ORIGINAL ARTICLE

TWO-HYBRID ANALYSIS IDENTIFIES PSMD11, A NON-ATPase SUBUNIT OF THE PROTEASOME, AS A NOVEL INTERACTION PARTNER OF AMP-ACTIVATED PROTEIN KINASE

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ABSTRACT

Mammalian AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase that acts as a sensor of cellular energy status. It interacts with a great variety of different substrates leading to short term (i.e. regulation of the activity of different enzymes by direct phosphorylation) and long-term effects (i.e. regulation of transcriptional activity of different transcription factors). In this work, we describe the use of the yeast two-hybrid technology to identify additional proteins that interact with the different subunits of AMPK. We have performed three yeast two-hybrid screenings of a human skeletal muscle cDNA library using three different baits: a constitutively active form of AMPKα2 (LexA-AMPKα2-T172D) co-expressed with AMPKγ1, LexA-AMPKβ2 and LexA-AMPKγ3. Our results identify novel interaction partners of AMPK in human skeletal muscle. We also further characterize the interaction of AMPK with one of these novel interacting proteins, the non-ATPase subunit of the proteasome PSMD11. Our results indicate that AMPK is able to interact physically with this subunit and modify its phosphorylation status, supporting a possible role for AMPK in regulating proteasome function.

Key words: AMPK, two-hybrid screening, proteasome, PSMD11, phosphorylation.

Abbreviations: AICAR: 5-aminoimidazole-4-carboxamide-1β-D-ribofuranoside; AMPK: AMP-activated protein kinase; PKA: protein kinase A; SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis; 2D-E: two-dimensional electrophoresis.

INTRODUCCION

Mammalian AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that acts as a sensor of cellular energy status. It is activated in an ultrasensitive manner by cellular stresses that deplete ATP, either by inhibiting ATP production (hypoxia, glucose deprivation, heat shock, mitochondrial oxidative phosphorylation inhibitors, etc.) or by accelerating ATP consumption (muscle contraction, etc.). Once activated, it switches on catabolic pathways and switches off many ATP-consuming processes (anabolic pathways) (see Hardie and Sakamoto, 2006; Hardie, 2007 for reviews). AMPK is an oligomeric complex composed of three different subunits: α, β and γ. Phylogenetic analysis of the three subunits indicates that all of them have orthologues in the eukaryotic kingdom, from yeast to humans, thus indicating that the function of this complex is well conserved throughout evolution (Hardie et al., 1998; Hardie et al., 2003). AMPKα is the catalytic subunit of the AMPK complex. Two isoforms of this subunit have been described, namely AMPKα1 and AMPKα2. Both isoforms are localized in the cytoplasm, although AMPKα2 shows also a nuclear localization (Salt et al., 1998). The AMPKγ subunit contains four tandem repeats of a structure module called CBS, described initially in cystathionine-β-synthase (Bateman, 1997), which is involved in AMP binding (Scott et al., 2004). Three isoforms of the γ-subunit, named AMPKγ1, γ2 and γ3, have been described. The three isoforms display little homology at the N-terminus, but the C-terminus (where the CBS domains are located) is conserved. Finally, the AMPKβ subunit functions as a scaffold to assemble the α and γ subunits and it also determines the subcellular localization and substrate specificity of the complex. Two isoforms of the β-subunit (AMPKβ1 and β2) have been described; they differ only at the N-terminus, but interact with the same efficiency with

the AMPKα and AMPKγ subunits (Thornton et al., 1998; Gimeno-Alcaniz and Sanz, 2003).

AMPK function has been implicated in multiple metabolic pathways. It interacts with a great variety of different substrates leading to short term (i.e. regulation of the activity of different enzymes by direct phosphorylation) and long-term effects (i.e. regulation of transcriptional activity of different transcription factors) (Hardie, 2007). In this work we describe the use of the yeast two-hybrid technology to identify novel binding partners for the different subunits of the AMPK complex. Of the interacting proteins indentified, we have focused our attention on PSMD11, a non-ATPase subunit of the lid of the proteasome.

Proteasomes are one of the major pathways of intracellular protein degradation in mammalian cells (Ciechanover, 2005; Hershko, 2005; Rose, 2005; Varshavsky, 2005). The 26S proteasome complex consists of the 20S catalytic core, where the proteins are degraded, plus one or two 19S regulatory complexes. The 19S complex is composed of at least 19 different subunits that form a lid and a base-like structure. The base includes six ATPases that interact with the 20S proteolytic core; these ATPases have chaperone functions and are required for the unfolding of substrates and their translocation to the 20S proteolytic chamber. The lid provides the binding sites for poly-ubiquitinated substrates and a deubiquitinating activity involved in recycling of ubiquitin moieties upon substrate degradation (Bajorek and Glickman, 2004; Wolf and Hilt, 2004;Meiners et al., 2008). The lid is clustered into two groups of proteins, one that is close to the base, which is formed by four subunits, PSMD3 (Rpn3), PSMD6 (Rpn7), PSMD8 (Rpn12) and SHFM1 (Rpn15) (in parenthesis, the name of the yeast orthologue subunits), and another which lays on top of the first cluster, which is composed of five subunits, PSMD7 (Rpn8), PSMD11 (Rpn6), PSMD12 (Rpn5),

PSMD13 (Rpn9) and PSMD14 (Rpn11) (Fu et al., 2001; Sharon et al., 2006; Murata et al., 2009). PSMD11 (Rpn6) is a non-ATPase subunit of the lid of the proteasome. It is a protein of 422 amino acids, initially defined as the p44.5 subunit of the lid of the human proteasome (Hoffman and Rechsteiner, 1997; Saito et al., 1997), which can complement the lethality of a yeast *rpn6* mutant (Santamaria et al., 2003). PSMD11 has been reported to interact with the C-terminus of NF-kB2/p100, but only when this domain is previously phosphorylated and ubiquitinated (Fong et al., 2002). We describe in this work that PSMD11 physically interacts with both the catalytic AMPKα2 and the regulatory AMPKβ2 subunits of the AMPK complex, and that AMPK can modify the phosphorylation status of PSMD11.

MATERIALS AND METHODS

1.- Microorganisms, culture conditions and genetic methods.

