BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 227–235 (1997)
ARTICLE NO. RC976757

Mouse Adhalin: Primary Structure and Expression during Late Stages of Muscle Differentiation *in Vitro*

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Received May 7, 1997

Adhalin, or α-sarcoglycan, is a 50-kDa glycoprotein that was originally characterized as a muscle membrane protein. The importance of adhalin is suggested by the diseases associated with its absence, notably the limb-girdle muscular dystrophies. However, the function of adhalin is unknown. To analyze the biological roles of adhalin, we cloned the mouse adhalin cDNA, raised peptide-specific antibodies to its cytoplasmic domain, and examined its expression and localization in vivo and in vitro. The mouse adhalin sequence was 80% identical to that of human, rabbit, and hamster. Adhalin was specifically expressed in striated muscle cells and their immediate precursors, and absent in many other cell types. Adhalin expression in embryonic mouse muscle was coincident with primary myogenesis. Its expression was found to be up-regulated at mRNA and protein levels during myogenic differentiation in vitro. The proper localization of adhalin to the muscle cell membrane was observed only in late stages of myotube maturation, coincident with the re-distribution of caveolin-3 and dystrophin. These data suggest that adhalin is highly specific for striated muscle and that it is linked with the formation of a fully functional muscle fiber.

Adhalin (α-sarcoglycan) is a component of the sarcoglycan complex in muscle cell membranes (1). This complex contains at least three other components termed β, γ, and δ-sarcoglycans (2-3). Primary defects in sarcoglycans are the causes of various limb-girdle muscular dystrophies (4-11), and mutations in all four proteins have been characterized (9,12-14). It is commonly observed that all sarcoglycans are absent or reduced in muscle when one of them is missing due to gene mutation (4,15-16). This indicates a close interdependence of expression and localization of the sarcoglycans at the sarcolemma. Deficiency in adhalin expression has been reported also in other muscular dystrophies such as Duchenne/Becker muscular dystrophy, caused by mutations in the dystrophin gene, as well as in diseases yet to be characterized at the molecular level (17-19). It appears then that adhalin, and the sarcoglycan complex, play a central role in muscle cell stability. Although a great deal is known about the biology and physiology of muscle and of many of its specialized proteins, much remains unclear or poorly understood about the molecular and cellular biology of sarcoglycans, as well as how sarcoglycans relate to the development and maintenance of muscle function. This is of special concern when considering the apparent importance and involvement of sarcoglycans in muscle disease.

Adhalin has been cloned and sequenced previously in rabbit, hamster, and human (20-22); the β-, γ-, and δ-sarcoglycans have been cloned recently as well in human (10,12,23). Based on sequence translations, adhalin is predicted to be a type I transmembrane protein whereas the other three sarcoglycans are type II membrane proteins. Splice variants coding for secreted forms of adhalin have been described (21).

As mouse is the experimental animal of choice, it would be valuable to establish sarcoglycan studies in this species. It would also be important to develop in vitro systems in which to study the potential activities of adhalin. To begin to elucidate the biology of sarcoglycans, we undertook the identification and characterization of adhalin in mouse. For this purpose, we cloned and sequenced a full length mouse adhalin cDNA, raised peptide-specific antibodies to it, and studied its expression in various tissues and cell types during development and differentiation. We found that the deduced mouse adhalin amino acid sequence is 80% identical to those of the human, hamster, and rabbit ad-

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0006-291X/97 $25.00

halins. Adhalin is highly specific for differentiated, striated muscle cells in vivo and in vitro. In culture, its expression increases as skeletal myoblasts differentiate into multinucleated myotubes. Most importantly, the appropriate localization of adhalin in the cell membrane was observed only in late stages of myotube maturation, coincident with the re-distribution of caveolin-3 and dystrophin and the formation of T-tubules (24). These data suggest that the function of adhalin relates to the terminal maturation of myofibers, and more specifically to the establishment of a fully functional muscle cytoarchitecture.

MATERIALS AND METHODS

Tissues. Tissues were collected from two months old FVB/N mice and from 12 and 14 day embryos (Taconic Farms, CA), frozen in liquid nitrogen, and stored at −70°C. Human muscle was obtained from surgery.

