Control of AMP deaminase 1 binding to myosin heavy chain

ICHIRO HISATOME,1 TAKAYUKI MORISAKI,1 HIROSHI KAMMA,2 TAKAKO SUGAMA,3 HIROKO MORISAKI,1 AKIRA OHTAHARA,3 AND EDWARD W. HOLMES1

Departments of Medicine, Genetics, and Biochemistry, University of Pennsylvania School of Medicine, Philadelphia 19104-4283; 2Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148; and 3The 1st Department of Medicine, Tottori University Faculty of Medicine, Yonago 683, Japan

AMPD is an allosteric enzyme, and in solution its activity is controlled by a number of intracellular metabolites, principally purine nucleotides and inorganic phosphate (2, 5, 20, 21, 33, 34). The activity of this enzyme is governed in a physiological sense by the adenylate energy charge of the cell (24), and it can be viewed as a sensor of the energy needs of the cell. When AMPD is activated and AMP is deaminated to IMP, the enzyme shifts the equilibrium of the adenylate kinase reaction, thereby increasing the adenylate energy charge (24). Activation of AMPD and the resultant increase in adenylate charge help to preserve viability of the cell under stressful conditions.

Recent studies have identified a novel mechanism for regulating the activity of AMPD in myocytes. In resting muscle, more than 90% of this enzyme is free in the sarcoplasm, and when free in the sarcoplasm the enzyme is inactive. After vigorous muscle contraction, 50–60% of this enzyme becomes bound to the myofibril (22, 23), and the activity of the bound enzyme increases as a consequence of a decrease in Michaelis-Menten constant for AMP and a decrease in inhibition by nucleotides and inorganic phosphate (22, 23). These studies have provided convincing evidence that the enzyme cannot be activated under the conditions existent in maximally stressed myocytes when it is not bound to the myofibril (22, 23).

The present study was undertaken to define the properties of the AMPD1 peptide that control binding of this isoform to the myofibril. AMPD1 has been demonstrated to bind to myosin heavy chain (MHC) in vitro, in particular the S2 subregion of MHC (1, 3, 9, 10). Presumably, this is the target of binding for this enzyme in the myofibril, but other macromolecules have also been proposed as binding sites for AMPD1 in the myofibril (11). Experiments reported here have taken advantage of a model in which expression of MHC in nonmyocytes leads to formation of filamentous structures that can be identified immunohistochemically (29, 30). Through cotransfection of epitope-tagged AMPD1 expression vectors containing deletion or point mutations in the AMPD1 peptide, this system has been exploited to assess binding of wild-type and mutant AMPD1 peptides to the myosin filament in vivo. This model has the advantage that other components of the myofibril are not present, excluding AMPD1 binding to other proteins that are present in the sarcomere. This approach is superior to in vitro binding experiments, since cell lysis and extraction of AMPD1 uniformly lead
to proteolysis with a resultant change in binding properties of the enzyme (13).

Results of the studies reported here demonstrate that at least three regions of the AMPD1 peptide influence binding of this enzyme to MHC. The central region of the native 727-amino acid peptide contains a myosin binding site (residues 178–333). The ATP binding site (residues 660–674) located in the carboxy terminus regulates binding in response to changes in the intracellular concentration of this nucleotide. The amino-terminal 65 residues of the AMPD1 peptide are essential to the intact molecule for mediation of ATP-induced inhibition of binding to MHC. The AMPD1 primary transcript is subject to tissue-specific and stage-specific alternative splicing in this region of the AMPD1 peptide, generating isoforms that contain exon 2-encoded sequences (E2+) or that exclude sequences encoded by exon 2 (E2−) (16). Thus alternative splicing of the AMPD1 primary transcript can eliminate four amino acid residues from the amino-terminal region of the AMPD1 peptide (16, 18), and this physiological amino acid residues from the amino-terminal 65 residues of the AMPD1 peptide are essential to the intact molecule for mediation of ATP-induced inhibition of binding to MHC under certain conditions.

**MATERIALS AND METHODS**

Plasmid constructs. pRC/CMV (Invitrogen) was used as the expression vector for both the AMPD1 and MHC cDNAs. The rat and human AMPD1 cDNAs used in this study have been described previously (25, 26). A full length of the rat cardiac MHC-α cDNA was provided by Dr. Lee Sweeney of the Physiology Department at the University of Pennsylvania. Deletions were made in the rat AMPD1 cDNA using standard restriction enzyme digestion techniques, and point mutations were introduced into the human AMPD1 cDNA as previously described (8). Decisions for selection of restriction sites to create the various deletion mutants were based on availability of convenient cloning sites that removed reasonable stretches of amino acid residues from the amino and carboxy termini. Rat/human chimeras were made by ligating the 5′ end of rat AMPD1 cDNA to the 3′ end of human AMPD1 cDNA. These chimeras were created to take advantage of the availability of ATP and catalytic site point mutations created previously in human AMPD1 (8). A double-stranded oligonucleotide encoding the human c-myc epitope, MEQKLISEEDL, was added to the amino terminus of all AMPD1 constructs. This epitope is recognized by an anti-Myc monoclonal antibody (MAb), 9E10 (6). It has the advantage of giving a low immunofluorescent background in untransfected cells or cells transfected with constructs that do not contain this epitope. All constructs were sequenced to confirm their primary structure.