Escherichia coli DH5α was used as the host strain for plasmid constructions and protein production. It was grown in LB (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.5) medium supplemented with 50 mg/l ampicillin. Yeast strains used in this work were CTY10-5d (*MAT*a *ade2 his3 leu2 trp1 gal4 gal80 URA3*::lexAop-lacZ; gift from R. Sternglanz, State University of New York, Stony Brook, USA) and TAT7 (*MAT*a *ade2 his3 leu2 trp1 gal4 gal80 LYS2*::*lexAop-HIS3*, *URA3*::lexAop-lacZ; gift from R. Sternglanz, State University of New York, Stony Brook, USA). Yeast transformation was carried out using the lithium acetate protocol (Ito et al., 1983). Yeast cultures were grown in synthetic complete (SC) medium lacking the corresponding supplements to maintain selection for plasmids (Rose et al., 1990).

2.- Plasmids.

Plasmids used in this study were pBTM116-AMPKα2, pBTM116-AMPKα2 (T172D), pBTM116-AMPKβ2, pACT2-AMPKβ2, pBTM116-AMPKγ1, pWS93-AMPKγ1 (Solaz-Fuster et al., 2006) and pBTM116-AMPKγ3A (34-489) (Viana et al., 2007). The pCMV-HA-AMPKα2 and pCMV-HA-AMPKβ2 plasmids were described in (Solaz-Fuster et al., 2006).

pACT2-PSMD11 was obtained in the two-hybrid screening. It contained the complete ORF of PSMD11 fused in frame to the Gal4 activation domain of the pACT2 plasmid (Legrain et al., 1994). The pCMV-myc-PSMD11 plasmid was constructed by digesting plasmid pACT2-PSMD11 with SfiI and XhoI and subcloning the fragment into plasmid pCMV-myc (BD-Biosciences) digested with the same enzymes. The pGEX6P1-PSMD11 plasmid was constructed by digesting plasmid pACT2-PSMD11 with SmaI and XhoI and subcloning the fragment into plasmid pGEX6P1 (Amersham Biosciences) digested with the same enzymes. The pBTM116-PSMD11 plasmid was obtained by digesting plasmid pACT2-PSMD11 with BamHI and SalI and subcloning the fragment into plasmid pBTM116 (Vojtek et al., 1997), digested with the same enzymes. Oligos PSMD11-5 (5'-GCATGGATCCCGATGGCGGCGGCGGCGGTGGTG-3') and PSMD11-2 (5'-GACCCTCGAGCTATGCCCGGTAATCTGTCAGAGC-3') were used to amplify by PCR a fragment comprising the first 311 amino acids of PSMD11, using pACT2-PSMD11 as a template. This fragment was sequenced to check that the Taq polymerase had not introduced undesired mutations, digested with BamHI and XhoI and subcloned into plasmid pBTM116 to obtain the pBTM116-PSMD11 (1-311) plasmid. Similarly, oligos PSMD11-3 (5'-GGCGGAATTCGAGCTCCGGGATGACCCAATC-3') and PSMD11-4 (5'-GACCCTCGAGCTATGTCAGTTTCTTGGCTTTG-3') were used to amplify a fragment comprising the C-terminal domain of PSMD11 (312-422). This

fragment was digested with EcoRI and XhoI and subcloned into pBTM116 to obtain plasmid pBTM116-PSMD11 (312-422).

The pBTM116-PSMD12 plasmid was obtained amplifying by PCR the PSMD12 ORF from a brain cDNA library with oligos PSMD12-1 (5'-GGCTGAATTCATGGCGGACGGCGGCTCGGAG-3') and PSMD12-2 (5'-GATCGTCGACTTATTGTAGATTATGTATCATC-3'), digesting the fragment with EcoRI and SalI and subcloning it into pBTM116. Similarly, a fragment containing the PSMD13 ORF was amplified using oligos PSMD13-3 (5'-GGGCCATGGAGAATTCTTATGAAGGACGTACCGGGCTTCC-3') and PSMD13-4 (5'-GGGCTCGAGCTAGGTGAGGATGTCATGGGCCTG-3'), digested with EcoRI and SalI and subcloned into pBTM116 to obtain plasmid pBTM116-PSMD13. Finally, a fragment containing the CSN2 ORF was amplified using oligos CSN2-1 (5'-GGGGGATCCTTATGTCTGACATGGAGGATGATTTCATG-3') and CSN2-2 (5'-CCCGTCGACTTAAGCCAGTTTACTGACTACAGCC-3'), digested with BamHI and SalI and subcloned into pBTM116 to obtain pBTM116-CSN2.

3.- Yeast two-hybrid screening.

A two-hybrid screening (Fields and Song, 1989) for proteins that interacted with LexA-AMPKα2-T172D (plasmid pBTM116-AMPKα2 (T172D), see above) was carried out in strain TAT7 (see above) co-expressing AMPKγ1 (plasmid pWS93-AMPKγ1; see above). The strain was transformed with a commercial human skeletal muscle cDNA library (Clontech). Transformants were selected in SC+2%glucose plates lacking tryptophan, leucine, uracil and histidine and were subsequently screened for β-galactosidase activity using a filter lift assay (Yang et al., 1992). Additional two-hybrid screenings of the same cDNA library were conducted using LexA-AMPKβ2 (plasmid

pBTM116-AMPKβ2) or LexA-AMPKγ3 (34-489) (plasmid pBTM116-AMPKγ3A (34-489)) as baits. β-galactosidase activity was measured in permeabilized yeast cells and expressed in Miller units as described by Ludin and collaborators (Ludin et al., 1998).

4.- Co-immunoprecipitation assay.

