Myopathy-associated αB-crystallin Mutants

ABNORMAL PHOSPHORYLATION, INTRACELLULAR LOCATION, AND INTERACTIONS WITH OTHER SMALL HEAT SHOCK PROTEINS

Received for publication, April 18, 2007, and in revised form, September 11, 2007 Published, JBC Papers in Press, September 25, 2007, DOI 10.1074/jbc.M703267200

Stephanie Simon‡, Jean-Marc Fontaine‡, Jody L. Martin§, Xiankui Sun¶, Adam D. Hoppe‡, Michael J. Welsh‡, Rainer Benndorf‡, and Patrick Vicart‡

From the §EA300 Stress et Pathologies du Cytosquelette, Université Paris 7, UFR de Biochimie, 75005 Paris, France, the Departments of ‡Cell and Developmental Biology and §Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan 48109, and the ‡Department of Medicine, Cardiovascular Institute, Loyola University Chicago, Maywood, Illinois 60153

Three mutations (R120G, Q151X, and 464delCT) in the small heat shock protein αB-crystallin cause inherited myofibrillar myopathy. In an effort to elucidate the molecular basis for the associated myopathy, we have determined the following for these mutant αB-crystallin proteins: (i) the formation of aggregates in transfected cells; (ii) the partition into different subcellular fractions; (iii) the phosphorylation status; and (iv) the ability to interact with themselves, with wild-type αB-crystallin, and with other small heat shock proteins that are abundant in muscles. We found that all three αB-crystallin mutants have an increased tendency to form cytoplasmic aggregates in transfected cells and significantly increased levels of phosphorylation when compared with the wild-type protein. Although wild-type αB-crystallin partitioned essentially into the cytosol and membranes/organelles fractions, mutant αB-crystallin proteins partitioned additionally into the nuclear and cytoskeletal fractions. By using various protein interaction assays, including quantitative fluorescence resonance energy transfer measurements in live cells, we found abnormal interactions of the various αB-crystallin mutants with wild-type αB-crystallin, with themselves, and with the other small heat shock proteins Hsp20, Hsp22, and possibly with Hsp27. The collected data suggest that each αB-crystallin mutant has a unique pattern of abnormal interaction properties. These distinct properties of the αB-crystallin mutants identified are likely to contribute to a better understanding of the gradual manifestation and clinical heterogeneity of the associated myopathy in patients.

αB-crystallin (αBC)2 is a ubiquitously occurring small heat shock protein (sHsp) with particularly high abundance in skeletal and cardiac muscles (1) in which it can be incorporated into the sarcomeric structure (2, 3). It is now well established that αBC is a major player in the function of muscular tissues, for example it protects cardiomyocytes from adverse conditions such as ischemic stress (4).

αBC and sHsps in general are widely believed to act as molecular chaperones, preventing the aggregation and precipitation of damaged or misfolded proteins in an ATP-independent way in stress conditions (5, 6). Consistent with this property, sHsps accumulate in human degenerative diseases, particularly in diseases involving abnormal protein aggregation (7). Usually, sHsps exist as polydisperse hetero-oligomers that change in size and/or organization when interacting with substrates or upon stress exposure (8–12). At the cellular level, sHsps protect cells from noxious conditions as diverse as toxicity promoted by aberrantly folded proteins, oxidative conditions, and proteasome inhibition. Distinct mechanisms have been proposed for the protective effects of sHsps, including chaperone-like activity, anti-apoptotic effects, or intracellular redox homeostasis (13–19). These distinct mechanisms are not exclusive and could occur concomitantly.

Mutations in sHsps are associated with the development of several degenerative diseases. A number of mutations in αB-crystallin were identified that lead to the degeneration of distinct tissues, including the lens of the eye and/or cardiac and skeletal muscles (20–26). Which of the tissues actually is affected depends on the specific mutation, and currently it is not known what causes this clinical heterogeneity. In addition, two others sHsps, Hsp22 and Hsp27, have been associated with the human degenerative diseases Charcot-Marie-Tooth and distal hereditary motor neuron diseases (27–33). The first discovered sHsp mutation is the missense mutation R120G in αBC (R120G/αBC) (20), and to date this is the best studied mutation. The R120G mutation in αBC results in dominant gain-of-function properties and causes a particular subtype of myofibrillar...
myopathy (desmin-related myopathy or αB-crystallinopathy) with associated cardiac involvement and cataract formation. The $\text{R}^{120G}_\text{αBC}$ protein exhibits changes in its secondary, tertiary, and quaternary structural features (34–37). It is more polydisperse than the wild-type $\text{αBC}$ (WT $\text{αBC}$) (36) and is inherently unstable in solution (37). These structural disturbances correlate with a decreased in vitro chaperone-like activity (36). A recent study has established that $\text{R}^{120G}_\text{αBC}$ directly promotes the aggregation of the desmin filament network and that desmin networks are differently affected, depending on the cellular backgrounds (38). In various cell lines, independent of desmin levels, $\text{R}^{120G}_\text{αBC}$ aggregates in a time-dependent process starting with the formation of multiple foci of insoluble proteins in the cytoplasm. Subsequently, these foci coalesce into large amorphous perinuclear aggregates in a microtubular network-dependent manner. Expression of $\text{R}^{120G}_\text{αBC}$ leads to the formation of a cage of type III intermediate filament proteins such as vimentin, but also of a cage of type II intermediate filament proteins such as keratins, suggesting a general response of the intermediate filament networks to the aggregate formation. Nevertheless, these intermediate filament proteins have not been found as components of the aggregates by themselves, in contrast to desmin (13). Moreover, $\text{R}^{120G}_\text{αBC}$ and its pseudophosphorylated mutants are unable to confer resistance to differentiation-induced apoptosis during C2C12 myoblast differentiation because of their impaired capacity to inhibit the proteolytic activation of caspase-3 (39). Overexpression of $\text{R}^{120G}_\text{αBC}$ in cardiomyocytes of transgenic mice results in a 100% mortality by early adulthood in high expressing lines, whereas a modest expression level results in a strikingly similar phenotype to that observed in patients with $\text{R}^{120G}_\text{αBC}$-associated cardiomyopathies (40). In these transgenic mice, the desmin network, myofibril alignment, mitochondrial-sarcoplasmic architecture, mitochondrial function, and the ubiquitin/proteasome system (UPS) were significantly impaired (41, 42). In addition, a hypertrophic response occurred, and the apoptotic pathways were activated (41). Taken together, it appears that all the known protective functions of $\text{αBC}$ are impaired. The two other $\text{αBC}$ mutations that are associated with myofibrillar myopathy are the nonsense mutation Q151X ($Q^{151X}_\text{αBC}$) and the frameshift mutation 464delCT ($464_\text{αBC}$) (22). Both mutants caused the formation of cytoplasmic aggregates in skeletal muscles, without cardiac or eye lens involvement. No further information on these two mutants is available. Recently, several studies have investigated the potential of $\text{SHsp}$ overexpression for the treatment of degenerative diseases. Hsp22, Hsp27, and WT $\text{αBC}$ revealed a high capacity to dissociate the aggregates formed by $\text{R}^{120G}_\text{αBC}$ expression in several cell lines (13, 43–45). Nevertheless, recent studies in cardiomyocytes suggest that the resulting hetero-oligomers of WT $\text{αBC}$/ $\text{R}^{120G}_\text{αBC}$ were more toxic for the cells than the homo-oligomers formed by the $\text{R}^{120G}_\text{αBC}$ alone (44). In contrast, both Hsp22 and Hsp25 (murine equivalent of Hsp27) co-expression with $\text{R}^{120G}_\text{αBC}$ rescued cell viability (45). So far, there is no explanation for this differential effect of $\text{αBC}$ and Hsp22 or Hsp27/Hsp25 to date.

