Plectin scaffolds recruit energy-controlling AMP-activated protein kinase (AMPK) in differentiated myofibres

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Summary
Plectin, a cytolinker protein greater than 500 kDa in size, has an important role as a mechanical stabiliser of cells. It interlinks the various cytoskeletal filament systems and anchors intermediate filaments to peripheral junctional complexes. In addition, there is increasing evidence that plectin acts as a scaffolding platform that controls the spatial and temporal localisation and interaction of signalling proteins. In this study we show that, in differentiated mouse myotubes, plectin binds to the regulatory γ1 subunit of AMP-activated protein kinase (AMPK), the key regulatory enzyme of energy homeostasis. No interaction was observed in undifferentiated myoblasts, and plectin-deficient myotubes showed altered positioning of γ1-AMPK. In addition we found that plectin affects the subunit composition of AMPK, because isoform α1 of the catalytic subunit decreased in proportion to isoform α2 during in vitro differentiation of plectin−/− myotubes. In plectin-deficient myocytes we could also detect a higher level of activated (Thr172-phosphorylated) AMPK, compared with wild-type cells. Our data suggest a differentiation-dependent association of plectin with AMPK, where plectin selectively stabilises α1-γ1 AMPK complexes by binding to the γ1 regulatory subunit. The distinct plectin expression patterns in different fibre types combined with its involvement in the regulation of isoform compositions of AMPK complexes could provide a mechanism whereby cytoarchitecture influences energy homeostasis.

Key words: Cytoskeleton, Cytolinker proteins, AMPK regulation, Myoblast differentiation, Z-lines

Introduction
The cytoskeleton determines the shape of cells, regulates cell movement and organises the cell interior, such as the compartmentalisation of intracellular signalling cascades (Janmey, 1998). Plectin, a versatile component of the cytoskeleton, is likely to have an important role in this scenario (for a review, see Wiche, 1998). The indispensability of plectin for muscle cell integrity became evident from the phenotypic analyses of plectin-deficient mice and the symptoms of EBS-MD patients suffering from skin blistering combined with muscular dystrophy (Andrä et al., 1997; Gache et al., 1996; McLean et al., 1996; Smith et al., 1996). Recent studies suggest that plectin acts as a scaffolding platform, determining the intracellular localisation and interaction of signalling proteins, thereby regulating the strength and speed of localised signalling events. In fact, a function of plectin in actin filament dynamics and an involvement of the protein in protein kinase C and FER kinase signalling cascades have been reported (Andrä et al., 1998; Lunter and Wiche, 2002; Osmanagic-Meyers and Wiche, 2004). In a recent screening of a cDNA library in the yeast two-hybrid system, we identified the γ1 subunit protein of AMP-activated protein kinase (AMPK), the key regulatory enzyme of energy homeostasis, as a new interaction partner of plectin. This is in line with a report by Larson et al. (Larson et al., 2002) showing naringin-sensitive phosphorylation of plectin in hepatocytes. Since the most important function of skeletal muscle is to convert chemical energy into physical movement, these findings raised our interest and prompted the present study.

The SNF1/AMPK cascade acts as a metabolic sensor or ‘fuel gauge’ of the cell (Carling, 2004; Hardie and Carling, 1997; Hardie et al., 1998; Hardie et al., 1999; Hardie et al., 2003; Winder, 2001). AMPK is a heterotrimeric protein, consisting of the regulatory γ subunit, the α subunit with the kinase domain, and the β subunit, which acts as a scaffold for the two other subunits (Hardie and Carling, 1997; Hardie et al., 1998). Homologues of all three subunits have been identified in mammals, fruitfly (Drosophila melanogaster), worm...
(Caenorhabditis elegans), yeast (Saccharomyces cerevisiae), plants (Arabidopsis thaliana) and the primitive protozoan Giardia lamblia, a degree of conservation that suggests that formation of the heterotrimeric complex is essential for functional kinase activity (Carling, 2004; Hardie et al., 2003). In mammals, each subunit is encoded by two or three genes (α1, α2, β1, β2, γ1, γ2, γ3). It seems that formation of the heterotrimeric complex is an essential requirement for at least some of the functions of the kinase (Carling, 2004). Differences in the tissue distribution of isoforms (Stapleton et al., 1999; Durante et al., 2002; Mahlapuu et al., 2004; Winder et al., 2003). The two different catalytic domains, suppressor LKB1, in a complex with STRAD and MO25, only recently been revealed, when it was shown that the tumour suppressor AMPK, leading to the activation of the kinase (Woods et al., 2004). AMP binding in turn promotes phosphorylation at Thr172 of its regulatory subunit (Thr172-P) AMPK were elevated in plectin+/− myotubes compared with their wild-type counterparts. Our findings suggest a novel role of plectin as a regulator of AMPK implying a mechanism where cytoarchitecture interacts with cellular energy homeostasis.

Results

AMPK γ1 subunit interacts with plectin

In a previous study, a C-terminal fragment of plectin comprising the intermediate filament-binding domain flanked by the repeat domains 5 and 6 (Ple-R 5-6, see Fig. 1A) was used as a bait for screening a 19-day-old mouse embryonic cDNA library in the yeast two-hybrid system (Osmanagic-Myers and Wiche, 2004). One of the positive clones identified contained the full-length cDNA of the AMPK regulatory subunit γ1. Among the three γ isoforms, which are encoded by different genes, γ1 (331 amino acid residues) lacks the long N-terminal domains present in γ2 (569) and γ3 (492) (Cheung et al., 2000). All of these subunits can form heterotrimeric complexes with catalytic α and regulatory β subunits.