Mammalian HEK293 cells were transfected with the corresponding plasmids by the calcium phosphate transfection protocol. Twenty-four hours after transfection, cells were harvested with lysis buffer [50 mM TricHCl pH. 7.5, 10 mM NaCl, 2 mM EDTA; 15% glycerol, 1% nonidet P40, complete protease inhibitor cocktail (Roche) and 1 mM PMSF]. Cells were lysed by successive rounds of freezing and thawing. Cell lysates were then centrifuged at 1000 x g for 15 min and the soluble fraction was collected for immunoprecipitation. Co-immunoprecipitation experiments were carried out with 300 μg of crude protein extracts, in a final volume of 500 μl of lysis buffer, and one μl of anti-HA monoclonal antibodies (Sigma Co) or one μl of preimmune serum, as in (Solaz-Fuster et al., 2006). Mouse monoclonal anti-myc (BD-Biosciences) antibodies were used for immunoblotting. Antibodies were detected by enhanced chemiluminescence (ECL) with ECL or ECL plus reagents (Amersham Biosciences).

5.- Expression of recombinant proteins in E.coli.

E.coli transformants harboring the pGEX6P1-PSMD11 plasmid were grown in 500 ml of LB/ampicillin. Transformants were grown at 37ºC until the absorbance at 600 nm reached a value of around 0.3. IPTG was then added to a final concentration of 0.1 mM, and cultures were maintained overnight at 25 ºC. Cells were harvested and resuspended in 20 ml of sonication buffer [50 mM HEPES-NaOH pH 7.0, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM DTT, 2 mM PMSF and complete

protease inhibitor cocktail (Roche)]. Cells were disrupted by sonication and the fusion proteins purified by passing the extracts through columns containing 1 ml bed volume of glutathione-sepharose (Amersham Biosciences). GST-fusion proteins were eluted from the column with 25 mM glutathione. Samples were stored at -80ºC.

6.- AMPK in vitro phosphorylation assay.

Two hundred ng of purified GST-fusion proteins were phosphorylated with 50 mU of AMPK (Upstate), in a final volume of 25 µl of a buffer containing 20 mM HEPES-NaOH pH 7.0, 1 mM dithiothreitol, 5 mM $MgCl_2$, 200 µM AMP and 100 µM of a mixture of γ-^{32}P-ATP (3000Ci/mmol) and cold ATP. The reaction was incubated at 30ºC for 1 hour and stopped by boiling the mixtures in sample buffer. Samples were analyzed by SDS-PAGE and autoradiography. 200 ng of GST-fusion proteins were analyzed by SDS-PAGE and stained with Coomassie blue. For stoichiometry analysis, similar samples were stopped after 5, 10, 20, 40 and 60 min and analyzed by SDS-PAGE and phosphorimaging for the measurement of ^{32}P incorporation. A GST-ACC fusion protein, a generous gift of Dr. Mhairi Towler (University of Dundee), was used as a control (Scott et al., 2002).

7.- 2D-electrophoresis.

Mammalian HEK293 cells were transfected with plasmid pCMV-myc-PSMD11 and 24 hours after transfection, extracts were prepared in urea lysis buffer (9.7 M urea, 4% CHAPS, 20 mM DTT). Myc-PSMD11 was analyzed by two-dimensional electrophoresis (2D-E) using an IPGphor (Amersham Bioscience, Uppsala, Sweden) instrument. For the first dimension (IEF), 50 µg of total protein (in 100 µl of 9.7 M urea, 4% CHAPS, 20 mM DTT and 0.5 % IPG buffer) were loaded on a 7 cm IPG strip (pH

range 4-7) using the following focusing conditions: 500 V for 30 min, 1000 V for 30 min and 5000 V for 80 min. Electrophoretic separation (second dimension) was performed using SDS-PAGE.

8.- *λ-phosphatase treatment.*

Mammalian HEK293 cells were transfected with plasmid pCMV-myc-PSMD11 and 24 hours after transfection, extracts were prepared in λ-phosphatase buffer (50 mM Tric-ClH pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij35) containing 2 mM $MnCl_2$. Fifty micrograms of crude extract were treated at 30ºC for 30 min with 50 Units of λ-phosphatase (New England Biolabs). Reaction was stopped by adding two volumes of urea lysis buffer and analyzed by 2-D electrophoresis as above.

RESULTS

1.- Identification of novel interaction partners of AMP-activated protein kinase in human skeletal muscle.

1.1.- AMPKα2(T172D) plus AMPKγ1 as bait.

Studies on the distribution of the different AMPK subunits in human skeletal muscle have demonstrated that the α2-β2 subunits account for the majority of the AMPK complexes. AMPKγ3 was found to be associated with α2-β2 and accounted for 20% of the total AMPK activity. The AMPKγ1 subunit was responsible for the remaining activity and AMPKγ2 was shown to play only a minor role (Wojtaszewski et al., 2005). In addition, it has been described that exercise activates α2 but not α1, indicating that α2-containing complexes may be involved in the metabolic response to exercise (Fujii et al., 2000; Winder, 2001). For these reasons and in order to identify novel interaction partners of the AMPK complex in human skeletal muscle, we

designed a yeast two-hybrid screening in which we used the constitutively active form of human AMPKα2 (AMPKα2 T172D) as bait. In order to obtain a proper AMPK trimeric complex, we overexpressed the human AMPKγ1 subunit in yeast cells. We chose this approach based on our previous results showing that AMPKα2 interacts with Gal83, the yeast AMPKβ subunit orthologue, but it does not interact with Snf4, the yeast AMPKγ subunit orthologue; in addition, AMPKγ1 can also interact with Gal83 (Gimeno-Alcaniz and Sanz, 2003; Sanz, 2008). In this way, a LexA-AMPKα2T172D-AMPKγ1-Sc.Gal83 complex could be formed. Yeast cells expressing these constructs were used in a two-hybrid screening using a commercial human skeletal muscle cDNA library. We screened 10^6 transformants obtaining 85 initial positive clones. Plasmids containing the cDNA sequence of the putative interaction partners were extracted and reintroduced into the reporter yeast strain in order to confirm the interaction. Additionally, they were introduced into yeast cells expressing AMPKγ1 and only LexA in order to check for self-activating properties of the corresponding cDNAs. Only those plasmids that did not have self-activating properties and maintained the interaction with the bait were further analyzed. The cDNA of these plasmids was sequenced and the sequences characterized by BLAST analysis (Altschul et al., 1997). Thirty three of the plasmids were excluded from further analysis because they are likely to represent false positives (i.e. the cDNA sequence was out of frame or corresponded to untranslated or genomic sequences). The remaining 10 positive clones encoded full-length or almost full-length in-frame cDNA inserts (Table I). We quantified the strength of the interaction by measuring the β-galactosidase activity in these selected transformants (Table I).