In this study we have determined properties of the three mutants of $\text{αBC}$ ($\text{MTT}_\text{αBC}$, $\text{R}^{120G}_\text{αBC}$, $Q^{151X}_\text{αBC}$, and of $464_\text{αBC}$, that are associated with myofibrillar myopathy. We show that all three mutant proteins form abnormal cytoplasmic aggregates in both cardiomyocytes and in COS-7 cells. Moreover, $\text{MTT}_\text{αBC}$ proteins expressed in COS-7 cells distribute into additional cell fractions as compared with WT $\text{αBC}$. We also show that all three mutant proteins are hyperphosphorylated in all the three known phosphorylation sites (serine residues 19, 45, and 59) when expressed in COS-7 cells. We also investigated the interaction properties of these three $\text{MTT}_\text{αBC}$ with themselves, with WT $\text{αBC}$, and with Hsp20, Hsp22, and Hsp27. These $\text{SHsp}$s are known as interaction partners of $\text{αBC}$ and are abundant in muscles (46–48). Using the yeast two-hybrid (TH) method, chemical cross-linking (CL), pulldown (PD) assays, and the quantitative fluorescence resonance energy transfer (qFRET) method in live mammalian cells, we show here that all three $\text{MTT}_\text{αBC}$ are able to interact with themselves, with WT $\text{αBC}$, and with the other $\text{SHsp}$. We have identified a unique interaction pattern for each mutant with the other $\text{SHsp}$. It is expected that these identified abnormal properties of the three studied $\text{MTT}_\text{αBC}$ forms will contribute to a better understanding of the molecular processes that lead to the associated diseases and to the design of therapeutic strategies.

**EXPERIMENTAL PROCEDURES**

**Vector Constructs**—TH vector constructs were made using the vectors pACT2 and pAS2 (Clontech). Cyan (CFP) and citrine (Citr) fluorescent fusion protein expression vectors were made using the vectors pECFP-N1, pECFP-C1 (both from Clontech), and pCit-N1 and pCit-C1 (49). Myc/His-tagged and untagged protein constructs were made using the vectors pcDNA3.1(+)/myc-His B and pcDNA3, respectively (both from Invitrogen). A list of all used constructs and more detailed cloning information is given in supplemental Table 1 (construct numbers as used in this study are given in square brackets). Additional information on the constructs used is also given in previous publications (20, 48, 50, 51).

**Two-hybrid Method**—Small scale sequential transformation of yeast strain AH109 was performed as described in the manufacturer’s instructions (Clontech). Colonies were selected on −Trp, −Leu, −His medium for the phenotypes His + (growth) and LacZ + (blue color). The interaction assays were considered positive if both reporter genes were activated. For negative controls, yeast were transformed with each used vector alone and tested on −Trp, −Leu, and −His medium (not shown). Additionally, yeast were co-transformed with each vector and with the ‘empty’ partner vector as indicated in the figure legends (C1−C11, cf. supplemental Figs. 1B, 2A, 3A, and 4A). In none of these controls were the reporter genes activated.

**Cell Culture and Transfections**—COS-7 cells were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen) and penicillin/streptomycin (Invitrogen) in a 5% CO₂, humidified atmosphere. One day prior to transfections, cells were trypsinized and plated as specified below. Transfections were carried out using FuGENE 6 (Roche Diagnostics) with 0.75 μg (single construct) or 1.5 μg (two constructs) of vector DNA for CFP or Cit constructs or 2 μg of vector DNA for pcDNA3 or pcDNA3.1(+)/myc-His B constructs.
Abnormal Properties of Myopathy-associated αBC Mutants

Cardiomyocyte Isolation, Culture, and Infection—Rat neonatal ventricular myocytes were isolated by standard batch collagenase digestion and subsequently perfused to remove the fibroblasts. Cardiomyocytes were then counted and plated on gelatin-coated dishes in serum-free PC-1 medium (BioWhittaker). Twenty four hours after plating, the cells were washed, and the medium was changed to DMEM/M199 (4:1) maintenance medium. The cells were then transduced with adenovirus expressing the appropriate CFP fusion at an multiplicity of infection of 50. Two days later the cells were fixed in 2% paraformaldehyde solution, and rhodamine-phalloidin was stained before imaging.

Immunofluorescence Microscopy—COS-7 cells were grown on coverslips, transfected, and 48 h later were washed twice with ice-cold PBS, fixed, and permeabilized at 4°C for 5 min with cold methanol/acetone (7:3). Subsequently, cells were incubated for 1 h each at room temperature with primary polyclonal rabbit antibody (diluted 1:200 in PBS, 2% FCS) directed against the first 10 residues of αBC (Abcam) and with secondary goat anti-rabbit antibody (diluted 1:1000 in PBS, 2% FCS) coupled to AlexaFluor 11034 (Invitrogen). The coverslips were mounted on slides using Mowiol (Sigma). Images were collected using a fluorescent microscope (Leitz) equipped with a digital camera ORCA-ER (Hamamatsu) and processed using the Simple PCI 6.0 software (Compix Inc. Imaging Systems).

Live Cell Imaging—COS-7 cells were grown in 6-well glass-bottom culture plates (MatTek Corp.). 48 h after transfection with the various CFP and Cit fusion protein vectors, cells were washed twice with PBS and kept in DMEM without phenol red (Invitrogen). For fluorescence microscopy an inverted epifluorescence microscope (Eclipse TE-2000 U; Nikon) was used equipped with a 100-watt mercury arc-lamp, exciter filters 430/25 and 500/20, a dichroic microscope filter 86002bs, and with a 505dxr Dual View Micro Imager MSML.DV.CC (Optical Insights) with the emission filters 470/30 and 535/30. Images were collected by a digital CoolSnap CCD camera (Photometrics) and processed using Metamorph image processing software version 6.2r5 (Molecular Devices).

For determination of the fraction of cells with aggregates, microscopic fields were selected randomly using a Plan fluor ELWD ×40/0.6 objective lens (Nikon). At least 100 cells per sample group were included in these evaluations.

