Utrophin Localization in Normal and Dystrophin-Deficient Heart

Françoise Pons, PhD; Agnès Robert; Eric Fabbrizio, PhD; Gerald Hugon; Jean-Christophe Califano, PhD; Jean Alain Fehrentz, PhD; Jean Martinez, PhD; Dominique Mornet, PhD

Background The localization of dystrophin at the sarcolemma of cardiac skeletal fibers and cardiac Purkinje fibers has been described. Dystrophin deficiency produces clinical manifestations of disease in skeletal muscles and hearts of patients with Duchenne and Becker muscular dystrophy. Utrophin (or dystrophin-related protein), a dystrophin homologous protein, was found to be expressed in fetal muscles and reexpressed in dystrophin-deficient skeletal muscle fibers. We therefore examined utrophin expression in normal and in dystrophin-deficient hearts.

Methods and Results The expression and subcellular distribution of utrophin was examined in cardiac muscle by immunoblot and immunofluorescence analysis in normal bovine heart compared with dystrophin. Utrophin expression was also examined in normal and dystrophin-deficient hearts of MDX mice. Three monoclonal antibodies reacting with dystrophin and utrophin solely or reacting with both proteins along with two polyclonal antibodies reacting with either utrophin or dystrophin and utrophin were tested. In normal bovine heart, utrophin was not expressed at the periphery of fibers but was strongly expressed in intercalated disks and in the cytoplasm of cardiac Purkinje fibers. In cardiocytes, utrophin was colocalized along transverse T tubules with dystrophin. Dystrophin was present at the periphery of cardiocytes and cardiac Purkinje fibers as well as in transverse T tubules but was absent or faintly expressed in intercalated disks. The results with monoclonal and polyclonal antibodies were identical. Western blot analysis revealed that the detected molecules corresponded only to a 400-kD protein band and not to possible shorter transcripts of utrophin or dystrophin (apo-utrophin or apo-dystrophin). In dystrophin-deficient hearts of MDX mice, utrophin alone was abundant but not organized in the same networklike distribution.

Conclusions This first localization of utrophin in normal heart (in Purkinje fibers, transverse tubules, and intercalated disks) showed a distinct subcellular localization of this protein with dystrophin, suggesting an important function of this protein in intercellular communication. In dystrophin-deficient hearts of MDX mice, utrophin alone is overexpressed as in skeletal muscle sarcolemma, an area normally occupied by dystrophin but not organized in the same networklike distribution. (Circulation. 1994;90:369-374.)

Key Words • utrophin • dystrophin • antibodies

Dystrophin, the protein encoded by the Duchenne’s muscular dystrophy gene, is expressed predominantly in skeletal and cardiac muscle. In patients with Duchenne’s and Becker-type tardive muscular dystrophy, mutations in the dystrophin gene cause clinical manifestations of heart disease with left ventricular fibrosis and ECG changes. However, histopathological changes in the heart do not qualitatively or quantitatively reproduce the skeletal muscle involvement reported in this disease. The physiological mechanisms that cause conduction defects in these patients are still poorly understood. The subcellular localization of dystrophin in heart is well documented. Its expression was found to be limited to the inner face of plasma membrane and transverse tubule. It is notably absent from membranes that overlie adherent junctions of intercalated disks. A brain isosform of dystrophin was also found in cardiac Purkinje fibers.

A chromosome 6 encoded protein homologous to the dystrophin, named DRP or “utrophin” and described as being expressed in many tissues, is overexpressed in dystrophin-deficient muscles and is suspected to compensate for the absence of dystrophin in skeletal muscle. To test this hypothesis in the heart, we comparatively analyzed the localization of utrophin in normal and dystrophin-deficient mouse heart. Polyclonal antibodies specific to utrophin or utrophin/dystrophin were used in normal and MDX mouse heart to fully analyze the localization of utrophin in the absence of dystrophin.

Methods

Tissues

Adult bovine and mouse hearts were dissected, quickly frozen in liquid nitrogen–cooled isopentane, and stored at −80°C. Cryosections (10 to 12 μm) were air-dried and either stored or used immediately.

Antibodies

Anti-dystrophin and anti-dystrophin/utrophin monoclonal antibodies were obtained by lymphocyte hybridizations from different mice immunized with various recombinant dystrophin fragments of human or chicken skeletal muscle dystrophin. The specificities of monoclonal and polyclonal antibodies to dystrophin or
utrophin were determined as previously described\(^8,9\) by their reactivities in Western blot analysis on skeletal muscle extracts of a Becker patient who, because of a large deletion, had a 300-kD truncated dystrophin and a 400-kD utrophin.

Monoclonal antibody C5S3 specific to dystrophin detected only the 300-kD band in the Becker patient.