In the screening, we obtained the AMPKβ2 subunit, which represents a good internal control for the procedure (Table I). We also identified a group of cDNAs

encoding different cytoskeleton binding proteins, such as Nebulin-related anchoring protein (NRAP), which performs an anchoring function linking the terminal actin filaments of myofibrils to protein complexes located beneath the sarcolemma (Luo et al., 1997); tropomodulin 1 (TMOD), a tropomyosin-regulating protein that binds specifically to the N-terminus of tropomyosin and blocks elongation and depolarization of tropomyosin-coated actin filaments (Fowler et al., 1993); and myozenin 1 (MYOZ1), a muscle-specific calcineurin interacting protein that co-localizes with the Z disc proteins alpha-actinin and gamma-filamin (filamin C) and tethers calcineurin to the sarcolemma (Takada et al., 2001). Moreover, we identified a group of cDNAs encoding for transcriptional regulators, such as pre-B-cell leukemia transcription factor interacting protein 1 (PBXIP1), which inhibits the binding of the PBX1-HOX complex to DNA and blocks the transcriptional activity of E2A-PBX1 (Abramovich et al., 2000). Two other transcriptional regulators indentified in the screening were: the SNW domain containing 1, also named SKI-interacting protein (SKIIP), a transcriptional co-activator that functions to facilitate vitamin D and other nuclear receptor-mediated transcriptional pathways (Baudino et al., 1998), and thyroid receptor interacting protein 6 (TRIP6), a transcriptional co-activator of NF-kB-regulated genes (Solaz-Fuster et al., 2006). It is worth pointing out that TRIP6 was previously identified by our group in an independent two-hybrid screening of a human pancreas cDNA library using LexA-AMPKα2 as bait (Solaz-Fuster et al., 2006). In addition, we identified a cDNA encoding the ubiquitin-conjugating enzyme UBE2I, a small ubiquitin-related modifier (SUMO)-conjugating enzyme that participates in the sumoylation of different proteins (Watanabe et al., 1996) and a cDNA encoding a hypothetical protein (C19orf47) of unknown function (Wiemann et al., 2004).

1.2.- AMPKβ2 subunit as bait.

Since it is thought that AMPKβ subunits determine the subcellular localization and confer substrate specificity to the AMPK complex (Sanz, 2008), we performed an additional yeast two-hybrid screening of human skeletal muscle using LexA-AMPKβ2 as bait. We screened 10^6 transformants and obtained 142 initial positive clones. As above, plasmids containing the cDNA sequence of the putative interaction partners were extracted and tested for reproducibility of the interaction, self-activating properties and their sequences characterized by BLAST analysis. Only 17 positive clones were further analyzed, since they encoded full-length or almost full-length in-frame cDNA inserts (Table II). We quantified the strength of the interaction by measuring the β-galactosidase activity in these selected transformants (Table II). In the screening, we identified the AMPKα2 and AMPKγ1 subunits, which validated the procedure. We also identified a group of cDNAs encoding regulators of other protein kinases, such as guanine nucleotide-binding protein beta2 like1 (GNB2L1), also named receptor for activated protein kinase C (RACK1), which is implicated in regulating the function of protein kinase C (PKC) (Ron et al., 1994); the casein kinase 2 beta subunit (CSNK2B), the regulatory subunit of casein kinase 2 complex (Rodriguez et al., 2008); and DNA damage inducible transcript 4-like (DDIT4L), also named protein regulated in development and DNA damage response 2 (REDD2) or hypoxia induced factor 1 (HIF1)-response protein RTP801-like, which has been recently described as a negative regulator of the mammalian target of rapamycin kinase (mTOR) pathway (Corradetti et al., 2005). Moreover, we identified a group of cDNAs encoding proteins related to the cytoskeleton, such as gamma filamin (FLNC), a member of the family of actin-binding proteins involved in reshaping of the cytoskeleton (Thompson et al., 2000) and dystonin 1 (isoform 2) (DST), a cytoskeleton linker protein capable of anchoring neuronal

intermediate filaments to the actin cytoskeleton (Leung et al., 2001). Additionally, we identified cDNAs encoding the non-ATPase subunit 11 of the 19S regulatory particle of the proteasome (PSMD11), which plays a crucial role in the organization of the lid of the proteasome (Hoffman and Rechsteiner, 1997), ferredoxin 1 (FDX1), an iron-sulfur protein involved in steroid, vitamin D and bile acid metabolism (Jefcoate et al., 1986) and a hypothetical protein (C7orf20) of unknown function (Wiemann et al., 2004).

1.3.- AMPKγ3 subunit as bait.

Although complexes containing AMPKγ3 account only for 20% of the total AMPK activity in human skeletal muscle, this subunit has been proposed to play a major role in regulating AICAR (an activator of AMPK) induced AMPK-dependent glucose uptake and in restoring glycogen levels after exercise (Barnes et al., 2004). In order to identify putative interaction partners of AMPKγ3, we performed a yeast two-hybrid screening of human skeletal muscle using this subunit as bait. However, we noticed that a fusion between LexA and full-length AMPKγ3 had self-activating properties that prevented this type of analysis. For this reason, we used an N-terminal truncated form of AMPKγ3, lacking the first 33 amino acids [AMPKγ3-A; (Viana et al., 2007)] which lacked the self-activating properties. We screened 10^6 transformants and obtained 44 initial positive clones. After the quality controls described above, only 7 clones were further analyzed and the strength of the interaction was quantified in these transformants by measuring the β-galactosidase activity (Table III). We identified cDNAs encoding alpha-crystallin (CRYAB), a member of the small heat-shock protein family (Dubin et al., 1989) and three proteins of unknown function: LIM and cystein domains rich 1 (LMCD1), brain protein I3 (BRI3) and neuronal outgrowth associated isoform 2 (NGRN) (Wiemann et al., 2004).

2.- Analysis of the physical interaction between AMPK and PSMD11, a non-ATPase subunit of the lid of the proteasome.