Quantitative Fluorescence Resonance Energy Transfer Measurements in Live Cells—The qFRET method was applied for quantification of apparent fluorescence resonance energy transfer efficiencies (AAFE) as indicators of protein interaction. The configuration of the microscope was as described above for live cell imaging using a Fluor ELWD ×40/1.3 oil Dic H objective lens (Nikon). Maintenance and transfection of COS-7 cells with the various CFP and Cit fusion protein vectors was as described for live cell imaging. In each cell to be analyzed, three cytoplasmic areas without protein aggregates were selected for qFRET measurements. Images from at least 30 microscopic fields per sample group were acquired and background/shading-corrected prior to computation by the qFRET algorithm. The calculated output data were expressed as $(E_A + E_D)/(E_A)$ (is apparent acceptor efficiency calculated from sensitized emission and dependent on the fraction of acceptor in complex; $E_D$ is apparent donor efficiency calculated relative to donor fluorescence and dependent on the fraction of the donor in complex). More details concerning qFRET are given in earlier publications (49, 51). As negative controls, the cells were transfected with the “empty” CFP (peCFP-N1) and Cit (peCit-N1) vectors (Fig. 3D) or with the empty Cit (peCit-N1/C1) and the various CFP constructs (supplemental Figs. 2C, 3C, and 4D). AAFE values that were significantly different from the control signals indicated interaction. Quantitative data are expressed as the mean of AAFE values ± s.e. The data between groups were analyzed using one-way ANOVA. When overall significance was detected, a post hoc multiple group comparison was conducted using Tukey HSD adjustment. Differences between groups were considered statistically significant if $p < 0.05$.

Analysis of the Phosphorylation Status of αB-crystallin—COS-7 cells were grown in 6-well plates. 48 h after transfection, cells were washed twice in ice-cold PBS and lysed using the ReadyPrep protein extraction kit (Bio-Rad) according to the manufacturer’s instructions. One volume of buffer A (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 400 mM dithiothreitol, 0.01% bromphenol blue) was added, and the samples were boiled for 3 min followed by SDS-PAGE/Western blotting. Equal loading of the samples was verified by visualization of vimentin on the same blots using a monoclonal anti-vimentin antibody diluted to 1:2000 (Sigma). Phosphorylation of the three known serine phosphorylation sites (Ser-19, Ser-45, and Ser-59) of αBC (52) was determined using phosphorylation site-specific polyclonal antibodies, diluted to 1:2000 (StressGen). After electrotransfer of the proteins, the polyvinylidene difluoride membrane was blocked with bovine serum albumin free of IgG (Interchim). For immunodetection, secondary goat anti-mouse or anti-rabbit horseradish peroxidase-coupled secondary antibodies diluted to 1:10,000 (Pierce) were used. The degree of phosphorylation of the various αBC species was quantified on scanned images using ImageJ software (53). The base-line signal was obtained from untransfected control cells.

Protein Fractionation—COS-7 cells were grown in 10-cm cell culture dishes and transfected with vectors coding for Myc/His-tagged αBC species [5–7]. 48 h after transfection, ~4 × 10⁶ cells were harvested, and the cell proteins were differentially extracted yielding the fractions of cytosolic proteins, membrane/organelle proteins, nuclear proteins, and cytoskeletal proteins using the ProteoExtract subcellular proteome extraction kit (Calbiochem) according to the manufacturer’s instructions. Each extract was dosed using the Dc-Kit (Bio-Rad) prior to mixing with 1 volume of buffer A. Equal aliquots of protein extracts (15 μg of protein) were analyzed by SDS-PAGE/Western blotting. A polyclonal rabbit anti-Myc primary antibody diluted to 1:10,000 (Sigma) and a goat anti-rabbit horseradish peroxidase-coupled secondary antibody (Pierce) were used for immunodetection. Nontransfected cells were used as negative control.

Cross-linking—COS-7 cells were grown in 6-well plates and transfected with vectors coding for Myc/His-tagged αBC species [5–8]. 48 h after transfection, cells were washed three times with ice-cold PBS and incubated for 30 min at room temperature with 0.5 mM of the homo-bifunctional amine-reactive cross-linker disuccinimidyl suberate (DSS; Pierce). The reac-
tion was stopped by adding Tris-HCl, pH 7.5, to a final concentration of 15 mM and incubating for 15 min at room temperature. Cells were lysed by adding 1 volume of buffer A. After brief sonication, samples were boiled for 3 min and analyzed by SDS-PAGE/Western blotting. A polyclonal rabbit anti-Myc primary antibody (Sigma) and a goat anti-rabbit horseradish peroxidase-coupled secondary antibody (Pierce) were used for immunodetection as described previously.

**Pulldown Assays**—COS-7 cells were grown in 6-well plates, and singly or doubly transfected with vectors coding for Myc/His-tagged [5–9] and CFP-tagged αBC or other sHsp species [10–13, 26, 28] as indicated in Fig. 3A and supplemental Figs. 1A, 2B, 3B, and 4B. 48 h after transfection, cells were collected, washed two times with ice-cold PBS, resuspended, and briefly sonicated. An aliquot of the protein extracts was mixed with 1 volume of buffer A, boiled for 3 min and analyzed by SDS-PAGE/Western blotting to determine the expression of the various transgenes. Equal loading was verified by visualization of vimentin on gels.

To immobilize Myc/His-tagged proteins, protein extracts were incubated on a rotary shaker at 4°C with pre-equilibrated nickel/nitritolactric acid beads (nickel beads; Qiagen) for 24 h (48 h for binding of Myc/His-tagged Hsp22). The nickel beads were then washed with ice-cold buffer C (50 mM NaH2PO4, NaOH, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20), and briefly sonicated. An aliquot of the protein extracts was mixed with 1 volume of buffer A, boiled for 3 min, and analyzed by SDS-PAGE/Western blotting to determine the expression of the various transgenes. Equal loading was verified by visualization of vimentin on gels. To immobilize Myc/His-tagged proteins, protein extracts were incubated on a rotary shaker at 4°C with pre-equilibrated nickel/nitritolactric acid beads (nickel beads; Qiagen) for 24 h (48 h for binding of Myc/His-tagged Hsp22). The nickel beads were then washed with ice-cold buffer C (50 mM NaH2PO4, NaOH, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20), and briefly sonicated. An aliquot of the protein extracts was mixed with 1 volume of buffer A, boiled for 3 min, and analyzed by SDS-PAGE/Western blotting to determine the expression of the various transgenes. Equal loading was verified by visualization of vimentin on gels.

**RESULTS**

**Intracellular Location and Phosphorylation Status of Myopathy-associated αBC Mutants in COS-7 Cells**—To determine possible differences between WT αBC and myopathy-associated MTT αBC species, we have investigated the intracellular location of untagged MTT αBC species [2–4] expressed in COS-7 cells by indirect immunofluorescence microscopy and compared it with the location of WT αBC [1]. Similarly to previous reports (13, 20), we found that 48 h after transfection R120G αBC localized in cytoplasmic aggregates in about 30% of the transfected cells, whereas expression of WT αBC resulted in cytoplasmic location without aggregate formation in essentially all cells (about 97%). Similarly, expression of Q151X αBC and 464αBC resulted in formation of cytoplasmic aggregates in about 30% of the transfected cells. Representative images of cells expressing WT αBC without aggregates (panel a) and of cells expressing MTT αBC without (panels b–d) and with aggregates (panels e–g) are shown in Fig. 1A. Occasionally, we also noted the presence of these αBC species in the nuclei as can be seen for R120G αBC and Q151X αBC in Fig. 1A (panels b and c, respectively).