Cell culture and transfection conditions. Human HeLa cells and Monkey kidney COS-1 cells were routinely grown in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO). Cells grown on glass coverslips in 30-mm dishes were transfected with 5 µg of a single expression plasmid or 5 µg of both plasmids for cotransfection studies, using a calcium phosphate coprecipitation technique (28). Plasmid DNA was added to HEPES-buffered saline composed of 140 mM NaCl, 1.5 mM Na2HPO4, and 25 mM Hepes, pH adjusted to 7.05 with HCl, and precipitated by adding CaCl2 with constant stirring. After the COS cells were incubated with the coprecipitate for 4 h and washed with PBS, cells were cultured for 44 h before analysis by immunofluorescence microscopy. After the HeLa cells were incubated with the coprecipitate for 24 h and washed with PBS, cells were cultured for 24 h before analysis by immunofluorescence microscopy.

Cell permeabilization. HeLa cells were permeabilized using a Trans Port Kit from GIBCO using conditions recommended by the manufacturer (GIBCO). Permeabilized cells were incubated for 60 min with cytosolic solution (in mM: 140 KCl, 0.38 K2HPO4, 2.13 K2HPO4, 1 MgSO4, and 0.1 CaCl2, pH adjusted to 7.2 with KOH) containing 0–10 mM MgATP. The metabolic inhibitors 2,4-dinitrophenol (50 mM) and 2-deoxyglucose (10 mM) were also included in the 0 mM MgATP cytosolic solution ("low ATP"). For solutions with ATP concentrations higher than ambient ATP concentration ("high ATP"), 10 mM MgATP was added to the cytosolic solution. Comparison experiments with Trypan blue (molecular weight 1,000) added to the cytosolic solution confirmed that >90% of the HeLa cells were permeabilized under the conditions used in this study.

Immunofluorescence microscopy. For indirect immunofluorescence, cells were fixed with 2% formaldehyde (Polyscience) in PBS for 30 min at room temperature followed by a 3-min incubation in cold acetone at −20°C. Fixed cells were incubated for 1 h at room temperature with either anti-Myc MAb (9E10 culture supernatant, an IgG1 class antibody; American Type Culture Collection) or anti-sarcomeric MHC MAb (MF20 culture supernatant, an IgG2b class antibody; Hybridoma Bank of University of Iowa). Cells were washed three times with PBS and incubated for 1 h at room temperature with either 1:500 dilution of FITC-conjugated anti-mouse IgG1 antibody in PBS containing 3% BSA for Myc staining or Texas red (TXRD)-conjugated anti-mouse IgG2b antibody in PBS with 3% BSA for MHC staining. After coverslips were washed three times with PBS, they were inverted and mounted on glass microscope slides. For double staining, both anti-Myc MAb and anti-sarcomeric MHC MAb were applied simultaneously to cells for 1 h. After cotransfectants were washed three times with PBS, they were incubated with a 1:12.5 dilution of FITC-conjugated anti-mouse IgG1 antibody (Southern Medical Association) and a 1:25 solution of TXRD-conjugated anti-mouse IgG2b antibody (Southern Medical Association) in PBS with 3% BSA.

Immunoblots of transfected cells. A confluent 30-mm petri dish of HeLa cells was transfected with 10 µg of plasmid DNA as described above. Cells were harvested 48 h after transfection by scraping into SDS sample buffer. Peptides were displayed on either 7.5% (MHC transfectants) or 12.5% (AMPD1 transfectants) SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes using the Phast-Gel system (Pharmacia). Membranes were blocked with 5% dried milk powder in TBST overnight at 4°C. The supernatant of either the anti-Myc MAb or the anti-MHC MAb was incubated with the membrane for 2 h at room temperature. After membranes were washed three times with TBST, they were incubated with 1:5,000 dilution of peroxidase-conjugated goat anti-mouse IgG antibody (Southern Medical Association) and a 1:25 solution of TXRD-conjugated anti-mouse IgG2b antibody (Southern Medical Association) in PBS with 3% BSA.