Among the interaction partners described above, PSMD11 (non-ATPase subunit 11 of the 26S proteasome), which was isolated from four independent clones, was the protein with a defined function that interacted stronger (higher levels of β-galactosidase activity) with a subunit of the AMPK complex (AMPKβ2). Since we have recently described an involvement of AMPK in regulating the activity of the proteasome (activation of AMPK with AICAR or metformin leads to a decrease in proteasome function) (Viana et al., 2008), we selected PSMD11 for further analysis. We first studied whether PSMD11, in addition to interacting with AMPKβ2, was able to interact with the other subunits of the AMPK complex. As shown in Table IV, yeast two-hybrid analyses indicated that PSMD11 was able to interact with AMPKα2, although to a lesser extent, but not with AMPKγ1. In order to confirm the physical interaction between PSMD11 and AMPKα2 and β2 subunits, we performed co-immunoprecipitation experiments. As shown in Fig. 1, when crude extracts from mammalian HEK293 cells expressing HA-AMPKα2 and myc-PSMD11 were immunoprecipitated with anti-HA antibodies, myc-PSMD11 was recovered in the immunoprecipitate. The same result was obtained when crude extracts from HEK293 cells expressing HA-AMPKβ2 and myc-PSMD11 were used in the assay (Fig. 1). The observed co-immunoprecitation was specific since it did not occur in crude extracts from HEK293 cells expressing myc-PSMD11 and an empty plasmid expressing only HA, nor did it occur if pre-immune serum was used in the assay instead of the anti-HA antibodies (Fig. 1). We also performed co-immunoprecipitation analyses in crude extracts from HEK293 cells expressing HA-PSMD11 and myc-AMPKα2 or myc-

AMPKβ2. Affinity purifying with anti-HA antibodies, we detected myc-AMPKα2 and myc-AMPKβ2 in the corresponding immunoprecipitates (not shown). Taken together, these results confirmed the physical interaction between PSMD11 and two subunits of the AMPK complex: AMPKα2 and AMPKβ2.

PSMD11 is a protein of 422 amino acids with a PCI (Proteasome, COP9, eIF3) domain at its C-terminus. PCI is a domain present in several subunits of the lid of the proteasome (PSMD11, PSMD12, PSMD13, PSMD3 and PSMD6) and in similar subunits of the COP9/signalosome and eIF3 (mediator of translation initiation) complexes. PCI domains are involved in protein-protein interactions and are essential for the correct assembly of the lid of the proteasome (Glickman et al., 1998; Fu et al., 2001; Glickman and Ciechanover, 2002). In order to map the interaction of the AMPKβ2 subunit within the PSMD11 protein, we constructed two truncated forms of PSMD11, one containing the N-terminal domain, but lacking the PCI domain [PSMD11 (1-311)] and the other comprising the C-terminus of the protein which contains the PCI domain [PSMD11 (312-422)]. Yeast two-hybrid analyses indicated that only the construct that contained the C-terminus of the protein was able to interact with AMPKβ2, although to a lesser extent (Table V). Since the PCI domain is present in other subunits of the lid of the proteasome, we tested whether AMPKβ2 was able to interact with some of these subunits. However, we could not detect any two-hybrid interaction between AMPKβ2 and PSMD12, PSMD13 or CSN2 (a subunit of the COP9/signalosome complex closely related to PSMD11) (Table V). These results suggest that AMPKβ2 interacts with the C-terminus of PSMD11 and that the interaction does not depend on the presence of a PCI domain itself, but on regions of PSMD11 not present in the other PCI-containing proteins tested.

Since PSMD11 interacted with the AMPK complex, we checked whether AMPK could phosphorylate this protein *in vitro*. We purified a GST-PSMD11 fusion protein expressed in bacteria for use in *in vitro* phosphorylation assays. As shown in Fig. 2A, AMPK phosphorylated GST-PSMD11 *in vitro*. We determined the stoichiometry of the reaction and compared it with the one obtained for a GST-fusion protein containing residues 60-94 of mammalian ACC (Acetyl-CoA Carboxylase), a known substrate of AMPK, which is phosphorylated at only one site (Scott et al., 2002). Our results indicated that PSMD11 was phosphorylated to a lesser extent than ACC, suggesting the presence of only one potential phosphorylation site in PSMD11 (Fig. 2B).

We next analyzed whether AMPK could modify the phosphorylation status of PSMD11 *in vivo*. Mammalian HEK293 cells were transfected with a plasmid expressing myc-PSMD11 and crude extracts were analyzed by two-dimensional electrophoresis. As shown in Fig. 2C, in the 2-D gels, myc-PSMD11 was detected as a single spot. However, if cells were treated with AICAR (an activator of AMPK), we observed the appearance of a second spot towards the positive pole, with a difference in isoelectric point of 0.11 units, which is compatible with the acquisition of one phosphate group (Fig. 2C). Similar results were obtained if the cells were co-transfected with a combination of plasmids that forms a constitutively active AMPK complex (AMPKα2 T172D, AMPKβ2, AMPKγ1; CA-AMPK) (Fig. 2C). We also treated the cells with phenformin, a stronger AMPK activator, and observed in this case only one spot that moved to the position of the right spot in the analysis described above (Fig. 2C). Interestingly, if the crude extracts from phenformin treated cells were dephosphorylated with an exogenous phosphatase (λ-phosphatase), one major spot was detected that moved leftwards to the position of the spot observed in the crude extracts from

untreated cells (Fig. 2C). These results indicate that AMPK activation can induce the phosphorylation of PSMD11 *in vivo*.

We then searched the PSMD11 protein sequence for putative AMPK consensus phosphorylation sites, Hyd-(Basic,X)-X-X-Ser/Thr-X-X-X-Hyd, where Hyd can be Leu, Met, Ile, Phe or Val, and Basic: Arg, Lys or His (Hardie et al., 2003). We found two sites that fulfilled these conditions: FQRAQS$_{14}$LLST and VERKLS$_{366}$QMIL. In addition, recent global phosphoproteome analyses indicated that Ser14, Ser79 and Ser272 of PSMD11 were phosphorylated under certain conditions (Olsen et al., 2006; Wang et al., 2007; Dephoure et al., 2008). In order to check if one of these residues was phosphorylated by AMPK, we constructed S14A, S79A, S272A and S366A mutants, expressed and purified them in bacteria as GST-PSMD11 fusion proteins and subjected them to *in vitro* phosphorylation by AMPK. Unfortunately, all the mutants were phosphorylated to the same extent as wild type, so the putative AMPK phosphorylation site of PSMD11 remains unknown.