The altered intracellular location of the MTT αBC species may be accompanied by altered partition of those proteins into subcellular fractions. Therefore, we determined the partition of two of these mutant proteins, R120G αBC and Q151X αBC, into the subcellular fractions of cytosol, membranes/organelles, nuclei, and cytoskeleton, using a method based on differential cell extraction. Myc/His-tagged WT αBC [5] expressed in COS-7 cells partitioned almost completely into the cytosol and membranes/organelles fractions (Fig. 1B). In contrast, Myc/His-tagged R120G αBC [6] and Q151X αBC [7] both partitioned additionally into the nuclear and cytoskeletal fractions, with the strongest signals being obtained in the cytoskeletal fraction. As
expected, no signals were observed in nontransfected control cells.

As nuclear import of WT αBC has been suggested to be phosphorylation-dependent (54), we also determined the phosphorylation status of the various Myc/His-tagged MT αBC species by SDS-PAGE/Western blotting using phosphoserine-specific antibodies that recognize the three known phosphoseraes, Ser-19, Ser-45, and Ser-59, in the WT αBC sequence (Fig. 1C). Quantitative measurements of the intensities of the bands on the Western blots (Fig. 1D) revealed that all three myopathy-associated MT αBC [6–8] are hyperphosphorylated on all three phosphorylation sites, as compared with WT αBC [5], with a particularly pronounced increase in phosphorylation of Ser-19. Quantification also revealed that the signal corresponding to the phosphorylation of Ser-45 in R120G αBC was greater as compared with Q151X αBC and 464 αBC. Equal expression of the various WT/MT αBC species and equal loading of the samples onto the gels was verified by visualizing WT/MT αBC (through its Myc tag) and vimentin, respectively. Taken together, expression of the myopathy-associated MT αBC in COS-7 cells revealed abnormal intracellular location (aggregate formation) and partition into subcellular fractions and an abnormal degree of phosphorylation (hyperphosphorylation).

Intracellular Location of Cit- and CFP-tagged αBC Mutants in Neonatal Cardiomyocytes and COS-7 Cells—Because all three MT αBC species which this study focuses on are associated with muscle disorders, we performed similar localization experiments in neonatal cardiomyocytes. The neonatal cardiomyocytes were refractory to transfection, and therefore the cardiomyocytes were infected with recombinant adenoviruses expressing the various fusion proteins. 48 hours after infection, ~11% of the cells expressing CFP-tagged WT αBC showed formation of cytoplasmic aggregates, thus defining the base-line level for this construct. Cells expressing R120G αBC, Q151X αBC, or 464 αBC formed cytoplasmic aggregates in significantly higher proportions of cells, ~42, ~21, and ~20%, respectively. Selected images of cells without and with aggregates are shown in Fig. 2A (panels a–e and f–h, respectively). These data suggest that the altered properties of MT αBC species result in abnormally increased aggregate formation in normal neonatal cardiomyocytes, similar to what was observed in COS-7 cells. Because of the limitations related to the work with cardiomyocytes, we used the experimentally more feasible COS-7 cells to determine further properties of the MT αBC species. Because Cit- and CFP-tagged αBC species were used in most of the assays shown below, we also determined the aggregate formation of these constructs after expression in COS-7 cells. Similar to what was observed in neonatal cardiomyocytes, a portion of COS-7 cells expressing the various WT/MT αBC species contained cytoplasmic aggregates, with MT αBC species exhibiting increased tendencies to form aggregates. For example, expression of CFP-tagged R120G αBC [11], Q151X αBC [12], and 464 αBC [13] resulted in ~35, ~25, and ~33%, respectively, of cells containing aggregates, as compared with ~15% for WT αBC [10], which defines the base-line level for this group of constructs. Selected images of COS-7 cells without and with aggregates, expressing Cit-tagged WT/MT αBC species [18–21], are shown in Fig. 2B (panels a–e and f–h, respectively).

We also estimated the possible impact of the expression of the MT αBC species on the location of co-expressed wild-type sHsps as was required for the qFRET method. When the various CFP-tagged sHsp constructs were co-expressed with CFP-tagged R120G αBC [19] (Fig. 2C, panels a–e), we observed the recruitment of CFP-tagged WT αBC [18], Hsp20 [26], Hsp22 [27], and Hsp27 [28], into the aggregates (Fig. 2C, panels g–j), similar to what has been described previously (13). Co-expressed CFP alone was not recruited into the aggregates suggesting specificity for this sHsp recruitment (Fig. 2C, panels a and f). Similar data were obtained for Q151X αBC [20] and 464 αBC [21] when co-expressed with the various CFP-tagged sHsps (data not shown). These experiments also demonstrate that most of the cytoplasmic areas of the cells (be it from cells with or without aggregates) do not contain aggregates. Such aggregate-free areas were selected for the qFRET measurements as described below.
Protein Interactions Involving Myopathy-associated Mutant and Wild-type αB-Crystallins—We have determined the ability of \( \text{MTT}_\alpha \text{BC} \) species to interact with \( \text{WT}_\alpha \text{BC} \) and with themselves using different methods to assay protein/protein interactions including the PD, CL, TH, and qFRET methods. Previously, \( \text{WT}_\alpha \text{BC} \) was shown to interact with itself (55–57), and this interaction served as a positive control in all assays.

For the PD assays, the various CFP- and Myc-His-tagged constructs were used with nickel beads. After controlling the proper functioning of the method (see supplemental Fig. 1A), PD assays were conducted to determine the ability of myopathy-associated \( \text{MTT}_\alpha \text{BC} \) species to interact with \( \text{WT}_\alpha \text{BC} \) and with themselves. Cells were doubly transfected with the various Myc/His- [5–8] and CFP-tagged [10–13] \( \text{WT/MTT}_\alpha \text{BC} \) species as indicated in Fig. 3A. All Myc/His-tagged (Fig. 3A, row I) and CFP-tagged (row II) \( \text{WT/MTT}_\alpha \text{BC} \) species were expressed in the COS-7 cells in similar amounts. After incubation of the cell extracts with the nickel beads, all Myc/His-tagged \( \text{WT/MTT}_\alpha \text{BC} \) species did bind, as shown by SDS-PAGE/Western blotting after elution (Fig. 3A, row III). Analysis of the same fractions for the presence of the various CFP-tagged \( \text{WT/MTT}_\alpha \text{BC} \) species revealed their presence (Fig. 3A, row IV). This co-elution of the CFP-tagged \( \text{WT/MTT}_\alpha \text{BC} \) species (that do not bind to the nickel beads by themselves; see supplemental Fig. 1A) suggests interaction in all tested combinations. After elution (Fig. 3A, rows III and IV), the intensity of the obtained bands resulting from interactions for all \( \text{MTT}_\alpha \text{BC} \) species with \( \text{WT}_\alpha \text{BC} \) (lanes 2–4) or from all \( \text{MTT}_\alpha \text{BC} \) proteins with themselves (lanes 5–7) was similar to that of the \( \text{WT}_\alpha \text{BC}/\text{WT}_\alpha \text{BC} \) interaction (lane 1). Again, visualization of vimentin was used as loading control (Fig. 3A, row V). The data in Fig. 3A suggest that all myopathy-associated \( \text{MTT}_\alpha \text{BC} \) forms interact both with \( \text{WT}_\alpha \text{BC} \) and with themselves, with no obvious differences between any of the \( \text{MTT}_\alpha \text{BC} \) forms and \( \text{WT}_\alpha \text{BC} \).

