH3). This truncated mutant still preserves the novel 168 bp fragment present in wild type CAP_{SM} and therefore the potential properties conferred to the adaptor molecule. Transfection with CAP_{SM} or CAPΔSH3 did not alter basal 2-DG uptake. This finding suggests that GLUT1 may not be regulated by CAP_{SM}, since this glucose transporter is thought to regulate basal glucose transport in L6 cells [24]. However, in contrast with the lack of effect on basal rates of transport, there was a significant reduction in maximal insulin-stimulated 2-DG uptake in L6 myotubes transfected with CAPΔSH3. In fact, the magnitude of the CAPΔSH3-induced reduction in insulin-stimulated 2-DG uptake may be even greater if we take into account the efficiency of the transfection in L6 cells (≈40–45%). Consistent with the regulation of 2-DG uptake, overexpression of CAPΔSH3 also influenced c-Cbl phosphorylation. Insulin increased the phosphorylation of c-Cbl by approximately twofold over basal, and this increase was blunted after overexpression of CAPΔSH3. This finding is similar to results in 3T3-L1 adipocytes [8], suggesting that CAP/Cbl signaling in L6 myotubes is functional and might contribute together with the PI 3-kinase pathway in enhancing glucose uptake in response to insulin.

In Jurkat cells, c-Cbl has the ability to interact with the p85 subunit of PI 3-kinase, by virtue of the c-Cbl multiple proline-rich motifs [25]. Therefore, we tested the possibility that Akt, a downstream substrate of PI 3-kinase in insulin signaling to glucose transport, would be regulated by CAP_{SM}. Consistent with the "two pathway" hypothesis, transfection of L6 cells with CAPΔSH3 had no effect on insulin-stimulated Akt phosphorylation. The lack of effect on Akt phosphorylation suggests that the signaling events triggered by the association of c-Cbl with CAP_{SM} are independent of a potential c-Cbl-PI 3-kinase interaction in L6 myotubes. In addition, insulin signaling to ERK was also unaffected by CAPΔSH3, suggesting that insulin regulation of this mitogen pathway is also not dependent on CAP_{SM}. However, it cannot be excluded that the insulin-stimulated PI 3-kinase signaling may be subjected to regulation or crosstalk by CAP_{SM}.

The existence of functional CAP/Cbl signaling in skeletal muscle opens the possibility that this pathway contributes to the mechanisms involved in regulating glucose uptake by other, insulin-independent stimuli such as hypoxia, DNP, and exercise. In recent years

there has been a good deal of evidence that AMPK signaling is a key regulatory mechanism for insulin-independent glucose uptake [26–29]. This raises the possibility that analogous to insulin, there may be two signaling pathways regulating these insulin-independent stimulators of glucose uptake. However, neither DNP- nor hypoxia-stimulated glucose uptake was affected by overexpression of CAPΔSH3, although there was a tendency for an increase in glucose uptake with these stimuli when wild type CAP_{SM} was overexpressed. These findings suggest that expression of a normal functional CAP_{SM} is not necessary for insulin-independent signaling to glucose uptake, but that high levels of wild type expression of this adaptor protein may be able to upregulate uptake. If there is regulation of glucose transport by CAP_{SM}, it probably lies downstream of AMPK as the phosphorylation of this kinase was unaffected by overexpression of either CAP_{SM} or CAPΔSH3 transfection. Given that c-Cbl phosphorylation was undetectable after 1 h hypoxia and that SH3-binding domains are well known to be promiscuous in associating with a broad range of proteins, we cannot rule out the possibility of proteins other than c-Cbl mediating CAP_{SM} action in acute hypoxia.