DISCUSSION

In this work we have used of the yeast two-hybrid technology to identify novel interaction partners for AMPK. The procedure appeared to be more successful when using AMPKα2 or AMPKβ2 subunits as bait than when using AMPKγ3. This result probably reflects the functions of the AMPKγ subunits to regulate the activity of the trimeric complex by binding allosteric regulators (AMP, ATP), rather than actively participating in conferring substrate specificity. Among the novel interactors, we have found that both AMPKα2 and AMPKβ2 interact with proteins related to the regulation of the cytoskeleton, suggesting that AMPK might play a role in this process. Moreover, we describe that AMPKα2 interacts with different components of the transcription

machinery, reinforcing the role of AMPK in transcriptional regulation (Leff, 2003; McGee and Hargreaves, 2008). An additional concept that arises from our results is that AMPKβ2 interacts with different regulatory components of alternative protein kinases, suggesting that AMPK could participate in the regulation of the activity of these proteins. However, all these results must be confirmed by alternative methods to verify the true role for AMPK in these new pathways.

Several approaches have been described so far to identify new AMPK interacting proteins, i.e. affinity purification coupled to mass-spectrometry (Al-Hakim et al., 2005; Ewing et al., 2007), multidimensional substrate-screen (Tuerk et al., 2007) or even two-hybrid screening in bacteria (Fu and Gao, 2009). However, when comparing our results with those reported previously, it is clear that depending on the technique used, a different set of interacting proteins are identified. In fact, when we consulted the major interaction protein databases, i.e. IntAct (Kerrien et al., 2007), BioGRID (Breitkreutz et al., 2008) and UniHI (Chaurasia et al., 2009), the majority of the entries for the different AMPK subunits were reported by only one method. This is probably a reflection of the fact that our knowledge regarding AMPK interacting proteins is still far from being complete.

In this work, we have further characterized the interaction of AMPK with PSMD11, a non-ATPase subunit of the lid of the 26S proteasome. We have confirmed this interaction by co-immunoprecipitation and observed that AMPK can modify the phosphorylation status of PSMD11, both *in vitro* and *in vivo*. We have tried to identify the AMPK phosphorylation site(s) by mutating residues in putative AMPK consensus sites but all the tested mutants were still phosphorylated by AMPK to the same extent as wild type. Additionally, we purified a His-tagged-PSMD11 protein from mammalian cells grown under conditions of AMPK activation (phenformin treatment) and subjected

it to mass spectrometry analysis, using an approach similar to that which we recently reported for another interaction partner of AMPK (R5/PTG) (Vernia et al., 2009). However, no phosphorylated peptides were identified. So, we do not have at the moment any information about the putative AMPK phosphorylation site in PSMD11.

As we have recently reported that AMPK downregulates the activity of the proteasome (Viana et al., 2008), perhaps it performs this function by affecting the phosphorylation status of PSMD11. Additionally, analysis of the data in the UniHI protein interaction database (Chaurasia et al., 2009), indicates that both the AMPKα and AMPKβ subunits interact with alternative non-ATPase subunits of the lid of the proteasome (PSMD6 and PSMD7), which may identify the lid of the proteasome as a novel target of AMPK action. In this way, AMPK could participate in the metabolic control of proteasome function, as was recently described for another metabolic kinase, protein kinase A (PKA) (Zhang et al., 2007a; Zhang et al., 2007b).

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FIGURE LEGENDS

Fig. 1: PSMD11 co-immunoprecipitates with AMPKα2 and AMPKβ2. Protein extracts (300 μg) were prepared from HEK293 cells, transfected with plasmids pCMV-myc-PSMD11 and pCMV-HA-AMPKα2, pCMV-HA-AMPKβ2 or the empty plasmid pCMV-HA. One μl of anti-HA or pre-immune serum (ϕ) was used to immunoprecipitate the extracts (IP). Pelleted proteins were analyzed by SDS-PAGE and immunodetected with anti-myc (upper panel) and anti-HA (lower panel) monoclonal antibodies. Proteins in the input crude extracts (CE; 30 μg) were also immunodetected with anti-HA and anti-myc antibodies. Molecular weight standards are indicated on the left on each panel.

Fig. 2: AMPK modifies the phosphorylation status of PSMD11. A) AMPK phosphorylates a GST-PSMD11 fusion protein *in vitro*. GST and GST-PSMD11 (200 ng) produced in bacteria were phosphorylated *in vitro* using 50 mUnits of AMPK (Upstate) and [γ-^{32}P]ATP, in the presence of 200 μM AMP. Samples were analyzed by SDS-PAGE, stained with Coomasie blue and analyzed by autoradiography. Molecular weight standards are indicated on the left. B) Stoichiometry analysis of the *in vitro* phosphorylation of GST-ACC (control) and GST-PSMD11 by AMPK (see Materials and Methods). Aliquots of the corresponding reaction mixtures were removed at the indicated times for SDS-PAGE and phosphorimaging for the measurement of ^{32}P incorporation (molecular weight standards are indicated on the left). Values are expressed as mol incorporated phosphate per mol of protein (lower panel). C) AMPK phosphorylates PSMD11 *in vivo*. Cell extracts from HEK293 cells transfected with plasmid pCMV-myc-PSMD11 were analyzed by 2D-electrophoresis and western blotting using anti-myc monoclonal antibodies (upper panel). Cell extracts from

HEK293 cells treated with AICAR (0.5 mM, 2h) or with phenformin (5 mM, 2h) were analyzed similarly. Cell extracts from phenformin treated cells were also dephosphorylated with λ-phosphatase as described in Materials and Methods and analyzed as above (lower panel). HEK293 cells were also co-transfected with plasmid pCMV-myc-PSMD11 and a combination of plasmids that reconstituted a constitutively active form of the AMPK complex (pcDNA3-AMPKα2 T172D, pcDNA3-AMPKβ2 and pcDNA3-AMPKγ1; CA-AMPK); cell extracts were obtained and analyzed similarly. Molecular weight standards are indicated on the right.