Monoclonal antibody HSAS3, reacting with dystrophin and utrophin molecules, detected both the 400-kD utrophin band and the 300-kD dystrophin band in the Becker patient.

Monoclonal antibody DRP1/12B6, designated as anti-utrophin, was generated with a synthetic peptide containing 11 C-terminal amino acids of utrophin\(^10-21\) and detected only the 400-kD utrophin band in the Becker patient.

Polyclonal antibodies were raised in rabbits. Two New Zealand White female rabbits were immunized as follows:

Polyclonal antibody C422 was generated with fusion proteins as described previously.\(^19\) It reacted with both dystrophin and utrophin and detected in the Becker patient both the 400-kD utrophin band and the 300-kD dystrophin band.

Polyclonal CUT 5 was generated with a maleoylated bovine serum albumin--conjugated peptide synthesized according to a procedure that has been successfully applied to the production of various antibodies.\(^22-25\) The heptapeptide maleoyl-Pro-Ser-Arg(Pmc)-Pro-Gln-Ala-Met-OrBu was synthesized in solution. It was purified by reverse-phase high-performance liquid chromatography on a C18 column, and its structure was ascertained by fast atom bombardment mass spectrometry and \(^1\)H nuclear magnetic resonance spectroscopy (360 MHz). It reacted only on utrophin, detecting only the 400-kD utrophin band in the Becker patient.

### Indirect Cytochemical Fluorescence Labeling

Ten-micrometer cryostat sections of unfixed bovine or mouse hearts were treated for epifluorescence as previously described.\(^26\) Sections were first labeled with either monoclonal antibodies (anti-dystrophin, anti-dystrophin/utrophin, and anti-utrophin) or polyclonal antibodies (C422 or CUT 5) and subsequently labeled with fluorescein-conjugated rabbit antimouse IgG and goat anti-rabbit IgG, respectively (1/50, from Jackson ImmunoResearch Laboratories).

### Western Blot Analysis

Twenty serial 10-μm-thick cryostat sections of heart muscle adjacent to sections sampled for immunofluorescence studies were homogenized in 40 μL of gel loading buffer containing SDS according to the microprocedure previously described.\(^26\) Five microliters of each extract was separated on a 6% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Alkaline phosphatase conjugated affinity-purified rabbit anti-mouse IgG (1/500, Jackson ImmunoResearch Laboratories) was used to detect the immobilized specific antidy tropphin antibody.

### Results

#### Urophin and Dystrophin Distribution in Normal Heart

We investigated localization of utrophin and dystrophin with two monoclonal antibodies specific to each protein. In bovine heart, cardiac Purkinje fibers are easily identified by their size and localization. As already observed, dystrophin was expressed at the periphery of cardiocytes and Purkinje fibers but not in the cytoplasm (Fig 1b). In longitudinal sections, periodic staining was visible following the T tubules, as previously noted by Yarom et al.\(^9\) and Klitsch et al.\(^10\) Contrary to the results of these authors, we sometimes also observed a faint staining of the intercalated disks (see Fig 3a). This slight discrepancy could have been due to either different antibody affinities or sensitivity of the detection system. In contrast, utrophin was not expressed at the periphery of fibers but was strongly expressed in the intercalated disks and the cytoplasm of Purkinje fibers (Fig 1a). In longitudinal sections of fibers, dystrophin and utrophin presented the same periodic staining patterns.

Dystrophin and utrophin genes have been described as having tissue-specific and developmentally regulated short transcripts.\(^11,27\) To test the hypothesis that the present immunofluorescence observation on cardiac Purkinje fibers was not due to dystrophin or utrophin shorter transcript, we analyzed areas with or without these fibers by Western blot techniques. The two extracts presented the same Western blot patterns with both anti-dystrophin and anti-utrophin antibodies. In addition, this hypothesis could be completely discarded.

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**Fig 1. Subcellular distribution of utrophin and dystrophin in cardiac bovine Purkinje fibers (PF) by immunofluorescence labeling.** Serial (longitudinal) cryosections of auricularventricular areas of adult bovine heart were labeled with either anti-utrophin (a) or anti-dystrophin (b) monoclonal antibodies as described in "Methods." Dystrophin staining was clearly seen at the periphery of cardiocytes (c) and Purkinje fibers (PF). The utrophin was observed in the cytoplasm of Purkinje fibers but not at their peripheries. Bar=30 μm.
In with anti-dystrophin/utrophin monoclonal antibodies. Muscle extract proteins were separated by 6% SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred to nitrocellulose. Nitrocellulose strips were labeled with specific antibodies: anti-dystrophin (a) and anti-utrophin (b) visualized with alkaline phosphatase-conjugated secondary antibody. Lane 1 contained 5 μL of normal skeletal muscle extract to visualize the normal 400-kD dystrophin band and 5 μL of prestained SDS-PAGE molecular weight standard (Biorad). Lanes 2 and 3 contained 5 μL of bovine ventricular extract with or without Purkinje fibers, respectively, as described in "Methods." Only a protein 400-molecular-weight band was visible in both extracts with both antibodies.

by the observation obtained with an antibody produced against the central part of the dystrophin molecule. The immunofluorescence detections obtained with these antibodies were essentially due to proteins of apparent molecular weight of 400-kD bands: ie, dystrophin and utrophin (Fig 2).