While we found significant, albeit modest, increases in insulin-stimulated c-Cbl phosphorylation in L6 myotubes, in preliminary experiments we have not observed significant changes in c-Cbl phosphorylation with insulin in adult skeletal muscle obtained from the rat (Alcazar and Goodyear, unpublished observations). These data are consistent with a very recent report showing that insulin does not stimulate c-Cbl phosphorylation in the gastrocnemius muscle of rats [30]. Interestingly, the authors could not detect CAP in the skeletal muscle by immunoblotting, and postulated that the absence of CAP may explain the lack of c-Cbl phosphorylation by insulin. Our identification of a novel CAP splice variant in mouse skeletal muscle is not entirely consistent with this interpretation. In fact, we have detected expression of CAP in mouse tibialis anterior muscle (Alcazar and Goodyear, unpublished data). In addition, Saltiel and co-workers [10] have reported high abundance of CAP transcripts in skeletal muscle using a mouse multiple-tissue Northern blot. Therefore, it appears that a mechanism other than CAP expression level may explain the lack of significant changes of c-Cbl phosphorylation in rat skeletal muscle.

In conclusion, we report the molecular cloning of a unique splice variant of CAP expressed in mouse skeletal muscle tissues (CAP_{SM}). In addition, we provide evidence that CAP_{SM} significantly contributes to the regulation of insulin-stimulated glucose uptake in L6 myotubes, in a c-Cbl-dependent manner. CAP/Cbl signaling is not necessary for insulin-independent stimuli that regulate glucose transport in L6 myotubes.

References

[1] B. Cheatham, C.J. Vlahos, L. Cheatham, L. Wang, J. Blenis, C.R. Kahn, Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation, Mol. Cell. Biol. 14 (1994) 4902–4911.

[2] M.F. White, C.R. Kahn, The insulin signaling system, J. Biol. Chem. 269 (1994) 1–4.

[3] S.A. Summers, V.P. Yin, E.L. Whiteman, L.A. Garza, H. Cho, R.L. Tuttle, M.J. Birnbaum, Signaling pathways mediating insulin-stimulated glucose transport, Ann. N. Y. Acad. Sci. 892 (1999) 169–186.

[4] R.J. Wiese, C.C. Mastick, D.F. Lazar, A.R. Saltiel, Activation of mitogen-activated protein kinase and phosphatidylinositol 3′-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes, J. Biol. Chem. 270 (1995) 3442–3446.

[5] S.J. Isakoff, C. Taha, E. Rose, J. Marcusohn, A. Klip, E.Y. Skolnik, The inability of phosphatidylinositol 3-kinase activation to stimulate GLUT4 translocation indicates additional signaling pathways are required for insulin-stimulated glucose uptake, Proc. Natl. Acad. Sci. USA 92 (1995) 10247–10251.

[6] A. Guilherme, M.P. Czech, Stimulation of IRS-1-associated phosphatidylinositol 3-kinase and Akt/protein kinase B but not glucose transport by beta1-integrin signaling in rat adipocytes, J. Biol. Chem. 273 (1998) 33119–33122.

[7] S.S. Martin, T. Haruta, A.J. Morris, A. Klippel, L.T. Williams, J.M. Olefsky, Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes, J. Biol. Chem. 271 (1996) 17605–17608.

[8] C.A. Baumann, V. Ribon, M. Kanzaki, D.C. Thurmond, S. Mora, S. Shigematsu, P.E. Bickel, J.E. Pessin, A.R. Saltiel, CAP defines a second signalling pathway required for insulin-stimulated glucose transport, Nature 407 (2000) 202–207.

[9] C.B. Thien, W.Y. Langdon, Cbl: many adaptations to regulate protein tyrosine kinases, Nat. Rev. Mol. Cell. Biol. 2 (2001) 294–307.

[10] V. Ribon, J.A. Printen, N.G. Hoffman, B.K. Kay, A.R. Saltiel, A novel, multifuntional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 adipocytes, Mol. Cell. Biol. 18 (1998) 872–879.

[11] S.H. Chiang, C.A. Baumann, M. Kanzaki, D.C. Thurmond, R.T. Watson, C.L. Neudauer, I.G. Macara, J.E. Pessin, A.R. Saltiel, Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10, Nature 410 (2001) 944–948.