Colocalization of AMPD1 and MHC. In cotransfected cells that expressed both the AMPD1 and MHC peptides, two patterns of immunofluorescence were observed. In some cells, the AMPD1 stain was distributed throughout the cytoplasm. The percentage of dually transfected cells exhibit-
ing colocalization of AMPD1 and MHC was determined for each independent transfection experiment. Two hundred randomly encountered cells expressing both peptides were scored as either diffuse AMPD1 stained or AMPD1 colocalized with MHC by examining each cell with a red filter first to detect MHC filaments, followed by examination with a green filter to determine the AMPD1 staining pattern. Marginal images that were difficult to score as colocalized vs. nonlocalized were classified as nonlocalized or diffuse. Three individuals evaluated the slides independently, and their scores or percent colocalized was concordant in all experiments.

Confocal microscopy and image reconstruction. Cytosolic distributions of AMPD1 and MHC peptides were examined using a confocal laser microscope illuminated with a krypton/argon laser (Bio-Rad, MRC 600). With the use of a ×60 oil immersion lens (Olympus, Splan Apo, 1.4 numerical aperture), optical sections were collected at intervals of 0.8-µm increments through the entire z-axis of each cell. A single optical section was selected from the midregion of the transfected cell to provide a cross-sectional view of the intracellular distribution of the AMPD1 and MHC peptides. The FITC-labeled distribution of AMPD1 and the TXRD-labeled distribution of MHC were then combined using Adobe Photoshop. The surface plot module of National Institutes of Health Image 1.01 freeware was used for the integrated density volume mapping to quantitatively assess the intracellular abundance of AMPD1 and MHC in transfected cells (4, 14). Surface density maps displaying these results are shown in Fig. 3 (bottom). The distributions of the AMPD1 and MHC peptides in the cytosol are illustrated by the peaks and troughs of high- and low-intensity staining.

RESULTS

Colocalization of AMPD1 and MHC peptides in transfected cells. Preliminary experiments were performed to demonstrate that the respective expression vectors and cell culture conditions lead to production of AMPD1 or MHC peptides of the appropriate size (Fig. 1). Immunoblots of protein extracted from HeLa cells transfected with the AMPD1 expression vector confirm the presence of an AMPD1 peptide with an estimated size of 80 kDa, the predicted size for the nonproteolyzed, full-length AMPD1 peptide (13). The MHC peptide produced from this expression vector has an estimated molecular mass of 200 kDa, the size predicted for the full-length MHC peptide. Transfection of COS cells with these same vectors gave similar results (data not shown).

Transfection of the expression vector encoding the epitope-tagged, native AMPD1 cDNA alone into HeLa cells produces an immunofluorescent staining pattern in which AMPD1 is distributed diffusely throughout the cytoplasm and excluded from the nucleus (Fig. 2, top left). Transfection of HeLa cells with the MHC expression vector alone gives an immunofluorescent staining pattern similar to that reported by Vikstrom et al. (30); MHC filaments aggregate to produce flecks scattered throughout the cytoplasm (Fig. 2, top right). Transfection of the expression vector alone, which contains neither AMPD1 nor MHC cDNA, produces no detectable immunofluorescent staining under the conditions employed. After cotransfection with both the AMPD1 and MHC expression vectors, many cells express both peptides (Fig. 2, bottom panels). In some cells, the AMPD1 staining is superimposed on the MHC filaments, and, in other cells with easily discernible MHC filaments, AMPD1 staining is distributed diffusely throughout the cytoplasm. Confocal laser microscopy confirms colocalization of AMPD1 and MHC in cotransfected HeLa cells (Fig. 3). In 17 independent transfections, 32 ± 2.3% of HeLa cells exhibited colocalization of AMPD1 and MHC.

Initially, it was perplexing why only a fraction of cells expressing both peptides exhibits colocalization of AMPD1 and MHC. Variability in experimental conditions was not considered to be a likely explanation, since the fraction of HeLa cells exhibiting colocalization of AMPD1 and MHC was very consistent in the different transfection experiments, as evidenced by the small coefficient of variation among the 17 independent transfections.

Cells that demonstrate colocalization of AMPD1 and MHC do not contain more or less AMPD1 than cells that do exhibit colocalization to the extent that this can be determined quantitatively by confocal laser microscopy. The relative abundance of the AMPD1 and MHC peptides in a given transfected cell was determined from the confocal laser images using a three-dimensional display as illustrated in Fig. 3 (bottom). The

Fig. 1. Immunoblots of HeLa cells transfected with the myosin heavy chain (MHC) or AMP deaminase 1 (AMPD1) expression vector. Lysates of HeLa cells transfected for 48 h with either 10 µg of AMPD1 or MHC expression plasmid were resolved on 7.5 or 12.5% SDS-polyacrylamide gels, respectively. After transfer to nitrocellulose membranes, AMPD1 and MHC peptides were identified by immunoblotting with the respective monoclonal antibodies (MAbs). AMPD1 expression vector produces a peptide that contains an epitope recognized by a Myc MAb added to the amino terminus of AMPD1 (see Fig. 4). MHC peptide was detected by an MAb to anti-sarcomeric MHC. Left: lane 1, lysate of HeLa cells transfected with MHC expression vector; lane 2, lysate of mock-transfected cells. Right: lane 1, lysate of HeLa cells transfected with AMPD1 expression vector; lane 2, lysate of mock-transfected cells.