In our conditions, the use of monoclonal antibodies on MDX mice was limited by cross-reactivity of the second antibody used for detection, since all anti-mouse IgGs recognize the endogenous IgGs expressed at the periphery of muscle fibers and also in necrotic fibers. We used polyclonal antibodies to avoid the difficulties in the interpretation of the result consequent to the above considerations. The specificity of these antibodies was demonstrated as previously reported and as described in the "Methods" section, by their reactivity in Western blot analysis on utrophin and on a truncated dystrophin band of a muscle extract from a Becker patient. In addition, we analyzed their reactivity on bovine heart and compared the results with those obtained with monoclonal antibodies. The anti-utrophin CUT 5 polyclonal antibody generated the same pattern as the anti-utrophin 12B6 monoclonal antibody (Fig 3b and 3d). Polyclonal antibody C422 produced the same staining pattern as the monoclonal antibody H5A3 characterized previously as being utrophin- and dystrophin-reactive. The two former antibodies represent the sums of staining localization for dystrophin and utrophin alone in these conditions; since both of these antibodies gave the same periodic staining (length between two lines is between 1.7 and 2 μm), utrophin could be colocalized with dystrophin in transverse T tubules.

**Utrophin in Dystrophin-Deficient Heart**

Since both monoclonal and polyclonal antibodies gave the same staining pattern in normal bovine heart and because of the limited performances of monoclonal antibodies on mouse cardiac muscle discussed earlier, only polyclonal antibodies were used to compare normal and MDX mouse hearts. The histoimmunostaining patterns of normal and MDX mouse heart were compared with anti-dystrophin/utrophin and C422 and anti-utrophin CUT 5 polyclonal antibodies (Fig 4). In normal mouse heart, as observed in bovine heart, utrophin was detected only in intercalated disks and transverse tubules but was not expressed at the fiber periphery (Fig 4a). The polyclonal antibody C422 detected utrophin and dystrophin. This antibody presented a similar periodic staining pattern and the same labeling in intercalated disks as CUT 5, but labeling at the periphery of fibers was due to the dystrophin (Fig 4c). In MDX mouse heart, utrophin was detected by anti-utrophin polyclonal CUT 5 and also by anti-dystrophin/utrophin polyclonal antibody C422. Utrophin was clearly expressed in the intercalated disks and at the periphery of fibers, but the periodic staining was hardly detectable, whereas the cytoplasmic background was increased. Western blot analysis with monoclonal antibody H5A3 on cardiac and skeletal muscles of MDX mice showed greater utrophin expression in heart, in concordance with the results presented by Matsumara.

In cardiac Purkinje fibers that were easily detected in many species (rabbit and chicken), we observed the same cytoplasmic reactivity with utrophin as observed in bovine heart, but we were not able to clearly identify this structure in mice, and MDX cardiac Purkinje fibers were not assessed.

**Discussion**

To further define utrophin properties, we investigated for the first time immunolocalization of this protein in cardiac muscle. Unlike dystrophin, utrophin was expressed in the cytoplasm of cardiac Purkinje fibers and in intercalated disks but not at the cell periphery. These results were obtained with monoclonal and polyclonal antibodies. Cross-reactivity of these antibodies with any short transcripts of both proteins was excluded because our data were identical to antibodies specific to the central part or to the C-terminal end of both proteins. However, the existence of short transcripts of both proteins was not excluded. Only antibodies specific to short transcripts could resolve this question. The results in bovine cardiac muscles were in agreement with those obtained in normal mouse heart. In MDX mice, all antibodies used detected only utrophin and showed the distribution of this molecule within different heart structures.