To confirm AMPK γ1-subunit–plectin binding using an alternative method, co-immunoprecipitation (Co-IP) experiments were performed. When plectin was immunoprecipitated from lysates of differentiated mouse myoblasts, the AMPK γ1 subunit was indeed found to be part of the immunocomplexes (Fig. 1B). A similar Co-IP experiment performed with lysates from a plectin-deficient myoblast cell line (derived from plectin- and p53-deficient double-knockout mice) demonstrated the specificity of this interaction.

Fig. 1. Interaction of AMPK γ1 subunit with plectin. (A) Schematic representation showing the domain structure of plectin and the fragment (ple-R5-6; I4218-G4543; GenBank accession number AY480038) used as bait in the yeast two-hybrid screening (Osmanagic-Myers and Wiche, 2004). The actin- and IF-binding domains (ABD, IFBD), the rod and C-terminal repeat domains 1-6 of plectin are indicated. The isolated AMPK γ1 subunit clone contained the entire coding sequence comprising four CBS domains. (B) Plectin was immunoprecipitated from lysates of wild-type (+/+ ) myotubes using anti-plectin antisera. Lysates (Lys) and immunoprecipitates (IP) were analysed by immunoblotting using antibodies immunoreactive with AMPK γ1 subunit and plectin. Note Co-IP of AMPK γ1, and absence of signal in control sample (C) run without precipitating antibodies and in sample from plectin-deficient myocytes (+/−). (C) Immunoprecipitation of α1, α2, and γ1 AMPK subunits from lysates of differentiated wild-type myoblasts (myotubes) using indicated antibodies, and detection of cosedimenting α1 and γ1 AMPK subunits by immunoblotting.
assay, as in this case neither plectin nor the AMPK γ1 subunit were detectable in the IP fraction (Fig. 1B, lane −/−). Co-IP experiments using antibodies to individual AMPK subunits also showed that AMPK γ1 subunits formed heterocomplexes not only with α1, but also with α2 subunits, confirming the formation of distinct AMPK heterotrimers in differentiated myotubes (Fig. 1C).

Relocation of overexpressed γ1 AMPK in the absence of plectin

To study the localisation of the AMPK γ1 subunit in living cells we C-terminally fused its open reading frame to that of the green fluorescent protein or the Flag-epitope, and inserted these fusion constructs into retroviral expression plasmids (pBabe-puro) (Morgenstern and Land, 1990). Such plasmids were transfected into packaging cells and retroviral particles (harvested 48 hours after transfection) were directly used to infect mouse myoblasts, which were then subjected to differentiation.

To confirm that GFP-γ1 fusion proteins became integrated into the native AMPK complexes of cells, infected myoblasts and multinucleated myotubes derived from them after 6 days of differentiation were subjected to immunofluorescence microscopy using anti-AMPK subunit α1 antibodies (Fig. 2A-F). In myoblasts the ectopically expressed γ1-GFP fusion protein showed a punctuated and in part diffuse intracellular distribution, similar to the staining pattern of endogenous AMPK subunit α1 (Fig. 2A-C). In partially differentiated myotubes, both the γ1-GFP fusion protein and endogenous α1 appeared to be arranged in a filamentous pattern along the multinucleated tubes, revealing a cross-striated staining pattern in submembraneous regions of the fibres (Fig. 2D-F). These data demonstrated that the GFP-γ1 subunit indeed colocalised with the α1 subunit in differentiated myotube cultures.

The plectin-deficient myoblast cell line derived from plectin−/− and p53−/− double-knockout mice was also used to examine whether plectin had an influence on the subcellular distribution of AMPK. In their undifferentiated state these cells revealed staining patterns of ectopically expressed γ1-GFP and of endogenous α1 subunits that were very similar to those of the wild-type cells shown in Fig. 2A (data not shown). However, contrary to wild-type cells, no filamentous and/or striated intracellular alignment of the AMPK subunits was observed in differentiated plectin-deficient myotubes (Fig. 2G-I). Instead, the γ1-GFP fusion protein was diffusely distributed within the cell body. This showed that the proper positioning of AMPK subunits in differentiated myotubes was dependent on plectin.

Plectin-AMPK-complex formation is differentiation- and subunit-dependent

To visualise AMPK complexes and plectin in their physiological context without ectopic overexpression, myocytes at distinct stages of differentiation were double immunolabelled using antibodies to the AMPK complex and to plectin. Antibodies to the α1 subunit of AMPK turned out to be suited best to this type of experiment, and because the γ1 and α1 subunits codistributed at all stages of myocyte differentiation (see Fig. 2, Fig. 1C), monitoring the α1 subunit was considered indicative of the whole AMPK complex. Although in undifferentiated myoblasts both plectin and AMPK revealed a strong signal in the perinuclear region, there was hardly any codistribution observed in the cytoplasmic region (Fig. 3A-C). However, as soon as the cells reached multinucleated stages, prominent colocalisation of plectin and AMPK became apparent in a cross-striated pattern (Fig. 3D-F). These results were confirmed by immunofluorescence microscopy of frozen tissue sections of skeletal muscle from mouse. As shown below (Fig. 5A-E), plectin and AMPK complexes both generated a cross-striated staining pattern of skeletal muscle fibres, suggesting that plectin, a well-established constituent of Z-discs (Reipert et al., 1999; Wiche et al., 1983), has a role in recruiting AMPK complexes to these structures.