To verify the ability of the various \( \text{MTT}_\alpha \text{BC} \) to form homodimers, we applied CL as an independent assay. COS-7 cells were transfected with Myc/His-tagged constructs [5–8] to express each of the \( \text{WT/MTT}_\alpha \text{BC} \) species as indicated in Fig. 3B or not transfected (control). Subsequently, cells were treated with the cross-linker DSS (Fig. 3B, right panel) or were not treated for control purposes (left panel). In the absence of DSS, only monomers of the \( \text{WT}_\alpha \text{BC} \) or \( \text{MTT}_\alpha \text{BC} \) were detected by SDS-PAGE/Western blotting. In contrast, after incubation with DSS, additional bands that correspond to homodimers were detected. No major differences between \( \text{MTT}_\alpha \text{BC} \) forms and \( \text{WT}_\alpha \text{BC} \) were found. Thus, all three \( \text{MTT}_\alpha \text{BC} \) forms were able to form homodimers similarly as \( \text{WT}_\alpha \text{BC} \).

TH assays were applied as a further method to independently verify these interactions among the various \( \text{WT/MTT}_\alpha \text{BC} \) species. In the negative TH controls, the reporter genes were not activated (see supplemental Fig. 1B) thus reducing the risk of false positive data. The data showed that all myopathy-associated \( \text{MTT}_\alpha \text{BC} \) forms, in combination with themselves or with \( \text{WT}_\alpha \text{BC} \), activated both reporter genes to a similar extent (Fig. 3C). In summary, the TH data also suggested interaction of all myopathy-associated \( \text{MTT}_\alpha \text{BC} \) forms with themselves and with \( \text{WT}_\alpha \text{BC} \), with no major differences between \( \text{MTT}_\alpha \text{BC} \) forms and \( \text{WT}_\alpha \text{BC} \).

The qFRET method using CFP and Cit fusion proteins was developed to determine subtle changes in the stoichiometry of protein interactions (49), and by this method abnormal interactions involving mutant sHsps were identified that could not be revealed by other methods (51). Therefore, we applied this more sensitive method to determine possibly abnormal interactions involving \( \text{MTT}_\alpha \text{BC} \) forms. Homodimer formation of \( \text{WT}_\alpha \text{BC} \) essentially involves the C-terminal parts of both interacting molecules (55–57). To avoid possible steric hindrance in the C-terminal regions, the qFRET experiments were conducted using fusion protein constructs with the CFP and Cit tags being fused to the N termini of the \( \text{WT/MTT}_\alpha \text{BC} \) forms [10–13, 18–21]. The determined AAFE values for all the tested interactions were significantly different from the negative control value using CFP/Cit (Fig. 3D). This indicated that all \( \text{MTT}_\alpha \text{BC} \) and \( \text{WT}_\alpha \text{BC} \) forms interacted with one another. For most of the interactions (\( \text{WT}_\alpha \text{BC}/\text{R120C}_\alpha \text{BC}, \text{WT}_\alpha \text{BC}/\text{Q151X}_\alpha \text{BC}, \text{R120C}_\alpha \text{BC}/\text{Q151X}_\alpha \text{BC}, \text{Q151X}_\alpha \text{BC}/\text{Q151X}_\alpha \text{BC} \), and \( \alpha \text{BC}/\alpha \text{BC} \)), the determined AAFE values were very similar to that of the \( \text{WT}_\alpha \text{BC}/\text{WT}_\alpha \text{BC} \) interaction. Despite this high degree of similarity, some minor differences to the \( \text{WT}_\alpha \text{BC}/\text{WT}_\alpha \text{BC} \) interaction were statistically significant as was revealed by one-way ANOVA followed by post hoc pairwise group comparisons (Fig. 3D). Interestingly, all \( \text{MTT}_\alpha \text{BC}/\text{MTT}_\alpha \text{BC} \) interactions showed a small increase as compared with the \( \text{WT}_\alpha \text{BC}/\text{WT}_\alpha \text{BC} \) interaction (−11%). Only the \( \text{MTT}_\alpha \text{BC}/\alpha \text{BC} \) interaction was moderately reduced (−17%) and significantly different from all other tested interactions shown in Fig. 3D.

Taken together, the data resulting from all applied methods strongly suggest that the myopathy-associated \( \text{MTT}_\alpha \text{BC} \) forms interact with themselves and with \( \text{WT}_\alpha \text{BC} \). The PD, CL, and TH assays did not reveal any differences in these interactions. Although the qFRET method, because of its greater sensitivity, revealed minor differences in most of these interactions as compared with the \( \text{WT}_\alpha \text{BC}/\text{WT}_\alpha \text{BC} \) interaction, the data suggest that these mutations do not have major consequences for the interaction of \( \alpha \text{BC} \) with itself at the level of dimers. The only significant consequence was that the \( \text{WT}_\alpha \text{BC}/\alpha \text{BC} \) interaction was moderately decreased by this mutation.

Interaction of αB-Crystallin Mutants with Hsp20—To determine potentially aberrant interaction properties of the myopathy-associated \( \text{MTT}_\alpha \text{BC} \) species with Hsp20, we conducted TH, PD, and qFRET assays (see supplemental Fig. 2, A–C, respectively). The interaction between \( \text{WT}_\alpha \text{BC} \) and Hsp20 has been reported previously (46, 47) and served as positive control. The TH data confirmed the occurrence of the \( \text{WT}_\alpha \text{BC}/\text{Hsp20} \) interaction. Additionally, the results suggested interaction between both \( \text{Q151X}_\alpha \text{BC} \) and \( \alpha \text{BC} \) with Hsp20 to similar degrees, whereas the interaction of \( \text{R120G}_\alpha \text{BC} \) with Hsp20 apparently was decreased as suggested by the weak activation of both reporter genes. All negative TH controls (supplemental Fig. 1B, C1–C4, and supplemental Fig. 2A, C9) provided negative results as expected, thus rendering false-positive interaction data unlikely.