[12] P.E. Scherer, R.Y. Lewis, D. Volonte, J.A. Engelman, F. Galbiati, J. Couet, D.S. Kohtz, E. van Donselaar, P. Peters, M.P. Lisanti, Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo, J. Biol. Chem. 272 (1997) 29337–29346.

[13] P. Chomcynski, N. Sacchi, Single-step method of RNA isolation by acid guaninium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.

[14] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.

[15] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.

[16] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.

[17] M. Okabe, M. Ikawa, K. Kominami, T. Nakanishi, Y. Nishimune, 'Green mice' as a source of ubiquitous green cells, FEBS Lett. 407 (1997) 313–319.

[18] M. Tokui, I. Takei, F. Tashiro, A. Shimada, A. Kasuga, M. Ishii, T. Ishii, K. Takatsu, T. Saruta, J. Miyazaki, Intramuscular injection of expression plasmid DNA is an effective means of long-term systemic delivery of interleukin-5, Biochem. Biophys. Res. Commun. 233 (1997) 527–531.

[19] G.F. Merrill, E.J. Kurth, D.G. Hardie, W.W. Winder, AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle, Am. J. Physiol. 273 (1997) E1107–E1112.

[20] T. Hayashi, M.F. Hirshman, N. Fujii, S.A. Habinowski, L.A. Witters, L.J. Goodyear, Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism, Diabetes 49 (2000) 527–531.

[21] T. Jiang, G. Sweeney, M.T. Rudolf, A. Klip, A. Traynor-Kaplan, R.Y. Tsien, Membrane-permeant esters of phosphatidylinositol 3,4,5-trisphosphate, J. Biol. Chem. 273 (1998) 11017–11024.

[22] W.H. Lin, C.J. Huang, M.W. Liu, H.M. Chang, Y.J. Chen, T.Y. Tai, L.M. Chuang, Cloning, mapping, and characterization of the human sorbin and SH3 domain containing 1 (SORBS1) gene: a protein associated with c-Abl during insulin signaling in the hepatoma cell line Hep3B, Genomics 74 (2001) 12–20.

[23] N. Patel, Z.A. Khayat, N.B. Ruderman, A. Klip, Dissociation of 5′ amp-activated protein kinase activation and glucose uptake stimulation by mitochondrial uncoupling and hyperosmolar stress: differential sensitivities to intracellular Ca(2+) and protein kinase c inhibition, Biochem. Biophys. Res. Commun. 285 (2001) 1066–1070.

[24] R. Robinson, L.J. Robinson, D.E. James, J.C. Lawrence Jr., Glucose transport in L6 myoblasts overexpressing GLUT1 and GLUT4, J. Biol. Chem. 268 (1993) 22119–22126.

[25] D. Hartley, H. Meisner, S. Corvera, Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-cbl, J. Biol. Chem. 270 (1995) 18260–18263.

[26] T. Hayashi, M.F. Hirshman, E.J. Kurth, W.W. Winder, L.J. Goodyear, Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport, Diabetes 47 (1998) 1369–1373.

[27] R. Bergeron, R.R. Russell III, L.H. Young, J.M. Ren, M. Marcucci, A. Lee, G.I. Shulman, Effect of AMPK activation on muscle glucose metabolism in conscious rats, Am. J. Physiol. 276 (1999) E938–E944.

[28] E.J. Kurth-Kraczek, M.F. Hirshman, L.J. Goodyear, W.W. Winder, 5′ AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle, Diabetes 48 (1999) 1667–1671.

[29] B.E. Holmes, E.J. Kurth-Kraczek, W.W. Winder, Chronic activation of 5′-AMP-activated protein kinase increases GLUT4, hexokinase, and glycogen in muscle, J. Appl. Physiol. 87 (1999) 1990–1995.

[30] A.C. Thirone, J.B. Carvalheira, A.E. Hirata, L.A. Velloso, M.J. Saad, Regulation of cbl-associated protein/cbl pathway in muscle and adipose tissues of two animal models of insulin resistance, Endocrinology 145 (2004) 281–293.