To demonstrate differentiation-dependent plectin-AMPK interaction on the biochemical level, Co-IP experiments were performed using detergent-solubilised cell lysates prepared

Fig. 2. Colocalisation of ectopically expressed GFP-AMPK γ1 subunit fusion protein with endogenous AMPK α1 subunit in wild-type (ple +/+ ) myocytes (A-F) and dislocation of AMPK in plectin-deficient (ple −/− ) myotubes (G-I). Wild-type or plectin-deficient mouse myoblasts transfected with expression plasmids encoding a GFP-γ1 subunit fusion protein were subjected to immunofluorescence microscopy either in their undifferentiated state (A-C), or after differentiation for 4 days (D-I), using antibodies to the α1 subunit of AMPK (A.D,G); the GFP-γ1 fusion protein was visualised directly (B,E,H). Note, colocalisation of the two AMPK subunits in wild-type cells (C,F), indicating that the overexpressed γ1-subunit became integrated into the native AMPK complex. In wild-type myocytes differentiated for 4 days, AMPK showed a filamentous arrangement (D-F) with a striated appearance in subsarcomembranous regions (see boxed area magnified in insert). In plectin−/− myotubes, expressing GFP-AMPK γ1 subunit fusion protein, the regular arrangement of AMPK (as seen in wild-type myotubes) is lost (G-I). Both, the overexpressed GFP-γ1 subunit fusion protein (H) and the endogenous AMPK α1 subunit (G) showed a more diffuse staining pattern compared with wild-type cells. Bars, 10 μm.
from undifferentiated and differentiated myocytes. Association of AMPK subunits with immunoprecipitated plectin was assessed by immunoblotting using antibodies to α1 and α2 AMPK subunits as a mixture, or individually. Since α subunits occur in the cell only in their heterotrimeric form (unpublished data), it could be assumed that the entire AMPK complex was pulled down. As shown using the antibody mixture (Fig. 4A,B), AMPK complexes co-precipitated with plectin only from differentiated myotubes, whereas they were absent from precipitates obtained from undifferentiated myoblasts. Thus this analysis correlated well with the immunolocalisation data. Interestingly, however, when immunoblotting was performed using antibodies to AMPK α1 and α2 subunits individually, it was revealed that only AMPK α1 and not α2 subunits co-precipitated with plectin (Fig. 4B). Complex formation of plectin specifically with the AMPK subunit α1 was confirmed by the reverse Co-IP experiment, where cosedimentation of plectin with immunoprecipitated AMPK subunit α1, but not with α2, was observed (Fig. 4C). These data strongly suggested that AMPK-plectin complex formation was not only differentiation-dependent but also subunit-dependent.

To confirm these data, we analysed skeletal muscle fibres from conditional muscle-specific plectin-knockout mice, generated by crossing mice carrying a floxed plectin gene with transgenic mice expressing Cre recombinase under the control of the muscle-specific creatine kinase promoter (MCK-Cre deleter mice). Tissue-specific disruption of the plectin gene...
resulted in up to 100% loss of the protein in mature skeletal muscle fibres, such as m. soleus (data not shown; details of the generation and genotypic characterisation of these mice will be published elsewhere). Such mice have the advantage over plectin−/− mice to be long-lived, providing an animal model in which the effects of plectin deficiency on muscle tissues can be studied at all stages of development. When cryosections of m. soleus from adult control wild-type mice were subjected to immunofluorescence microscopy, the staining pattern of AMPK α1 showed a high degree of overlap with that of plectin, as was particularly evident along Z-discs (Fig. 5A-E). In muscle fibres of corresponding plectin−/− mice, the staining intensity of AMPK α1 was much lower, and the striated staining pattern was considerably deteriorated in comparison to wild-type fibres (Fig. 5F-J). In marked contrast to AMPK α1, AMPK α2 did not colocalise with plectin at Z-discs in wild-type muscle fibres. In fact both staining patterns were striated, but mutually exclusive (Fig. 5K-O). Moreover, the staining pattern of AMPK α2 remained largely unaffected in the absence of plectin (Fig. 5P-T). These data were fully consistent with the Co-IP experiments, showing that only AMPK heterotrimers comprising α1 but not α2 subunits bind to plectin in differentiated myotubes (see Fig. 4).