To confirm these results by an independent method, we conducted PD experiments. When CFP-tagged Hsp20 was co-expressed with any of the Myc/His-tagged \( \text{WT/MTT}_\alpha \text{BC} \) species, Hsp20 was co-eluted from the nickel beads together with the
Abnormal Properties of Myopathy-associated αBC Mutants

A

![Image 153x393 to 193x427]

B

![Image 221x603 to 251x617]

C

![Image 237x393 to 319x427]

D

![Image 254x603 to 284x617]

FIGURE 3. PD, CL, TH, and qFRET assays to determine interactions of MTMαBC species with WTαBC and themselves. A, PD assays to determine binding of each MTMαBC species to WTαBC (lanes 2–4) and with themselves (lanes 5–7). COS-7 cells were doubly transfected with the various Myc- and CFP-tagged αBC species as indicated and were harvested 48 h later. The expression of all transgenes in the cell extracts was verified using Myc- and GFP/CFP-specific antibodies as indicated (rows I and II). Myc/His-tagged αBC species in cell extracts were bound to nickel beads, and after washing, bound proteins were eluted with buffer A and analyzed by SDS-PAGE/Western blotting, again using the Myc- and GFP/CFP-specific antibodies (rows III and IV). The results revealed that all probed CFP-tagged αBC species that did not bind by themselves to the nickel beads were bound through interaction with the Myc/His-tagged αBC species (rows III and IV). Vimtenin was visualized by an anti-vimentin (anti-Vim) antibody to verify equal sample loading (row VI). B, CL of WTαBC and MTMαBC species. COS-7 cells were transfected to express Myc/His-tagged WTαBC, R120GαBC, Q151XαBC, and 464αBC. 48 h later, and cells were resuspended in PBS and treated with 0.5 mM of DSS (right panel) or not (left panel) prior to analysis by SDS-PAGE/Western blotting using a polyclonal anti-Myc antibody. CL resulted in similar homodimer formation of WTαBC and all MTMαBC species. The position of molecular mass marker proteins (kDa) is indicated on the left. C, TH assays. Yeast was co-transformed with pairs of the various αBC species as indicated. In all combinations, the αBC species activated both reporter genes (His, growth; LacZ, β-galactosidase resulting in blue color) indicating interaction. D, qFRET assays. The AAFE was determined in selected areas of doubly transfected COS-7 cells co-expressing CFP- and Cit-tagged αBC species as indicated. COS-7 cells co-expressing CFP and Cit were used as negative control. #, indicates significant differences of the AAFE values from negative control. *, indicates significant differences of the AAFE values from the WTαBC/MYMαBC interaction. All sample values were significantly different from this negative control thus indicating interaction. A minor increase in the interaction of MYMαBC/MYMαBC is observed, whereas the WTαBC/MYMαBC showed a moderate decrease as compared with WTαBC/MYMαBC.
tested WT/MTT αBC species (supplemental Fig. 2B, row IV, lanes 2–5). Thus, the PD assays suggest interaction between the tested MTT αBC species and Hsp20. The R120G αBC/Hsp20 interaction appeared to be weaker when compared with the WT αBC/Hsp20 interaction, which is in agreement with the TH data. To quantify possible changes in the interactions resulting from the mutations, we conducted qFRET experiments using Cit-tagged WT/MTT αBC species (with the Cit moiety fused to the N termini of the WT/MTT αBC species) [18–21] and CFP-tagged Hsp20 [26] (supplemental Fig. 2C). The determined AAFE values for all tested interactions were significantly different from the negative control value (CFP-tagged Hsp20 and Cit alone) suggesting that all WT/MTT αBC forms interacted with Hsp20 thus confirming the results obtained by the TH and PD assays. For the R120G αBC/Hsp20 interaction, a substantial decrease of the AAFE by ~50% was measured, whereas the Q151X αBC/Hsp20 and 464αBC/Hsp20 interactions showed a moderate decrease by ~15 and ~22%, respectively, as compared with the WT αBC/Hsp20 interaction. Results from one-way ANOVA and post hoc pairwise group comparisons revealed significant differences in AAFE values between all groups. To avoid potential artifacts because of steric hindrance by the Cit moiety of the fusion proteins, a separate set of experiments was conducted with Cit fused to the C terminus of the WT/MTT αBC species [22–25]. The obtained data were similar to those using N-terminal fusion constructs thus confirming these results (data not shown).

Taken together, the data collected from the TH, PD, and qFRET experiments showed that all myopathy-associated MTT αBC forms are able to interact with Hsp20. By all three methods, the R120G αBC/Hsp20 interaction was found to be greatly diminished. Additionally, the qFRET method revealed somewhat decreased interactions of Q151X αBC and 464αBC with Hsp20.

Interaction of α-B-Crystallin Mutants with Hsp27—Finally, experiments were conducted to determine potentially aberrant interaction properties of the MTT αBC with Hsp27 (see supplemental Fig. 4). Both WT αBC and Hsp27 are interacting proteins [46, 47, 56], and this WT αBC/Hsp27 interaction served as positive control. The TH data confirmed the interaction between WT αBC [29] and Hsp27 [39] (supplemental Fig. 4A). In addition, the TH results suggest interaction between all MTT αBC species [30–32] and Hsp27 with no major differences in the interactions. Again, all negative TH controls (supplemental Fig. 1B, C1–C4; Fig. 4A, C1I) provided negative results.

To confirm these interactions, corresponding PD experiments were conducted (supplemental Fig. 4B). PD assays confirmed interaction between all the tested MTT αBC species and Hsp27. Those interactions appeared to be slightly increased when compared with the WT αBC/Hsp27 interaction. Finally, qFRET experiments were conducted with the intent to quantify possible differences in binding of the various MTT αBC species to Hsp27. Two series of experiments were performed using CFP-tagged Hsp22 [28], and Cit-tagged WT/MTT αBC species with the Cit moiety fused to the C terminus of the WT/MTT αBC species (vector group I, constructs 22–25) (supplemental Fig. 4C) or Cit-tagged WT/MTT αBC species with the Cit moiety fused to the N terminus of the WT/MTT αBC species (vector group II, constructs 18–21) (supplemental Fig. 4D). Using both vector groups, all determined AAPE values were significantly greater than the negative control (CFP-tagged HSP27/Cit alone). This confirmed that all WT/MTT αBC species indeed did interact with Hsp27.
Abnormal Properties of Myopathy-associated αBC Mutants

However, the results were not consistent using the constructs of vector groups I and II. When WT/MTT αBC species of the vector group I were used, all MTT αBC showed increased interaction with Hsp27 when compared with the WT αBC/Hsp27 interaction (by ~81, ~58, and ~60% increase for R120G αBC, Q151X αBC, and 464 αBC, respectively; see supplemental Fig. 4C). This set of data is consistent with the slight increase in interaction that was observed for all MTT αBC forms by the pulldown assays (supplemental Fig. 4B). In contrast, when WT/MTT αBC species of the vector group II were used, WT αBC and R120G αBC showed a similar interaction with Hsp27, whereas the Q151X αBC and 464 αBC mutants showed moderately decreased interactions when compared with the WT αBC/Hsp27 interaction (by ~25 and ~19% decrease for Q151X αBC and 464 αBC, respectively; supplemental Fig. 4D). Using one-way ANOVA and subsequent post hoc pairwise group comparisons indicated statistical significance for the data obtained by both vector groups. This inconsistency was probably caused by steric hindrance involving the CFP and Cit tags. For that reason, no conclusion regarding possible differences between WT αBC and MTT αBC in the interaction with Hsp27 can be drawn at this time.