Fig. 2. Confocal laser images of AMPD1 and MHC peptide distributions. (a) Lane 1, lysate of HeLa cells transfected with both the AMPD1 and MHC expression vectors; lane 2, lysate of mock-transfected cells. (b) Lane 1, lysate of HeLa cells transfected with both the AMPD1 and MHC expression vectors; lane 2, lysate of mock-transfected cells.
integrated density volumes were calculated for the AMPD1 and MHC peptides, respectively (4, 14). These analyses were repeated in triplicate, and the ratio of the AMPD1 peptide relative to that of the MHC peptide in a given cell was determined. For the native AMPD1 peptide, the ratio of AMPD1 to MHC was determined to be 1.09 ± 0.02 (Table 1), and this ratio was not appreciably different in cells that exhibited colocalization of AMPD1 and MHC vs. those that exhibited a diffuse staining pattern for AMPD1. These findings suggest that some factor other than variation in the intracellular level of AMPD determines the percentage of cells demonstrating colocalization.

A potential explanation for why some cells exhibit colocalization and others do not is suggested by previous studies. AMPD binding to MHC in myocytes is reversible and varies depending on the intracellular concentration of ATP or the adenylate energy charge (13, 22, 23). Results that are presented later in this study demonstrate that increasing or decreasing the intracellular content of ATP reproducibly decreases or increases, respectively, the fraction of cells exhibiting colocalization of AMPD1 and MHC. Point mutation of the ATP binding site in AMPD1 also reproducibly increases the fraction of cells that exhibit colocalization. We conclude from these observations that conditions in nonperturbed HeLa cells are near the equilibrium for dissociation of AMPD1 from MHC. If more of the AMPD1 is bound than free, staining over the MHC filaments is more intense and these cells are scored as localized AMPD1 in this assay. Perturbations of the intracellular environment or mutations in the AMPD1 peptide shift this equilibrium, and the percentage of cells exhibiting colocalization increases or decreases as a reflection of the shift between bound and free AMPD1. Thus the fraction of cells exhibiting colocalization of AMPD1 and MHC provides a method for quantitating AMPD1 binding to MHC in the intact cell.

To confirm the utility of this cotransfection model for assessing AMPD1 binding to MHC, another cell line was also utilized. COS cells transfected with the AMPD1 expression vector alone give a diffuse immunofluorescent staining pattern, COS cells transfected with the MHC expression vector give a filamentous staining pattern, and COS cells transfected with both vectors provide a method for quantitating AMPD1 binding to MHC in the intact cell.

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Fig. 2. Immunofluorescent staining of transfected HeLa cells. HeLa cells grown on coverslips were transfected with the AMPD1 expression vector alone (top left) or the MHC expression vector alone (top right) or cotransfected with both expression vectors (bottom). Cells shown on left were stained with the Myc MAb, which detects the Myc epitope-tagged AMPD1 peptide. Cells shown on right were stained with anti-sarcomeric MHC MAb. TXRD, Texas red.

Fig. 3. Confocal laser microscopy of transfected HeLa cells. HeLa cells were prepared as in Fig. 2 and photographed with confocal laser microscopy. Top left: cells were transfected with the AMPD1 expression vector alone and stained with the Myc MAb, which detects the Myc epitope-tagged AMPD1 peptide. Top right: cells were transfected with MHC expression vector alone and stained with MHC MAb. Middle: a single cell cotransfected with both expression plasmids visualized with a green filter (epitope-tagged AMPD1 peptide; left) and visualized with a red filter (MHC peptide; right). Bottom: 3-dimensional display of integrated density volume calculated for the AMPD1 peptide (left) and for the MHC peptide (right). TXRD, Texas red.
give one of two AMPD1 staining patterns: diffuse or colocalization with MHC. In four independent cotransfection experiments, 58 ± 14% of COS cells exhibited colocalization of AMPD1 and MHC. Although fewer experiments were performed with COS cells, mutations of the AMPD1 peptide that increase or decrease binding to MHC in HeLa cells also increase or decrease binding in COS cells. Thus binding of AMPD1 to MHC is not restricted to the environment of the HeLa cell, and the model reflects qualitatively similar changes in binding in both cell types.

Transfection experiments with the AMPD1 expression vectors were also attempted with a soleus 8 myoblast cell line to assess binding of AMPD1 to intact myofibrils. Although the AMPD1 peptide was easily detectable in myoblasts 48–72 h posttransfection, expression was extinguished or the myoblasts killed after 72 h, before myotube formation. Subsequent experiments have confirmed that expression of the AMPD1 peptide during the late myoblast stage is deleterious to the differentiation process.