Synthetic peptides and antisera. A synthetic peptide representing the sequence of the 14 most C-terminal amino acids of mouse adhalin was synthesized at the Analytical Core Facility, Shriners Research Unit (Portland, OR). Peptides representing the C-termini of utrophin (25) and dystrophin (26) were synthesized at The Burnham Institute, La Jolla, CA. A portion of each peptide was coupled to Keyhole limpet hemocyanin (KLH) with m-Maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL) as described by O'Sullivan et al. (27), and some peptide was mixed with methylated BSA (Sigma, Louis, MO). The KLH-conjugated and BSA-complexed peptide preparations were mixed with adjuvant and injected subcutanously into rabbits at monthly intervals. Antisera were collected 10 days after the second and all subsequent injections. Caveolin-3 antiserum was purchased from Transduction Laboratories (Lexington, KY). An antiserum to myosin heavy chain and the mAb AE-53 directed to sarcomeric α-actinin were purchased from Sigma (St. Louis, MO).

Cell lines and cell culture. PC12 rat phaeochromocytoma cells, 3T3 and 10T1/2 mouse fibroblasts, P19 mouse embryonal carcinoma cells, L6 rat myoblasts, RD human rhabdomyosarcoma cells, embryonic stem (ES) cells (line E14), and B16 mouse melanoma cells were obtained from the American Type Culture Collection (Rockville, MD). PF HR-9 mouse endodermal cells (28) were also used. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO/BRL, Gaithersburg, MD) containing 20% fetal calf serum (FCS; Gibco/BRL), 10 mM HEPES, 4 mM glutamine, 1 mM sodium pyruvate, 100 u/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO_2 in air. ES cells were cultured as described (29). Cultures were refed every 24 hr. Myogenic differentiation of C2C12, L6, and RD cells was induced at confluence by replacing the growth medium with DMEM containing only 1% FCS (30). Myogenic differentiation of 10T1/2 cells was induced by treatment with 5-azacytidine as described (31). The induction of differentiation of ES cells was as described (29).

For contraction studies, C2C12 mouse myoblasts were plated onto 35-mm dishes (Nunc, Napierville, IL) coated with rat tail collagen type I (50 μg/ml; Collaborative Biomedical Products, Bedford, MA), and cultured in the same conditions as above but without antibiotics. In this case, the differentiation of the C2C12 cells was initiated by replacing the growth medium with DMEM containing 2% horse serum (HS) and 15 mM Hepes. These modifications in culture conditions allowed C2C12 cells to differentiate into actively contracting myotubes (32-33).

Isolation of cDNA encoding mouse adhalin. Total RNA was isolated from human muscle by the acid guanidinium thiocyanate phenol-chloroform extraction method (34-35). To generate a probe, oligonucleotides spanning nt 341-360 (5′-GTCATTGAGGTCACAG-3′) and 945-966 (5′-CGGCAGCACATGACAT-3′) of the human adhalin sequence (14) were used as primers in RT-PCR (see below). The RT-PCR was carried out with 1.0 μg of total RNA, using the First-Strand cDNA Synthesis kit (Pharmacia, Uppsala, Sweden). PCR products were separated by electrophoresis on 1.0 % agarose gels prestained with ethidium bromide, using a 100-bp DNA ladder (GIBCO/BRL, MD) as standard. The purified adhalin DNA fragment was radiolabeled with ^{32}P and used as a probe to screen a mouse skeletal muscle cDNA library (Clontech, Palo Alto, CA).

Northern blotting. Total mouse RNA was isolated from stomach, leg muscle, spleen, liver, heart, testis, ovary, thymus, lung, brain, kidney, and whole embryo. A panel of mouse RNA samples isolated from spleen, liver, heart, testis, ovary, thymus, lung, brain, kidney, and midterm embryo was purchased from Ambion (Austin, TX). RNA samples were separated on a 1.0 % agarose/formaldehyde gel, transferred to Hybond-N (Amersham, Arlington Heights, Il), and fixed at 80°C for 2 hr. Hybridization was performed by using a ^{32}P-labeled 1.6 Kb mouse cDNA probe containing the full length adhalin sequence (10^7 cpm/ml) at 42°C overnight in a buffer containing 50% (vol/vol) formamide, 5× standard saline citrate (SSC), 5× Denhardt's solution, and herring sperm DNA at 200 μg/ml. After hybridization, the nylon filter was washed three times at a final stringency of 0.2 × SSC, 0.1% SDS at 50°C for 30 min. The filter was exposed to Kodak XAR film at −70°C for 24 hr with intensifying screen.

Indirect immunofluorescence. Mouse tissues were sectioned (10 μm) on a cryostat and fixed in methanol for 10 min at +4°C. Sections were incubated with primary antibodies at 1:100 dilution for 1 hr at 37°C. After washing with PBS three times, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA) at room temperature for 1 hr and washed. The sections were then mounted with Vecta-Shield (Vector, Burlingame, CA) and examined under an Axiovert 405M fluorescence microscope (Zeiss).