**Absence of plectin alters α subunit composition of AMPK complexes in differentiated myotubes**

The two catalytic subunits of AMPK, α1 and α2, expressed in skeletal muscle tissue, were reported to have distinct physiological functions (Cheung et al., 2000; Thornton et al., 1998; Viollet et al., 2003). Furthermore, compensatory

![Fig. 5. Plectin deficiency alters the staining pattern of AMPK catalytic subunits α1 in skeletal muscle fibres. Frozen tissue sections of m. soleus isolated from 3-month-old wild-type (A-E,K-O; ple +/+ ) and muscle-specific plectin knockout (F-J,P-T; ple −/− ) mice were immunolabelled using antibodies to AMPK α1 (A,F) and α2 (K,P) subunits, and plectin (B,G,L,Q). Note that plectin-specific staining in knockout tissue is restricted to connective tissue and blood vessels; only AMPK α1, but not α2, displayed extensive colocalisation with plectin (compare magnified boxed areas E and O); and fibres without plectin showed reduced AMPK α1 staining, and the striated staining pattern of the α1, rather than the α2 AMPK subunit was considerably deteriorated compared with wild-type (magnified boxed areas D, I and N, S, respectively). Bar, 10 μm.](image-url)
changes in expression levels of α subunits have been described in AMPK α2 knockout mice (Viollet et al., 2003). To investigate whether plectin-AMPK interaction in muscle cells has any functional significance, in particular for the α isoform composition of AMPK and to assess whether the observed changes in the spatial organisation of AMPK in the absence of plectin are reflected in subunit composition of AMPK complexes, we performed immunofluorescence microscopy and immunoblotting analyses of plectin+/+ and plectin−/− myoblasts, before and during differentiation to myotubes, using antibodies specific for subunits α1 and α2, and γ1. Interestingly, immunofluorescence microscopy showed that in undifferentiated myoblasts from either type of myocytes hardly any significant signal was obtained using anti-α2 subunit-specific antibodies, whereas the α1 subunit was found to be expressed in both types of myoblasts and throughout myotube differentiation. However, at later stages of differentiation, α2 subunits could also be detected in both cell types (data not shown). When individual subunit expression levels (corresponding to α1, α2 and γ1) were quantified by immunoblotting of cell lysates at different time points of differentiation and normalised against total AMPK, levels of α1 and γ1 subunits in plectin−/− myotubes gradually decreased, whereas in wild-type cells their expression levels remained unchanged (Fig. 6A,B,D). In agreement with immunofluorescence microscopy data (not shown), α2 subunit expression levels showed an increase during differentiation, without any significant difference between plectin+/+ and plectin−/− myocytes (Fig. 6A,C). To demonstrate the same extent of differentiation in plectin+/+ and −/− myoblasts, the expression levels of three different markers, caveolin 3, myogenin and MyoD, were monitored in parallel. No differences between the two cell types were detectable (Fig. 6A). Taken together, these results clearly indicated plectin-dependent alterations in α isoform composition of AMPK in the course of myocyte differentiation.

Distinct isoforms of α, β and γ AMPK subunits show muscle fibre type-specific expression patterns (Winder et al., 2003). The factors determining these patterns are unknown. Interestingly, similar to the AMPK α1 subunit (Winder et al., 2003), we found that plectin was more abundantly expressed in mitochondria-rich slow oxidative type I (Fig. 7), compared with glycolytic type II muscle fibres. These data, too, were consistent with a role of plectin in AMPK α subunit composition in differentiated muscle cells, suggesting that plectin may stabilise α1-AMPK complexes.

Increased phosphorylation of AMPK in differentiated plectin−/− myotubes

AMPK complexes containing the catalytic α2 subunit were found to be activated to a greater extent by AMP than α1 complexes (Cheung et al., 2000; Salt et al., 1998). An alteration in the subunit composition of AMPK, as observed in plectin-deficient myotubes, might therefore have been reflected by changes in the activity of the enzyme. Phosphorylation of AMPK on Thr172 of its catalytic activity (Hawley et al., 2003; Stein et al., 2000). Therefore, the levels of activated AMPK in plectin+/+ and −/− myocytes were measured by immunoblotting of cell lysates using antibodies specific to Thr172-P, along with antibodies recognising all α isoforms irrespective of their

![Fig. 6.](image-url) Plectin-deficient myocytes display differentiation-dependent changes in AMPK subunit expression. (A) Total cell lysates, prepared from differentiating wild-type (+/+)) and plectin-deficient myocytes (−/−) at the days (d) indicated, were subjected to immunoblotting using AMPK α1-, α2- and γ1 subunit-specific antisera, as well as antibodies immunoreactive with all AMPK α subunit isoforms (total AMPK). Equal amounts of proteins were loaded in each lane and protein bands were visualised as described in the text. The extent of differentiation of plectin+/+ and −/− myoblasts was monitored in parallel using antibodies to caveolin 3, myogenin and MyoD.

(B-D) Signal intensities of AMPK subunit bands, densitometrically determined in three independent experiments (including that shown in A), were normalised to total AMPK. Error bars represent the s.e.m. of three independent experiments. Differences between values in wild-type (ple+/+) and plectin-deficient (ple−/−) cells were determined using an unpaired Student’s t-test; *P<0.05.
phosphorylation state. As shown in Fig. 8A,C, in plectin+/+ myocytes a burst in the level of Thr172-P 5AMPK was observed within the first 24 hours of differentiation (day 1), followed by a massive decrease upon further differentiation. Similar to wild-type cells, lysates from plectin−/− myocytes also contained a high proportion of Thr172-P AMPK after one day of differentiation. However, the level of activated AMPK decreased thereafter considerably more slowly than in wild-type myocytes, as significantly higher levels of activated AMPK were still detectable on day 3 of differentiation, when cells had already become multinucleated (Fig. 8A,C). These findings were further corroborated by a similar analysis of skeletal muscle (diaphragm) and heart tissue homogenates from newborn wild-type and plectin-knockout mice. Similar to cultured myocytes, the levels of Thr172-P AMPK were found to be increased in the tissues of plectin-deficient mice (Fig. 9A). Quantification of these data and normalisation to the total
AMPK content of the samples showed a more than twofold greater proportion of Thr172-P AMPK in plectin−/− compared with wild-type tissues (Fig. 9B).