Identification of the MHC binding domain in AMPD1. As illustrated in Fig. 4, a series of amino- and carboxy-terminal deletions were made in the AMPD1 peptide. The diagram of the AMPD1 peptides illustrated in Fig. 4 has been broken into sections to indicate the location of the Myc epitope tag; the nonconserved, noncatalytic region of AMPD1; the conserved catalytic domain; and the positions of the catalytic and ATP binding sites (8). The expression vectors encoding these deletion constructs produce AMPD1 peptides of the predicted size in HeLa cells as demonstrated in Fig. 5.

The wild-type peptide used for comparison in this set of experiments is the full-length, exon 2 plus (E2+1 isoform of AMPD1. Deletion of the amino-terminal 65 residues of AMPD1 (NΔ65) virtually abolishes binding of this peptide to MHC; the percentage of HeLa cells exhibiting colocalization falls from 31.7 ± 2.3% to 2.2 ± 0.9% (P < 0.01) (Fig. 4). This truncated peptide accumulates in amounts relative to MHC that are comparable to that for the wild-type peptide (Table 1), excluding peptide abundance as the explanation for its reduced binding to MHC. Prior studies have shown that deletion of these amino-terminal residues has only a minor effect on the catalytic activity of AMPD1 (8), providing evidence that this truncated form of AMPD1 retains some structural features similar to that of the native enzyme.

Deletion of another 113 residues (NΔ178) from the amino terminus not only restores binding but cells

<p>| Table 1. Ratio of AMPD1 to MHC in HeLa cells (integrated density volume determinations) |
|---------------------------------|---------------------------------|</p>
<table>
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<tr>
<th>AMPD1/MHC (10% FBS)</th>
<th>AMPD1/MHC (2.5% FBS)</th>
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<tr>
<td>E2+</td>
<td>1.09 ± 0.02</td>
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<tr>
<td>E2-</td>
<td>1.33 ± 0.01</td>
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<tr>
<td>NΔ65</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>NΔ178</td>
<td>1.31 ± 0.03</td>
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<tr>
<td>NΔ333</td>
<td>1.11 ± 0.24</td>
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Values are means ± SD; determinations were performed in triplicate. The integrated density volume was determined for AMP deaminase 1 (AMPD1) and myosin heavy chain (MHC) in single HeLa transfectants using confocal laser microscopy, as illustrated in Fig. 3. HeLa cells were routinely cultured in 10% fetal bovine serum (FBS) for all constructs; for E2+ and E2− constructs, cells were also cultured in 2.5% FBS.
expressing this truncated peptide exhibit an even higher percentage of colocalization than observed in cells expressing the wild-type peptide (60 ± 2.7% vs. 31.7 ± 2.3%, P < 0.05) (Fig. 4). Deletion of an additional 155 residues (NΔ333) from the amino terminus reduces binding of AMPD1 to MHC to very low levels; only 8.6 ± 0.9% of cells expressing this truncated peptide exhibits colocalization (P < 0.05 compared with the wild-type peptide). These truncated peptides accumulate in cells in amounts relative to MHC which are comparable to that observed for the wild-type peptide (Table 1), excluding abundance of the mutant AMPD1 peptides relative to that of MHC as the explanation for observed differences in binding.

Deletions from the carboxy-terminal region can also affect AMPD1 binding to MHC. Removal of 85 residues (CΔ85) from the carboxy terminus has no discernible effect on binding: 31.4 ± 4.4% of cells expressing this truncated peptide exhibits colocalization, a value comparable to that observed in cells expressing the wild-type peptide (P > 0.5). Deletion of an additional 118 residues (CΔ118) removes both the catalytic site and the ATP binding site (8). Cells expressing this truncated peptide exhibit a significantly higher percentage of colocalization of AMPD1 and MHC compared with cells expressing the wild-type peptide (61.8 ± 4.2% vs. 31.7 ± 2.3%, P < 0.05). Deletion of another 426 residues (CΔ426) from the carboxy terminus does not appreciably change binding from that observed with the CΔ118 construct; 77.6 ± 1.8% of cells that express the CΔ426 peptide exhibits colocalization. As with the other mutant constructs, abundance of the AMPD1 peptides relative to that of MHC in the transfected cells does not explain the observed differences in binding observed with these mutants (Table 1).

These deletion constructs define a minimal region within the AMPD1 sequence that is encompassed by residues 178–333. To confirm that the region encompassed by residues 178–333 includes a myosin binding site, these sequences were ligated into the expression vector to make a construct called N178–333. In three experiments with the N178–333 construct, colocalization of this AMPD peptide with MHC was observed in 90% of dually transfected cells (Fig. 4).