Table I: Positive clones identified in two-hybrid screening of a human skeletal muscle cDNA library using LexA-AMPKα2(T172D) as bait (co-expressed with AMPKγ1). Yeast TAT7 cells transformed with plasmids pBTM116-AMPKα2-T172D and pWS93-AMPKγ1 were co-transformed with a human skeletal muscle cDNA library. Positive clones were subjected to BLAST analysis and protein interaction estimated by measuring the β-galactosidase activity.

Clon nº	Encoded protein (gene name)	NCBI mRNA accession nº	Covered protein fragment	β-Galactosidase (Units)
pA-41	Beta2 AMPK subunit (PRKAB2)	NM_005399	Complete ORF	318
pA-3	Nebulin related anchoring protein (NRAP)	NM_198060	Last 1477/1730 aa	196
pA-33	Tropomodulin I (TMOD)	BC002660	Complete ORF	183
pA-12	Ubiquitin-conjugating enzyme E2I (UBE2I)	NM_003345	Complete ORF	108
pA-46	Pre-b-cell leukemia homeobox interacting protein1 (PBXIP1)	NM_020524	Last 196/731 aa	76
pA-38	SNW domain containing 1 (SKIIP)	NM_012245	Complete ORF	65
pA-13	Thyroid receptor interacting protein 6 (TRIP6)	BC028985	Last 310/476 aa	59
pA-37, pA-40	Myozenin1 (MYOZ1)	NM_021245	Complete ORF	41
pA-17	Hypothetical protein in Chr19 (C19orf47)	NM_178830	Complete ORF	39
pA-0	none	-	-	25

Table II: Positive clones identified in two-hybrid screening of a human skeletal muscle cDNA library using LexA-AMPKβ2 as bait. Yeast TAT7 cells transformed with plasmid pBTM116-AMPKβ2 were co-transformed with a human skeletal muscle cDNA library. Positive clones were subjected to BLAST analysis and protein interaction estimated by measuring the β-galactosidase activity.

Clon nº	Encoded protein (gene name)	NCBI mRNA accession nº	Covered protein fragment	β-Galactosidase (Units)
pB-3/ pB-124	Alpha2 AMPK subunit (PRKAA2)	NM_006252	Complete ORF	694
pB-8/ pB-14	Hypothetical protein in Chr7 (C7orf20)	NM_015949	Complete ORF	580
pB-37	Gamma1 AMPK subunit (PRKAG1)	NM_002733	Complete ORF	546
pB-5/ pB-55/ pB-123/ pB-125	Proteasome 26S non-ATPase subunit 11 (PSMD11)	NM_002815	Complete ORF	460
pB-48	Ferredoxin 1 (FDX1)	NM_004109	Last 163/184 aa	313
pB-44/ pB-128	Guanine nucleaotide binding protein, beta polypeptide 2-like 1 (GNB2L1)	NM_006098	Last 138/317 aa and last 169/317 aa	250/ 43
pB-45/ pB-50	Gamma filamin (FLNC)	NM_001127487	Last 518/2725 aa and last 457/2725 aa	176/ 218
pB-141	Dystonin (DST)	NM_001144769	Last 524/3214 aa	73
pB-126	Casein kinase2, beta subunit (CSNK2B)	NM_001320	Complete ORF	72
pB-7	DNA damage inducible transcript 4-like (DDIT4L)	NM_145244	Complete ORF	34
pB-0	none	-	-	10

Table III: Positive clones identified in two-hybrid screening of a human skeletal muscle cDNA library using LexA-AMPKγ3(34-489) as bait. Yeast TAT7 cells transformed with plasmid pBTM116-AMPKγ3(34-489) were co-transformed with a human skeletal muscle cDNA library. Positive clones were subjected to BLAST analysis and protein interaction estimated by measuring the β-galactosidase activity.

Clon nº	Encoded protein (gene name)	NCBI mRNA accession nº	Covered protein fragment	β-Galactosidase (Units)
pG-22, pG-26	Alpha (B) crystallin (CRYAB)	NM_001885	Complete ORF	31
pG-5, pG-6	LIM and cystein domains rich 1 (LMCD1)	NM_014583	Last 129/365 aa	30
pG-11	Brain protein I3 (BRI3)	NM_015379	Complete ORF	28
pG-3, pG-33	Neugrin (NGRN)	NM_001033088	Last 79/219aa	14
pB-0	none	-	-	10

Table IV: Two-hybrid interaction of PSMD11 with the different AMPK subunits. Yeast TAT7 cells transformed with plasmids pBTM116 (empty), pBTM-AMPKα2, pBTM-AMPKβ2 and pBTM-AMPKγ1 were co-transformed with plasmid pACT2-PSMD11. Protein interaction was estimated using the yeast two-hybrid system, by measuring the β-galactosidase activity. Values correspond to means from 4-6 different transformants (standard deviation lower than 15% in all the cases).

Plasmids	β-Galactosidase (Units)
pBTM-AMPKα2 + pACT2-PSMD11	10
pBTM-AMPKβ2 + pACT2-PSMD11	460
pBTM-AMPKγ1 + pACT2-PSMD11	<1
pBTM116 + pACT2-PSMD11	<1

Table V: Two-hybrid interaction of AMPKβ2 with different domains of PSMD11. Yeast CTY10.5d cells transformed with plasmid pACT2-AMPKβ2 were co-transformed with plasmids pBTM116-PSMD11 (1-422, full length), pBTM116-PSMD11 (1-311) or pBTM116-PSMD11 (312-422). Additionally transformants containing plasmid pACT2-AMPKβ2 were also co-transformed with plasmids pBTM116-PSMD12, pBTM116-PSMD13 or pBTM116-CSN2 Protein interaction was estimated using the yeast two-hybrid system, by measuring the β-galactosidase activity. Values correspond to means from 4-6 different transformants (standard deviation lower than 15% in all the cases).