In summary, the data collected from the TH, PD, and qFRET assays showed that all myopathy-associated MTT αBC forms are able to interact with Hsp27. Possible differences in the interactions between MTT αBC forms and WT αBC could not be unambiguously verified.

DISCUSSION

In this study, we focused our work to examine the interaction properties of the three known myofibrillar myopathy-associated MTT αBC species R120G αBC, Q151X αBC, and 464 αBC. First, we examined the cellular localization of the mutant proteins in transfected cells. We established that all the three mutant proteins have an increased tendency to form cytoplasmic aggregates in both COS-7 cells and in cardiomyocytes. This result confirmed the tendency of myopathy-associated αBC mutants to form aggregates in cultured cells in the presence or absence of desmin. This aggregate formation may result from misfolding of the mutant proteins, as was shown for R120G αBC (37). No data are available about the stability of Q151X αBC and 464 αBC; however, previous studies have established that the affected C-terminal arm of αBC is particularly important for the stability and function of αBC (4, 55, 57). This increased tendency to form aggregates may impair the cellular function of all MTT αBC species, as was shown for the cytoprotective ability of R120G αBC (34, 36, 38–40, 54, 58).

In cell fractionation experiments using transfected COS-7 cells, we demonstrated that R120G αBC or Q151X αBC associated with the nuclear and especially the cytoskeletal fractions, whereas WT αBC did not. This finding strongly suggests that those MTT αBC forms have an abnormally increased affinity for cytoskeletal components. For R120G αBC, a previous study has demonstrated increased affinity for desmin intermediate filaments that are specifically expressed in muscle (38). Thus, Q151X αBC and perhaps also 464 αBC, may share this enhanced desmin-binding property and thus lead to similar clinical phenotypes as the desmin-related myopathies.

The intracellular location of WT αBC has been found to be related to its phosphorylation state (52, 54, 59–65). The only mutant studied to date, R120G αBC, was seen to be hyperphosphorylated at all three phosphorylation sites in muscles of transgenic mice or at two phosphorylation sites when it was expressed in HeLa cells (54). Using COS-7 cells, we observed that all three myopathy-associated MTT αBC forms indeed are hyperphosphorylated at all three phosphorylation sites as compared with WT αBC. This αBC hyperphosphorylation could be a specific feature of myopathies caused by mutation in αBC gene as hyperphosphorylation of Hsp27 was described in myopathies caused by desmin mutation (66). Interestingly, we observed a higher tendency to be phosphorylated in Ser-45 for R120G αBC than for Q151X αBC or 464 αBC mutants. Considering that R120G αBC is also implied in cardiomypathy development whereas Q151X αBC or 464 αBC are only linked to myopathies, this result suggest a differential effect of Ser-45 phosphorylation in skeletal and cardiac muscles. Our findings reinforced the hypothesis suggesting that hyperphosphorylation of MTT αBC may be part of the disease mechanism, as has been previously proposed for R120G αBC (54).

Second, we examined if the MTT αBC species have altered properties in regard to interactions with themselves, with WT αBC, and with the other sHsps that are abundant in muscle. It is now well established that composition and size of the oligomeric structures formed by the various sHsps are important for both properties and functions of the sHsp complexes in cells (67). Indeed, the significance of protein-protein interactions is not only to form protein complexes for cellular functions but could also be implied in protein stability or solubility (19).

Our data show that the interactions of MTT αBC with WT αBC were not affected or were only slightly affected, with the exception of the 464 αBC/WT αBC interaction that was moderately decreased. A minor increase was also observed in the interaction of the MTT αBC species with themselves. Recent studies showed that despite its capacity to reduce the formation of R120G αBC aggregates, WT αBC is unable to reduce the cytotoxicity induced by expression of the mutated protein (44, 45). Our results showed that the effect on aggregation of WT αBC is probably because of a passive mechanism (there is not an active recruitment of WT αBC) leading to the formation of more soluble heterocomplexes of WT αBC/MMT αBC than homocomplexes of MTT αBC without a real chaperone mechanism involved.

Our data also show that the interactions of all MTT αBC species with Hsp20 were decreased as compared with the WT αBC/Hsp20 interaction. It is interesting to note that Hsp20, which is known as an sHsp with relatively low chaperone function, exhibits only decreased interaction with all MTT αBCs (68). We measured a major decrease in R120G αBC/Hsp20 interaction and moderate decreases in the Q151X αBC/Hsp20 and 464 αBC/Hsp20 interactions. Considering that R120G αBC induces myopathy with cardiac involvement, whereas Q151X αBC and 464 αBC result in myopathy without cardiac involvement (20, 22), and that Hsp20 is involved in cardiac protection (69), our data suggest that decreased R120G αBC/Hsp20 interaction may play a role in the manifestation of cardiac involvement of the disease.
Our data also reveal distinct differences in the interaction of the various MTT αBC species with Hsp22. R120G αBC and Q151X αBC showed greatly increased or decreased, respectively, interactions with Hsp22 as compared with the WT αBC/Hsp22 interaction, whereas the interaction between 464 αBC and Hsp22 remained essentially unchanged. This increased R120G αBC/Hsp22 interaction confirms the results of Chavez Zobel et al. (13). Recently, several studies have suggested that Hsp22 overexpression may be an efficient therapeutic tool to restore the functions of R120G αBC (13, 45). Following this argument, the decreased or unchanged interactions of Q151X αBC and 464 αBC, respectively, with Hsp22 would not support the idea that overexpression of Hsp22 might be a useful method to neutralize the adverse effects of these two MTT αBC species in myofibrillar myopathies.

Our results collected by various methods concerning the interaction of the different MTT αBC species with Hsp27 confirm the interaction between both proteins. However, the qFRET data were inconsistent with respect to possibly increased or decreased interactions of MTT αBC species as compared with WT αBC, depending on the constructs used. Although the qFRET experiments using C-terminally tagged WT/MTT αBC species showed a pronounced increase in interactions for all three MTT αBC species, the experiments using N-terminally tagged WT/MTT αBC species showed unchanged or decreased interactions resulting from the mutations. Consistent with this latter observation is that the N-terminally tagged MTT αBC species as used in the PD assays also indicated slightly increased interactions. Although the reason for this vector-dependent inconsistency is not known, steric hindrance of the N-terminally tagged MTT αBC species is a plausible explanation.

In summary, this study for the first time characterizes the myopathy-associated mutants Q151X αBC and 464 αBC and compares these mutants with the previously characterized R120G αBC. We established that these three myopathy-associated MTT αBC proteins show both abnormal protein aggregation and abnormal subcellular localization. Moreover, we show that all three mutants are hyperphosphorylated at the three serine phosphorylation sites. These data reinforce the emerging concept of a key role of sHsp phosphorylation in the development of degenerative diseases (39, 54, 62, 66).