ATP modulates AMPD1 binding to MHC. Prior studies with resting and stimulated skeletal muscle (1, 23) and isolated MHC and AMPD1 peptides (13) indicate that ATP, or some derivative of the adenylate energy charge, modulates binding of AMPD1 to MHC. Two approaches were taken in the present study to define the role of ATP in the control of AMPD1 binding to MHC.
and to identify regions of the AMPD1 peptide that control binding of AMPD1 to MHC in response to changes in the intracellular concentration of ATP. One series of experiments employed the AMPD1 mutants described above. These mutants were transfected into HeLa cells, and the cells were subsequently permeabilized to permit external control of the ATP concentration in the cell.

As illustrated in Fig. 6, binding of wild-type AMPD1 peptide to MHC in permeabilized HeLa cells responds to changes in the intracellular concentration of ATP. At lower than ambient ATP concentrations, the percentage of cells exhibiting colocalization of AMPD1 and MHC increases from 31.7 ± 2.3% to 70.6 ± 3.4% (P < 0.05); at higher than ambient ATP concentrations, the percentage of cells exhibiting colocalization decreases from 31.7 ± 2.3% to 20.3 ± 1.8% (P < 0.1). Deletions in the carboxy-terminal region of AMPD1 (CΔ426 and CΔ118), which eliminates the segment of this peptide containing the ATP binding site and the catalytic site (Fig. 4), render the truncated AMPD1 peptides resistant to changes in ATP concentrations with respect to their binding to MHC (Fig. 6). A more restricted deletion of carboxy-terminal residues (CΔ85) that preserves the region of AMPD1 that contains the ATP binding site and the catalytic site produces a peptide that exhibits binding characteristics in response to changes in the intracellular concentration of ATP similar to those observed with the wild-type peptide.

The above studies with the carboxy-terminal deletion mutants are consistent with the hypothesis that the ATP binding site located in this region of the peptide is responsible for the observed effects of ATP in modulating binding of AMPD1 to MHC, but these experiments have several limitations. As demonstrated in Fig. 5, the CΔ85 and especially the CΔ118 mutants are subject to proteolysis. We cannot exclude that this proteolysis occurred in the cell and that the resultant mixture of native and degraded peptides contributed to some of the observed differences in ATP responsiveness of these carboxy-terminal deletion mutants. Furthermore, these deletion constructs removed residues in addition to the ATP binding site. Therefore, another set of experiments was performed that utilized AMPD1 peptides engineered to contain point mutations in the ATP binding site or the catalytic center of AMPD1. Chimeric rat/human AMPD1 cDNAs were used for these studies to take advantage of point mutants in human AMPD1 characterized previously (8). As illustrated in Fig. 7, substitution of a glycine for aspartate at position 650, a mutation known to destroy the catalytic center of this enzyme (8), has no effect on binding of this chimeric AMPD1 peptide to MHC at ambient ATP concentrations in HeLa cells (32 ± 13.8% vs. 31.7 ± 2.3% for the mutant and wild-type peptides, respectively, P > 0.5). In contrast, mutation of residue 663 (glutamine to lysine) within the ATP binding site of the rat/human chimeric AMPD1 peptide (8) results in a peptide that exhibits increased binding to MHC at ambient ATP concentrations in HeLa cells (82 ± 8.3% vs. 31.7 ± 2.3% colocalization of the mutant chimera vs. the wild-type rat peptide, P < 0.05). When HeLa cells containing the 663 glutamine-to-lysine mutation are permeabilized and exposed to media containing different ATP concentrations, colocalization of the ATP binding site mutant is not appreciably altered by either higher or lower than ambient ATP concentrations (data not shown). These two chimeric peptides accumulate in amounts relative to MHC that are comparable to that observed for the wild-type rat AMPD1 peptide, and their sizes are comparable to that of the native rat AMPD1 peptide (data not shown).

Effect of the amino terminus on AMPD1 binding to MHC. Results presented in Fig. 4 demonstrate that residues in the amino-terminal region of AMPD1 have a profound effect on the binding of this peptide to MHC. Deletions within the amino terminus appear to have paradoxical effects on binding of AMPD1 to MHC; the NΔ65 mutant reduces binding at ambient ATP concentrations to essentially background levels, whereas deletion of an additional 113 residues (NΔ178) results in binding levels at ambient ATP concentrations that are greater than that observed with the wild-type peptide. This paradox is explained by results obtained from experiments that examine binding of the various amino-terminal deletion mutants at different ATP concentrations (Fig. 8).

The NΔ65 mutant exhibits colocalization with MHC in only 2.2 ± 0.9% of HeLa cells at ambient ATP concentrations. However, when the intracellular ATP
concentration is reduced in permeabilized cells, the percentage of cells exhibiting colocalization increases to 20 ± 4.0% (P < 0.05). Thus this deletion mutant is capable of binding to MHC, but binding is reduced at all ATP concentrations. These results suggest that the amino-terminal 65 residues of AMPD1 influence binding of this peptide to MHC through enhancing sensitivity to ATP-induced inhibition of binding.