Plasmids	β-Galactosidase activity (Units)
pACT2-AMPKβ2 + pBTM116-PSMD11 (1-422)	90
pACT2-AMPKβ2 + pBTM116-PSMD11 (1-311)	<1
pACT2-AMPKβ2 + pBTM116-PSMD11 (312-422)	30
pACT2-AMPKβ2 + pBTM116-PSMD12	<1
pACT2-AMPKβ2 + pBTM116-PSMD13	<1
pACT2-AMPKβ2 + pBTM116-CSN2	<1

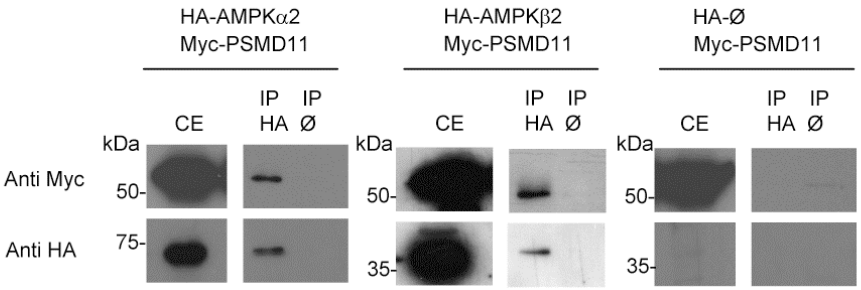
HA-AMPKα2
Myc-PSMD11
HA-AMPKβ2
Myc-PSMD11
HA-Ø
Myc-PSMD11
CE
IP HA
IP Ø
kDa
Anti Myc
Anti HA
50-
75-
35-

Fig. 1

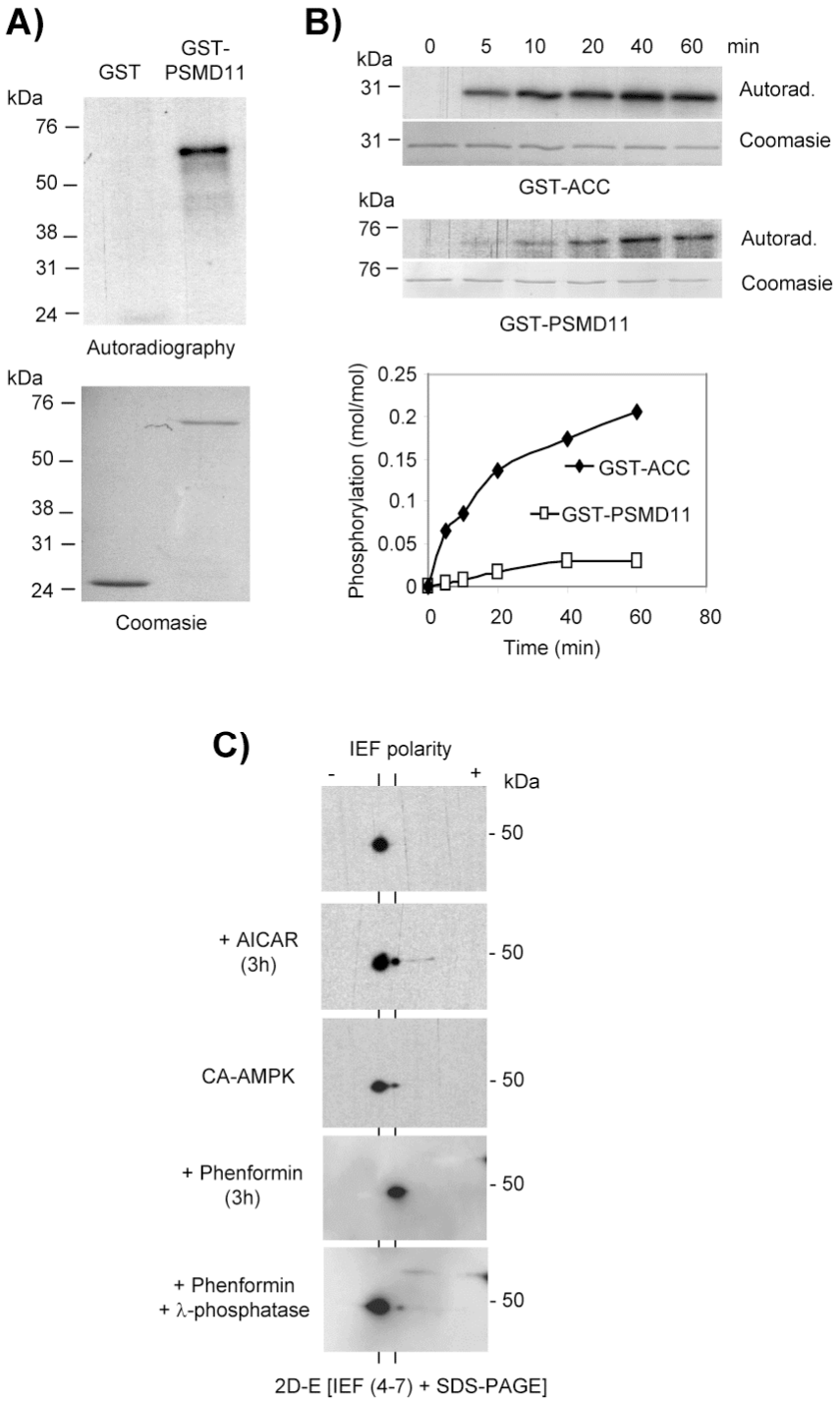
A)
GST
GST-PSMD11
kDa
76
50
38
31
24
Autoradiography
kDa
76
50
38
31
24
Coomasie
B)
0 5 10 20 40 60 min
kDa
31
Autorad.
31
Coomasie
GST-ACC
kDa
76
Autorad.
76
Coomasie
GST-PSMD11
Phosphorylation (mol/mol)
0.25
0.2
0.15
0.1
0.05
0
GST-ACC
GST-PSMD11
0 20 40 60 80
Time (min)
C)
IEF polarity
- +
kDa
- 50
+ AICAR (3h)
- 50
CA-AMPK
- 50
+ Phenformin (3h)
- 50
+ Phenformin + λ-phosphatase
- 50
2D-E [IEF (4-7) + SDS-PAGE]

Fig. 2