Three classes of phosphatases, namely PP1, 2A and 2B, were shown to dephosphorylate AMPK (Davies et al., 1995; Samari et al., 2005). To exclude the possibility that the observed increase in AMPK phosphorylation in plectin−/− cells resulted from the deregulation of these phosphatases, the activities of PP1, 2A, and 2B were individually evaluated in undifferentiated myoblasts, myoblasts differentiated for 3 days, and fully differentiated (6 days) myotubes (Fig. 8E-G). Although slightly lower activities of all phosphatases tested were measured in lysates from plectin-deficient myoblasts compared with wild-type myoblasts, at least at the first two time points (0 days and 3 days), there was no correlation between the differential time courses of AMPK activities and the phosphatase activities measured in plectin-deficient and wild-type cells.

To assess the specificity of AMPK activation in plectin-deficient myocytes, we measured the activity of p38 MAP kinase, another kinase related to myoblast differentiation (Baeza-Raja and Munoz-Canoves, 2004; Wu et al., 2000). As shown in Fig. 8B,D, p38 activities of both plectin+/+ and plectin−/− myoblasts gradually increased during differentiation at a similar rate, reaching maxima in fully differentiated myotubes. Only at this stage did plectin−/− myoblasts show a slightly higher activity compared with their wild-type counterparts. In any case, the time courses of p38 MAP kinase activation were quite similar in both cell types and thus did not correlate with the different time courses of AMPK phosphorylation observed in these cells. Showing a specific elevation of AMPK activity levels in plectin-deficient myocytes, in all, these data were consistent with the observed shift in AMPK subunit composition from α1 to the more readily activated α2 subunit.

AMPK has been postulated to have a role as a sensor and regulator of the cellular adenylate nucleotide pool defined as energy charge (ATP + 1/2ADP) / (ATP + ADP + AMP) (Atkinson, 1968; Hardie and Hawley, 2001). To examine whether the increased phosphorylation of AMPK observed in plectin−/− myocytes correlated with changes in energy charge, we measured the nucleotide contents of plectin+/+ and −/− myocytes at distinct stages of differentiation (Fig. 10). In both cell types energy charge remained within the physiological range and no significant differences were observed (Fig. 10A). The critical physiological parameter controlling AMPK kinase is the intracellular AMP:ATP ratio, with increased AMP and decreased ATP levels leading to phosphorylation and activation of the kinase (Hardie and Carling, 1997). Similar to energy charge, there was no significant difference between plectin+/+ and −/− myocytes regarding this parameter (Fig. 10B). In both cell types the ratio of AMP:ATP was found to be high during the first 24 hours of differentiation and to decrease thereafter. This correlated well with the changes in AMPK Thr172-P observed in wild-type cells, but not with those in plectin−/− myocytes. Since there were no significant differences observed in the nucleotide ratios of plectin+/+ and −/− cells at later stages of differentiation, we conclude that the higher levels of Thr172-P AMPK detected in plectin-deficient myotubes for up to 3 days, and possibly longer (see Fig. 8C), were not due to an altered AMP:ATP ratio.

Fig. 9. Elevated proportion of Thr172-P AMPK in muscle of newborn plectin−/− mice. (A) Immunoblot of skeletal muscle (diaphragm) and heart tissue homogenates prepared from plectin+/+ and plectin−/− newborn mice using antibodies to plectin and plectin–/– newborn mice using antibodies to plectin and plectin–/–. (B) Signal intensities of phosphorylated AMPK protein bands were densitometrically determined in three independent experiments (including that shown in A), and normalised to total AMPK. Error bars represent the s.e.m. of these experiments. Differences between plectin+/+ and plectin−/− values were determined using an unpaired Student’s t-test; **P<0.01; †P<0.001.

Fig. 10. Cellular energy state. The ATP, ADP and AMP nucleotide pools in lysates of wild-type (ple +/+ ) and plectin-deficient (ple −/− ) myocytes were determined by HPLC at various stages of differentiation and the corresponding energy charge (ATP + 1/2ADP) / (ATP + ADP + AMP) values (A) and the AMP:ATP nucleotide ratios (B) were calculated. Error bars represent the s.e.m. of three independent experiments.
Discussion
Plectin as a targeting platform for AMPK in differentiated myotubes

In line with our present finding, a large intracellular pool of plectin has been found associated with the Z-discs of myofibres (Reipert et al., 1999; Wiche et al., 1983). Plectin has been shown to appear at these structures rather early on during muscle differentiation (Schröder et al., 2000). Given the multitude of plectin interaction partners identified to date, the specific localisation of plectin in myofibres might be essential for the spatiotemporally coordinated positioning of specific signalling molecules at the Z-discs. Plectin could regulate the access of specific intracellular substrates to the AMPK complex allowing selective phosphorylation of downstream AMPK target proteins. It could also hold the kinase in the vicinity of its regulatory molecules and its upstream kinase, or within the sphere of influence of the local nucleotide ratio. Plectin might be involved in the selective stabilisation of AMPK complexes composed of distinct isoforms, thereby affecting the physiological function of the kinase.