In contrast to the NΔ65 mutant, the NΔ178 is less sensitive to ATP-induced inhibition of binding to MHC. This mutant exhibits a level of binding to MHC at ambient ATP concentrations that is comparable to that observed with the wild-type peptide at reduced ATP concentrations. Moreover, the percentage of cells exhibiting colocalization of the NΔ178 mutant with MHC is unchanged at low, ambient, and high ATP concentrations (Fig. 8). These results suggest that residues within the region of the AMPD1 peptide encompassed by amino acids 66–177 are necessary for ATP to inhibit AMPD1 binding to MHC.

The results with the amino-terminal deletion mutants indicate that the more proximal regions of the AMPD1 peptide have the potential to influence binding of this enzyme to MHC through modulation of the sensitivity to ATP-induced inhibition of binding. For this region of the peptide to exert a regulatory effect on binding, there would need to be a physiological mechanism for altering the structure of the amino-terminal domain of AMPD1. The second exon of AMPD1, which encodes residues 8–12 of this peptide, is retained or excluded from the mature messenger RNA derived from the AMPD1 primary transcript in response to stage-specific and tissue-specific signals (16, 18). These sequences fall within the region of the amino terminus that influences the sensitivity of the AMPD1 peptide to ATP-induced inhibition of binding.

To determine if the alternative isoforms of AMPD1 exhibit differences in binding to MHC, an expression vector was constructed that encodes an AMPD1 peptide...
that excludes exon 2-derived sequences (E2−) for comparison with the parent construct that includes exon 2-derived sequences (E2+). Binding to MHC was assessed under several culture conditions following cotransfection with the MHC expression vector (Fig. 9).

Routine culture conditions for the studies described above utilized medium containing 10% FBS. Under these culture conditions, the percentage of HeLa cells exhibiting colocalization of AMPD1 and MHC is comparable for the E2+ and E2− isoforms (31.1 ± 2.3% vs. 25.6 ± 3.4% P > 0.5) (Fig. 9A). In the course of testing different culture conditions, it was noted that the serum concentration of the medium has a profound effect on the binding of the E2− isoform to MHC at ambient ATP concentrations. Binding of the E2− isoform increases from 25.6 ± 3.4% to 61.5 ± 1.7% (P < 0.05) in 2.5% serum, whereas binding of the E2+ isoform is essentially unchanged (21 ± 9.2% vs. 31.7 ± 2.3%, P > 0.5). The rate of growth of HeLa cells in 2.5% and 10% serum was comparable, excluding this variable as an explanation for the difference in binding observed with the two AMPD1 isoforms. The abundance of the E2+ and E2− peptides relative to that of MHC is essentially unchanged when the serum concentration is reduced from 10 to 2.5% FBS (Table 1). In addition, supplementation of medium containing 2.5% serum with epidermal growth factor, endogenous growth factor, insulin, transferrin, triiodothyronine, progesterone, 17β-estradiol, testosterone, and hydrocortisone does not suppress binding of the E2− isoform at ambient ATP concentrations (Fig. 9).

Because of the results obtained with the aminoterminal deletion mutants, the alternatively spliced isoforms of AMPD1 were evaluated for differences in sensitivity to ATP-induced inhibition of binding. After permeabilization of HeLa cells cultured in either 10% serum (Fig. 9B) or 2.5% serum (Fig. 9C), the percentage of cells exhibiting colocalization was determined at low, ambient, and high ATP. In 10% serum, the two isoforms of AMPD1 exhibit comparable changes in binding to MHC in response to alterations in the intracellular concentration of ATP. However, in 2.5% serum, the E2−, but not the E2+, isoform of AMPD1 exhibits reduced sensitivity to ATP-induced inhibition of binding. The E2− isoform, in 2.5% serum, resembles the Nα178 deletion mutant in its binding characteristics in that it is relatively insensitive to ATP-induced inhibition of binding.

**DISCUSSION**

Results of this and prior studies suggest that there are a number of domains within the AMPD1 peptide that interact to control the activity of this enzyme in response to the physiological needs of the myocyte (Fig. 10). The peptide can be subdivided into a nonconserved amino terminus that is unique to the AMPD1 gene product and a conserved carboxy terminus that is shared among the various members of this multigene family (24). The catalytic site and an ATP binding site are located in the conserved, carboxy-terminal region of the AMPD1 peptide (8, 13, 15). The present study has
identified a myosin binding domain in the amino terminus that resides within the region encoded by residues 178–333. These studies do not exclude the presence of other myosin binding domains in the AMPD1 peptide, and the presence of one or more additional MHC binding domains could contribute to the low level of binding observed with the N1333 mutant. A portion of the amino-terminal region of AMPD1 that encompasses the myosin binding domain, residues 182–209, shares sequence similarity with another myosin binding protein, titin (Table 2). This region of titin, referred to as the PEVK domain, is thought to disrupt or destabilize local tertiary structures because of the reduced complexity of this repeating amino acid sequence and the cluster of negative charges in the PEVK domain (12). These conserved sequences could participate in the formation of the MHC binding domain in AMPD1 or play a role in regulating accessibility of the MHC binding domain through their influence on local tertiary structure. The latter is an attractive hypothesis because of the data presented in this report, which suggest that a secondary or higher-order structure of the intact AMPD1 peptide influences binding to MHC in response to changes in ATP concentration, mediated by a site remote to the MHC binding domain, and through alternative splicing in a region of the amino terminus distinct from the MHC binding domain.