Modifications in the interaction level between sHsps caused by pathological mutation in a sHsps (Hsp22) have been established previously (51). It has been suggested that those modifications could participate in the manifestation of the related diseases. Our data on the interactions of the MTT αBC species have also established that each myopathy-associated mutant has a specific pattern of abnormal interactions, be it with WT αBC (464 αBC), with themselves (R120G αCB, Q151X αBC, 464 αBC), with Hsp20 (R120G αCB, Q151X αBC, 464 αBC), or with Hsp22 (R120G αCB, Q151X αBC). A scheme of these abnormal interactions is presented in Fig. 4. Further studies will establish if altered interactions between sHsps is a common feature in pathologies linked with sHsps mutations. The abnormal properties of MTT αBC species may contribute to the slowly progressing manifestation of the associated myopathies, e.g. by destabilization of the structure MTT αBC-containing sHsp oligomers. The heterogeneity observed for the abnormal phosphorylation and interaction patterns of the three MTT αBC forms may be of relevance with regard to the observed heterogeneity of the associated disease phenotypes. These different abnormal properties may differentially impair the function(s) of MTT αBC such as chaperoning, be it in response to environmental stress factors or in a tissue-specific way. This work provides a significant advance in understanding the heterogeneity in the clinical manifestations of muscular diseases caused by αBC mutations.

REFERENCES
1. Iwaki, T., Kume-Iwaki, A., and Goldman, J. E. (1990) J. Histochem. Cytochem. 38, 31–39
2. Lutsch, G., Vetter, R., Offhaus, U., Wieske, M., Grone, H. J., Klemenz, R., Schimke, I., Stahl, J., and Benndorf, R. (1997) Circulation 96, 3466–3476
Abnormal Properties of Myopathy-associated αβC Mutants

3. Golenhofen, N., Perg, M. D., Quinlan, R. A., and Drenckhahn, D. (2004) Histocompatibility Testing. Cell Biol. 122, 415–425

4. Martin, J. L., Bluhm, W. F., He, H., Mestril, R., and Dillmann, W. H. (2002) Am. J. Physiol. 283, H85–H91

5. Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) J. Biol. Chem. 268, 1517–1520

6. Ganea, E. (2001) Curr. Protein Pept. Sci. 2, 205–225

7. Sun, Y., and MacRae, T. H. (2005) FEBS J. 272, 2613–2627

8. Horwitz, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449–10453

9. Carver, J. A., Guerreiro, N., Nicholls, K. A., and Truscott, R. J. (1995) Biochim. Biophys. Acta 1252, 251–260

10. Ehrnsperger, M., Lilie, H., Gaestel, M., and Buchner, J. (1999) J. Biol. Chem. 274, 14867–14874

11. Lee, G. J., Roseman, A. M., Saibil, H. R., and Vierling, E. (1997) EMBO J. 16, 659–671

12. Wang, K., and Spector, A. (2000) Eur. J. Biochemistry. 267, 4705–4712

13. Chavez Zobel, A. T., Loranger, A., Marceau, N., Theriault, J. R., Lambert, H., and Landry, J. (2003) Hum. Mol. Genet. 12, 1609–1620

14. Stamler, R., Kappé, G., Boelens, W., and Slingsby, C. (2005) J. Mol. Biol. 353, 68–79

15. den Engelsman, J., Keijers, V., de Jong, W. W., and Boelens, W. C. (2003) J. Biol. Chem. 278, 4699–4704

16. Kamradt, M. C., Chen, F., and Cryns, V. L. (2001) J. Biol. Chem. 276, 16059–16063

17. Kamradt, M. C., Mu, L., Werner, M. E., Kwan, T., Chen, F., Strobecker, A., Oshita, S., Wilkinson, J. C., Yu, C., Oliver, P. G., Dukett, C. S., Buchsbaum, D. J., LoBuglio, A. F., Jordan, V. C., and Cryns, V. L. (2005) J. Biol. Chem. 280, 11059–11066

18. Arriag, A. P., Virot, S., Chauffour, S., Firdaws, W., Kretz-Remy, C., and Diaz-Latoud, C. (2005) Antioxid. Redox Signal. 7, 414–422

19. Liu, S., Li, T., and Xiao, X. (2007) Biochem. Biophys. Res. Commun. 354, 109–114

20. Vicart, P., Caron, A., Guicheny, P., Li, Z., Prevost, M. C., and Welsh, M. J. (2002) Nature Genet. 29, 10449–10453

21. Kato, K., Goto, S., Inaguma, Y., Hasegawa, K., Morishita, R., and Asano, T. (2001) Biophys. J. 80, 741–748

22. Inaguma, Y., Ito, H., Iwamoto, I., Saga, S., and Kato, K. (2001) J. Biol. Chem. 276, 16095–16099

23. Carver, J. A., Guerreiro, N., Nicholls, K. A., and Truscott, R. J. (1995) Biochim. Biophys. Acta 1252, 251–260

24. Ehrnsperger, M., Lilie, H., Gaestel, M., and Buchner, J. (1999) J. Biol. Chem. 274, 14867–14874

25. Liu, S., Li, J., Tao, Y., and Xiao, X. (2007) Biochem. Biophys. Res. Commun. 354, 109–114

26. Vicart, P., Caron, A., Guicheny, P., Li, Z., Prevost, M. C., and Welsh, M. J. (2002) Nature Genet. 29, 10449–10453

27. Caron, A., Guicheney, P., Li, Z., Prevost, M. C., Faure, A., Chateau, D., Chapon, F., Tomez, F., Dupret, J. M., Paulin, D., and Fardeau, M. (1998) J. Biol. Chem. 273, 11059–11065

28. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

29. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

30. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

31. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

32. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

33. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

34. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

35. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

36. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

37. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

38. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

39. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664

40. Benndorf, R. (2004) Eur. J. Biochemistry. 271, 3652–3664
Abnormal Properties of Myopathy-associated αBC Mutants

62. Kato, K., Inaguma, Y., Ito, H., Iida, K., Iwamoto, I., Kamei, K., Ochi, N., Ohta, H., and Kishikawa, M. (2001) J. Neurochem. 76, 730–736
63. Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y., Garcia-Mata, R., Sztul, E., and Kato, K. (2002) J. Biochem. (Tokyo) 131, 593–603
64. Gaestel, M. (2002) Prog. Mol. Subcell. Biol. 28, 151–169
65. den Engelsman, J., Bennink, E. J., Doerwald, L., Onnekink, C., Wunderink, L., Andley, U. P., Kato, K., de Jong, W. W., and Boelens, W. C. (2004) Eur. J. Biochem. 271, 4195–4203
66. Clemen, C. S., Fischer, D., Roth, U., Simon, S., Vicart, P., Kato, K., Kaminski, A. M., Vorgerd, M., Goldfarb, L. G., Eymard, B., Romero, N. B., Goudeau, B., Eggermann, T., Zerres, K., Noegel, A. A., and Schroder, R. (2005) FEBS Lett. 579, 3777–3782
67. Giese, K. C., and Vierling, E. (2002) J. Biol. Chem. 277, 46310–46318
68. van de Klundert, F. A., Smulders, R. H., Gijsen, M. L., Lindner, R. A., Jaenicke, R., Carver, J. A., and de Jong, W. W. (1998) Eur. J. Biochem. 258, 1014–1021
69. Fan, G. C., Chu, G., and Kranias, E. G. (2005) Trends Cardiovasc. Med. 15, 138–141