The proper localisation of AMPK is of utmost importance in muscles, where the kinase cascade allows matching of the rate of ATP production with increased energy requirements during exercise. Adenylate kinase (AK) and creatine phosphokinase (CK) are two enzymes which are responsible for maintaining relatively stable ATP concentrations in the muscle cell as it begins to contract and use ATP at increased rates (Neumann et al., 2003a). AMPK reduces CK activity by approximately 60% (Ponticos et al., 1998), probably to prevent the backward reaction of CK at high creatine levels, leading to the consumption of scarce ATP for resynthesis of phosphocreatine (Neumann et al., 2003b). In this scenario, the improper localisation of AMPK resulting from plectin deficiency could lead to inefficient energy usage. Furthermore, fixing AMPK to a subcellular site, where its regulatory molecules, creatine phosphate (Ponticos et al., 1998) and AMP/ATP (Carling et al., 1989; Hardie and Carling, 1997; Mitchellill et al., 1994; Sullivan et al., 1994), are locally concentrated, is probably one of the most important primary conditions for the kinase to monitor the energy state of the muscle cell and trigger metabolic processes.

Fleming et al. (Fleming et al., 2005) revealed shear stress-induced phosphorylation of AMPK to be controlled by an unknown signaling pathway. It is conceivable that such a pathway involves regulation based on AMPK-plectin interaction, as plectin and other members of the plakin protein family seem to have a crucial role as stabilising elements of cells against mechanical stress. Plectin-kinase scaffolding might thus represent a prerequisite for the integration of mechanical stimuli with signaling pathways and may have consequences going beyond energy metabolism.

The C-terminal part of plectin, containing six globular repeat domains (R1-R6) connected by flexible linker regions, can interact with various types of intermediate filament (IF) proteins, including epidermal keratins, vimentin, desmin, GFAP, the neurofilament triplet proteins and lamin B (for a review, see Wiche, 1998). The vimentin-binding site has been mapped to the R5-R6 linker region (Nikolic et al., 1996), which probably also serves as an interface to other IF proteins. This domain may also harbour the docking site for RACK1, the receptor for activated C kinase 1 (Osmanagic-Myers and Wiche, 2004), as well as that for AMPK (this study). Whether these interactions are mutually exclusive or can occur simultaneously remains to be shown.

The selective stabilisation of AMPK α1 complexes in myotubes places plectin-AMPK interaction in a physiological context

In mammals, several different isoforms of all three AMPK subunits act to fine-tune the adapted energy homeostasis in distinct cell types (Cheung et al., 2000; Hardie and Hawley, 2001; Salt et al., 1998; Stapleton et al., 1996; Winder et al., 2003; Woods et al., 1996b). Our study, which we believe is the first on AMPK α subunit expression during in vitro differentiation of myocytes, revealed that co-expression of both catalytic subunit isoforms (α1 and α2) occurred only at later stages of differentiation. Our immunoprecipitation and immunofluorescence microscopy data imply that plectin tethers exclusively AMPK complexes comprising α1 subunit to Z-discs. Together with the finding that cell lysates prepared from plectin−/− myocytes contained less α1 isoform compared with wild-type controls, these results support a role of plectin in stabilising kinase complexes containing specifically this catalytic isoform. Stabilisation of α and γ subunits of the kinase has been reported to be carried out by the β subunit of AMPK, increasing the half-life of the kinase approximately twofold (Crute et al., 1998). AMPK β subunits act in a manner similar to the yeast Sip1/Sip2/Gal83 protein family members, which anchor Snf1 (homologue of AMPK α subunit) and Snf4 (homologue of AMPK γ subunit) into high molecular mass complexes (Hardie et al., 1998). The fine-tuning of the wide palette of AMPK functions in mammalian cells is likely to implicate other proteins serving as anchorage sites. Plectin may selectively stabilise γ1-α1 kinase complexes in differentiated myotubes via a conformational change of its binding partner, the γ1 regulatory subunit. The physiological function of the AMPK holoenzyme depends on the particular isoform present in the complex (Stein et al., 2000; Thornton et al., 1998). Some physiological differences between the α1 and α2 catalytic subunits, such as α2 but not α1 being involved in glucose homeostasis (Viollet et al., 2003), correlate with their fibre-type-specific expression patterns. Subunit α1 is predominantly expressed in oxidative fibres, whereas α2 is more abundantly detected in glycolytic fibres (Winder et al., 2003), suggesting a role of the kinase in the metabolic switch between oxidative and glycolytic pathways. From this point of view the influence of plectin on AMPK subunit composition would infer a role of plectin in the maintenance of cell metabolism.

How does the altered activity of AMPK in plectin−/− myotubes fit into this concept?

Elevated levels of Thr172-P AMPK in plectin−/− myotubes could have been explained by an enhanced AMP:ATP ratio in these cells. However, HPLC measurements of the nucleotide contents did not show any significant difference between plectin−/− and wild-type control cells, supporting the notion that plectin directly regulates AMPK activity. Since several classes of phosphatases were shown to regulate AMPK in vivo and in vitro (Davies et al., 1995; Samari et al., 2005), increased AMPK activity could have resulted from their general deregulation in plectin-deficient myocytes. Activities of PP1, PP2A and PP2C were indeed slightly lower in plectin−/−.
myocytes at distinct stages of differentiation. However, the differences were of low significance and they did not correlate with the activation state of AMPK in plectin+/− and +/- myocytes in the course of differentiation. Moreover, in contrast to AMPK, the phosphorylation levels of p38 MAP kinase, another kinase involved in myoblast differentiation, did not diverge during differentiation of plectin+/− and +/- cells, again supporting the notion that alterations in phosphatase activity were not responsible for the differences in AMPK activities.