The model illustrated in Fig. 10 suggests a mechanism for regulating the binding of AMPD1 to MHC and thereby controlling the activity of this enzyme in the myocyte. This model may be oversimplified because transfections were performed in nonmyocytes for the express purpose of limiting the complexity of AMPD1 interaction with other myocyte-specific proteins. In addition, the model is based on forced expression of AMPD1 and MHC using a transient transfection assay, and there is no assurance that the relative abundance and/or higher-order structure of the AMPD1 and MHC peptides produced in these nonmyocytes might not influence the interaction between these peptides. Although these recognized limitations to the assay used in the present study exist, none of the results obtained with the transient expression of the mutant AMPD1 peptides in this model system is at variance with prior studies that have examined the control of AMPD1 by ATP binding to the nucleotide regulatory site in this enzyme. During strenuous exercise when ATP levels in the myocyte fall below a critical level following intense muscle contraction, the inhibitory effect of ATP on binding of AMPD1 to MHC is diminished. Under these circumstances, the E2+ isoform of AMPD1 translocates to the myofibril where it is activated. Deamination of AMP by AMPD shifts the adenylate kinase equilibrium toward ATP formation, and this increases the adenylate energy charge (24). Thus activation of the E2+ isoform of AMPD1 through binding to MHC could help to preserve the adenylate energy charge at a time when energy production is limited. This function of AMPD could protect the myocyte from irreversible injury, which would ensue if the ATP or adenylate energy charge fell below a critical level following intense muscle contraction.

Another function that has been ascribed to AMPD in myocytes is the role this enzyme plays in controlling flux through the purine nucleotide cycle (24). When IMP is formed as a consequence of AMP deamination, IMP can be condensed with aspartate, subsequently leading to the formation of fumarate and AMP. This
series of reactions is referred to as the purine nucleotide cycle (in which SAMP is succinyl AMP)

In myocytes, fumarate production via the purine nucleotide cycle is the only known pathway for expanding the pool of citric acid cycle intermediates during muscle contraction (24), and flux through the purine nucleotide cycle is inhibited when GTP levels are reduced (24). The simultaneous requirement for a high level of GTP and activation of AMPD presents a metabolic dilemma if the E2 isoform of AMPD1 is needed for flux through the purine nucleotide cycle. As discussed above, the E2+ isoform is activated only after the adenylyl energy falls, and such a fall in the adenylyl energy charge will also lead to a drop in GTP levels in the cell. Under these conditions, the two arms of the purine nucleotide cycle are not coordinated.

The different myosin binding properties of the E2– isoform provide a potential mechanism for bypassing this metabolic dilemma. The E2– isoform of AMPD1 can bind to MHC in the presence of high-ATP concentrations under specified conditions, and activation of AMPD1 under these conditions would lead to flux through the purine nucleotide cycle and fumarate production. In vitro, the E2– isoform can overcome ATP-induced inhibition of binding to MHC in response to a signal produced by some component of the tissue culture medium. In vivo, a hormonal or neural stimulus may provide a signal to the E2– isoform of AMPD1, which releases it from ATP-induced inhibition of binding to MHC. The relative abundance of the E2– isoform of AMPD1 in different skeletal muscle fiber types is consistent with a potential role of the E2– isoform in controlling flux through the purine nucleotide cycle and generation of citric acid cycle intermediates. Fast-twitch glycolytic fibers have the lowest amount of the E2– relative to the E2+ isoform, and slow-twitch oxidative fibers have the highest amount of the E2– relative to the E2+ isoform (16, 18). Through regulation of the alternative splicing pathway, the myocyte can produce varying ratios of the E2– and E2+ isoforms of AMPD1. Binding of these two AMPD1 isoforms to MHC with subsequent activation of this enzyme could be differentially regulated to meet the particular needs of different fiber types under various physiological conditions.

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Present addresses: I. Hisatome, 1st Department of Medicine, Tottori University Faculty of Medicine, Nishimachi 36-1, Yonago 683, Japan; H. Kamma, Department of Pathology, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki, 305-8575, Japan; H. Morisaki, Department of Bioscience, National Cardiovascular Center Research Institute, Osaka, Japan.

Address for reprint requests: E. W. Holmes, Stanford Univ. School of Medicine, Office of the Dean, M-121, 300 Pasteur Drive, Stanford, CA 94305-5119.

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