Soon after the discovery of dystrophin, a homologous protein was identified. The full sequences of both dystrophin and utrophin molecules showed large sequence homologies. Both proteins are reported to form a complex with similar glycoproteins. In addition, their balanced pathological and developmental expression marks the similarity between these two mol-
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FIG 3. Subcellular distribution of dystrophin and utrophin in histoimmunofluorescence analysis on bovine ventricle; comparison of reactivities of monoclonal and polyclonal antibodies. Longitudinal cryostat sections of bovine ventricle stained with anti-dystrophin (C5G5, a), anti-utrophin (12B6, b), or anti-utrophin/dystrophin (H5A3, c) monoclonal antibodies and with anti-utrophin (CUT 5, e) or anti-utrophin/dystrophin (C422, f) polyclonal antibodies. Detections were performed as described in “Methods” with anti-mouse and anti-rabbit IgG fluorescein-conjugated antibodies. Controls were performed by omitting the primary antibody; secondary antibodies alone gave the same negative pattern (d). Dystrophin is distributed at the cell periphery and in transversely oriented strands present in the center of I bands where T tubules are present in cardiac muscle but faintly present in intercalated disks (arrows). Utrophin with both monoclonal and polyclonal antibodies presented the same staining patterns, the same transversely oriented strands, and intercalated disks. Anti-utrophin/dystrophin monoclonal and polyclonal antibodies gave the same staining patterns corresponding to the sum of staining localization for dystrophin and utrophin. Bar=20 μm.

ecules. This suggests that both proteins may have similar functions. However, the present results suggest that specific functions could be related either to dystrophin or utrophin and sometimes to both. In normal adult tissues, localization of dystrophin has already been well documented. Its function is assumed to be the maintenance of muscle fiber integrity, but its mechanical processes still remain unclear.12 For utrophin, besides wide distribution in muscular and nonmuscular tissues, its abundance is generally related to organ vascularization. However, utrophin is predominant not only in vessel walls31 but also at the neuromuscular junctions32 and now in cardiac Purkinje fibers and in intercalated disks. These latter localizations are intimately associated with maintenance of intercellular connections; the function of utrophin may be completely different from that of dystrophin. The absence of any known mutation in the utrophin gene may be due to a lethal effect of such defects. Despite their homology, substitution of utrophin in areas where dystrophin is absent does not maintain fiber integrity in Duchenne’s muscular dystrophy patients.32 This is another indication of the diversity of dystrophin and utrophin functions.

The second aspect of this study concerned the codistribution of dystrophin and utrophin in normal heart. Previous reports have localized dystrophin and the glycoprotein complex in transverse T tubules.8 Utrophin was found here to be colocalized with dystrophin in the same subcellular structure and could be one element of this complex in heart. The absence of dystrophin leads

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to a sequence of events including reduction or disappearance of several components of the dystrophin/glycoprotein complex and fiber necrosis. Although this necrosis seems to affect skeletal muscle more systematically than cardiac muscle and could vary from species to species, it was of interest to investigate the expression of utrophin in heart in the absence of dystrophin. In dystrophin-deficient heart, Western blot analysis indicated overexpression of utrophin, but in immunofluorescence analysis, this expression was detected only in intercalated disks, at the periphery of cardiocytes, or increasing the cytoplasmic background, while absent or only faintly present in transverse T tubules. In this context, a tight association between dystrophin and utrophin organized in heterodimers could explain these observations. The autoassociation of dystrophin was first proposed by Byers et al. and isolated molecules supported this model. A heterodimeric association could have a different function from the regular dystrophin homodimer, localized at the cell periphery. These transverse T tubules were also described as a region containing calcium terminal cisternae. Dystrophin/utrophin heterodimers could have a specific role in this particular structure, maintaining the stability of calcium channels involved in muscle contraction.

Dystrophin and utrophin were observed in skeletal muscle at the normal neuromuscular junction but were not strictly colocalized. In dystrophin-deficient skeletal or cardiac muscles, the utrophin was able to replace dystrophin, but this substitution did not completely provide a compensatory function. Heart transverse tubules may be the primary structure in which dystrophin and utrophin were coexpressed and codistributed. The proposed hypothetical dystrophin/utrophin heterodimers would thus be essential to control calcium channel stability. In the absence of dystrophin, at least in the heart, utrophin alone could not provide the same stability; it could supply a pathway for calcium to leak into the cell. Further investigations on the subcellular distribution of dystrophin and utrophin and their colocalization could reveal the function of these putative homodimeric or heterodimeric associations of both proteins. In skeletal muscle of MDX mice transgenic for the dystrophin gene, the dystrophin/glycoprotein association is restored. It would be interesting to investigate whether the expression of utrophin is reorganized in transverse T tubules in the heart of these transgenic mice and also whether injection of part of the dystrophin gene via adenovirus in cultured dystrophin-deficient cardiac cells would also form the same network.

Fig 4. Comparison of utrophin expression in normal and in dystrophin-deficient hearts. Longitudinal cryostat sections of ventricle of BL10 mice stained with either polyclonal antibodies anti-utrophin (CUT 5, a) or anti-utrophin/dystrophin (C422, c). Longitudinal cryostat sections of MDX ventricle were stained in the same conditions with CUT 5 (b) or C422 (d). Arrows point out the intercalated disks. Bar=20 μm.
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