AMPK exists in cells as a heterogeneous pool of isoenzymes, that respond differently to changes in the AMP:ATP ratio (Cheung et al., 2000). It was reported that the AMP dependence of specific complexes depends on the relative stability of the α-γ subunit interactions (Cheung et al., 2000), implying that the AMP dependence of the kinase is controlled by both, the γ and the α subunit isoforms present in the complex. Since AMP activates the kinase allosterically, the reason for differences in the dependence on this mononucleotide could lie in distinct tertiary structures of the different isoforms associated in the active complex. Interestingly, it has been reported that an engineered mutation in the γ1 subunit resulted in a constitutively active form of the α1β1γ1 complex that was less dependent on AMP and more highly phosphorylated, and therefore more active under basal conditions (Hamilton et al., 2001). On the other hand it has been shown that complexes containing α2 are activated to a greater extent by AMP than α1 complexes (Cheung et al., 2000; Durante et al., 2002; Salt et al., 1998; Stein et al., 2000). This considered, the elevated level of active AMPK detected in plectin-deficient myotubes compared with wild-type control cells, correlated well with other data and probably reflected a shift in AMPK subunit composition towards a higher α2 subunit proportion.

In conclusion, we have obtained strong evidence for an interaction of plectin with AMPK and its effects on the activity of the kinase. Some aspects of our concept of the physiological role of this interaction remain speculative and need further investigation. Strikingly, SIK and QSK, two kinases closely related to AMPK were recently shown to interact with the adaptor protein 14-3-3 (Al-Hakim et al., 2005). Similar to our observations, this suggests that scaffolding might be an evolutionarily conserved regulatory mechanism of signaling pathways involving the AMPK kinase protein family. It will be of particular interest to examine the mechanisms by which plectin may determine and modulate the formation and stabilisation of AMPK complexes. In further investigations, new tools such as antibodies specific to distinct AMPK γ isoforms and AMPK subunit-deficient cell lines will be indispensable.

Materials and Methods

cDNA constructs and transfection of myoblasts

The cDNA of the AMPK γ1 subunit isolated in a yeast two-hybrid screening (Osmangan-Myers and Wiche, 2004) was amplified by PCR and cloned into a pENTR/D-Topo vector (Invitrogen) by directional TOPO cloning. In this system PCR products are directionally cloned by adding four bases to the forward primer. The overhang in the cloning vector invades the 5' end of the PCR product, anneals to the added bases, and stabilises the PCR product in the correct orientation. Topoisomerase I from V. vinifera virus (Invitrogen) was used for the formation of covalent bonds between the insert and vector DNAs. Three positive clones were selected and inserts combined with the original AMPK γ1 subunit sequence to eliminate mutations. The entry clone (pENTR/D-Topo) with the AMPK γ1 subunit insert was then mixed with the destination vector (pBabe containing genes of GFP or FLAG fusion proteins) and Gateway LR Clonase enzyme mix (Invitrogen) to generate the expression clone. Expression of the fusion proteins AMPK γ1-GFP or AMPK γ1-FLAG was performed using the Phoenix retroviral system. This system has the advantage of stable integration ensuring that in cell types which undergo multiple maturation steps the retrovirus construct will remain resident and continue to express. The day before transfection 2 × 10^6 Phoenix cells were seeded per 6 cm culture dish. Phoenix cells were cultivated in DMEM/high glucose, 10% FCS with penicillin/streptomycin. The cells were transfected with retroviral constructs using Lipofectamine 2000 reagent (Invitrogen) by adding 4 μg plasmid DNA and 10 μl lipofectamine in 500 μl serum-free medium (Optimem). Cells were incubated for 7 hours, in transfection medium, then the medium was replaced by fresh antibiotic-free DMEM/FCS. After incubation overnight, medium was removed, 2 ml fresh antibiotic-free medium was added and cells were grown for 30 hours at 32°C. The supernatant containing the infectious retroviral particles was mixed 1:1 with fresh medium, filtered and polybrene (1:1000 dilution of 5 mg/ml stock) was added to enhance viral uptake of target cells. The infection mix was added to myoblasts (see below) subcultivated the day before infection on 6 cm culture dishes. Cells were grown in the infection medium for 48 hours at 37°C.

Immunofluorescence microscopy of cells and tissues

Mammalian cells were isolated, following established protocols (Rando and Blau, 1994), from the hindlimbs of 2- to 3-day-old plectin−/− mice (Andrë et al., 1997) and their wild-type littermates, both crossed into a p53−/− background (Jacks et al., 1994) to achieve immortalisation of cells. Isolated myoblasts were purified based on α7 integrin expression, following a published protocol (Blanco-Bose et al., 2001), and were grown in F-10DMEM-based myoblast growth medium (Gibco) on collagen-coated culture plates. Differentiation to myotubes was induced by replacement of myoblast growth medium with DMEM supplemented with 5% horse serum. For immunofluorescence microscopy, myoblasts and myotubes adhering to collagen-coated culture dishes were fixed in methanol for 90 seconds at −20°C. BSA (5%) or horse serum (5%) were added to the fixed cells to block non-specific binding sites, and cells were then washed five times for 5 minutes with PBS. As primary antibodies we used sheep antisera to AMPK α1 and AMPK α2 subunits (Woods et al., 1996a), and rabbit anti-plectin antisera #46 (Wiche and Baker, 1982); incubations were for 1 hour at room temperature (RT). Dishes were then washed thoroughly with PBS, incubated with secondary antibody (donkey anti-sheep Cy3, from Chemicon; donkey anti-rabbit Cy5, from Jackson Laboratories) for 1 hour at RT, washed again with PBS, and finally rinsed with water and mounted in mowiol. For viewing cells in the microscope, the rims of the culture dishes were removed. Unfixed frozen longitudinal or cross sections (7 μm-thick) of skeletal muscle tissues were prepared from mouse or rat (specified in the text) and placed on coverslips. Samples from mouse were incubated (1 hour, at RT) with normal goat serum (1:30 in PBS) before incubation with primary antibodies. Immunoreagents and processing of specimens for microscopy were similar to those of cells, except that in the case of rat tissue mouse mAb 10P6 to plectin (Foisner et al., 1994) and mouse 14-3-3 antisera (Jackson Laboratories), were used as primary and secondary antibodies, respectively. All immunlabelled specimens were visualised using an Axiovert 100M laser-scanning microscope (Zeiss).

Enzyme histochemical staining of muscle fibres

For detection of type I and type II muscle fibres on unfixed frozen sections of skeletal muscle we used NADH-tetrazolium reductase staining. Specimens were incubated in 0.2 M Tris-HCl, pH 7.4, containing 1.5 mM NADH and 1.5 mM nitro blue tetrazolium, for 30 minutes at 37°C. Afterwards superfluous staining was removed by washing with 30, 60, 90, 60 and 30% acetone (2 minutes, each step). Finally slides were rinsed in water and mounted in Entellan.

Phosphatase activity measurement

Cultured myocytes were differentiated for the times indicated in the text and then collected and homogenised in 1 ml of 50 mM imidazole, 0.2 mM EGTA, 0.02% β-mercaptoethanol, 1% Triton X-100, 2 mM PMSF, 20 μg/ml aprotinin and 12.5 μg/ml leupeptin. Lysates were centrifuged for 1 hour at 100,000 × g and subjected to phosphatase activity measurements using EnzChek phosphatase assay kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

Immunoprecipitation, immunoblotting and protein determination

For immunoprecipitation experiments, myoblasts and myotubes from plectin wild-type and plectin-deficient mice were mixed twice with cold PBS and lysed in 50 mM HEPES/HCl, pH 7.0, 5 mM MgCl2, 1 mM EGTA, 100 mM NaCl (lysis solution), supplemented with 0.5% Triton X-100, 0.1 mM DTT, 0.5 mM MgCl2, 1% Triton X-100 (Boehringer Mannheim), 0.2 mg/ml RNase A (Serva), 1 mM PMSF, 10 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml pepstatin/Aeq1, and phosphatase inhibitor cocktail 1 (1:100, Sigma-Aldrich). Cell suspensions were incubated for 10 minutes at RT to achieve optimal activities of added enzymes. Afterwards, Triton X-100 was added to a final concentration of 1% and lysates were left for further 5
minutes at RT. Lysates of myotubes were passed 10x through a 1 ml pipette tip and insoluble matter was sedimented by centrifugation in an Eppendorf microfuge at 14,000 rpm (20 minutes, 4°C). Supernatants from samples to be quantitatively compared were adjusted to comparable protein concentrations (see below), and then precleared by incubation (30 minutes) with 7 μl (per 100 μl of supernatant) of a 10% solution of protein A (or G) Sepharose in PBS. After removal of beads, supernatants were incubated with antisera to p38 (54C6) or mAbs raised in sheep using a synthetic peptide (amino acids 12-25) of rat AMPK γ1 subunit for 3 hours at RT, or overnight at 4°C. To recover antibody-antigen complexes, protein A (or G) beads were added (conditions similar to above) and suspensions rotated for 2 hours at 4°C. Beads were sedimented and washed three times with lysis solution containing 1% Triton X-100. Centrifugation steps were carried out by slowly increasing the speed to 6000 rpm in an Eppendorf microfuge. Finally the beads were resuspended in 20 μl of 5x SDS electrophoresis sample buffer (containing 0.001% Bromophenol Blue), heated for 5 minutes at 95°C, and stored frozen until further analysis.

Aliquots of resuspended immunoprecipitates, cell lysates, and tissue homogenates (diaphragm and heart of newborn mice) were subjected to SDS PAGE and electrophoretic analysis as described (Andrá et al., 1997). After protein transfer, membranes were blocked with 5% milk powder or 5% horse serum in PBS-Tween, for 50 minutes at RT. They were then incubated with primary antibodies diluted in PBS-Tween for 1 hour at RT, or overnight at 4°C, washed briefly in PBS-Tween, and overlaid with secondary antibodies for 1 hour at RT. For detection of immunoreactive bands, the SuperSignal System (Pierce) was used. Signal and overlaid with secondary antibodies for 1 hour at RT. For detection of phospho-kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEB. LSS. 377, 421-425.

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