Immunofluorescence was also performed on cell cultures grown in 35 mm dishes (Nunc, Naperville, Il). Cells were washed in PBS, fixed for 10 min in precooled methanol at −20°C, washed in PBS, and permeabilized for 5 min with 0.5% Triton X-100 in PBS. Fluorescent staining was then performed as above.

Immunoblotting analysis. SDS-PAGE was performed on 4-12% gels (NOVEX, San Diego, CA). High molecular mass markers (14.3-200 KD range; GIBCO/BRL) were used as standards. Protein contents of all samples were estimated using the BioRad (Hercules, CA) protein assay. Total protein (25-50 μg/well) from tissues or cell cultures were solubilized in sample buffer (35), separated by electrophoresis, and electrotransfered to nitrocellulose membranes (Nitrocellulose-1; GIBCO/BRL) for subsequent immunoblotting. Antisera were used at 1:300 dilution. Goat anti-rabbit IgG conjugated with peroxidase were used at 1:2000 dilution (Calbiochem, San Diego, CA). Immunoreactive bands were visualized using the enhanced chemiluminescence method (ECL system; Amersham, Arlington Heights, IL).

RESULTS

Cloning of Mouse Adhalin

Mouse adhalin cDNA clones were isolated from a mouse skeletal muscle cDNA library using a probe generated by PCR from human RNA with primers based on the human adhalin sequence. Nine cDNA clones were isolated and 3 were sequenced in both directions (Fig. 1). The longest clone contained 1164 bp and encoded the full length adhalin cDNA.

The deduced amino acid sequence of mouse adhalin is shown in Fig. 2. An open reading frame encodes a

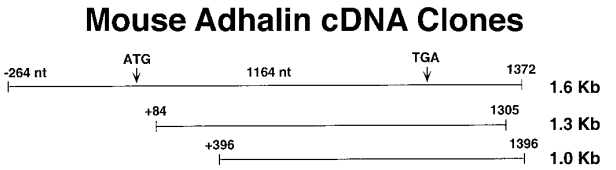

FIG. 1. Schematic representation of three mouse adhalin cDNA clones of 1.6, 1.3 and 1.0 kb. The longest clone containing 1164 bp encoded the full length mouse adhalin cDNA.

387 amino acid protein of predicted molecular mass of 43 kDa. Hydropathy analysis of the deduced amino acid sequence showed two hydrophobic domains, which suggested an amino-terminal signal sequence and a transmembrane domain close to the C-terminus (underlined in Fig. 2). Comparison of mouse, human, hamster, and rabbit adhalins shows that they are approximately 80 % identical at the amino acid level. The two consensus sites for N-linked glycosylation and all the five extracellular cysteines are conserved in all four species.

Tissue- and Cell-Type Specificity of Adhalin Expression

Adhalin has been shown to be present only in striated muscle tissue in human (21,36). To confirm the tissue-specific expression of adhalin in the mouse, the 1164 bp mouse cDNA clone was used as probe in Northern analysis. An ~1.6 kb adhalin mRNA was detected in striated muscle, both skeletal and cardiac, but not in stomach, spleen, liver, testis, ovary, thymus, lung, brain, or kidney (Fig. 3). In cardiac muscle, an additional band of ~2.8 kb that hybridized with the probe was also observed. Similar results were obtained with 4-7 independent samples of each type of tissue. In addition, a panel of commercial RNA samples provided the same results. When the adhalin message was detected

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seq_1Mouse      -MAAAVTWIPLLAGLLAGLKDTKAQQTTLHLLVGRVFVHPLEHATFLRLPEHVAVPPTVR  60
seq_2Human      -MAETLFWTPLLVVLLAGLGDTEAQQTTLHPLVGRVFVHTLDHETFLSLPEHVAVPPAVH
seq_3Hamst      -MAATLTWILLFVGLLAGLRDTKAQQTTLYPLVGRVFVHPLEHATFLRLPEHIAVPPTVR
seq_4Rabbi      MAAAALLWLPLLVGCLAGPGGTEAQQTTLYPLVGRVFVHTLEPASFLHLPEH-AAPATIP
                  * .. *  *    ***   * ******  ******** *.  .** **** * * ..

seq_1Mouse      LTYHAHLQGHPDLPRWVHYTQRSPYNPGFLYGSPTPEDRGYQVIEVTAYNRDSFDTTRQR 120
seq_2Human      ITYHAHLQGHPDLPRWLRYTQRSPHHPGFLYGSATPEDRGLQVIEVTAYNRDSFDTTRQR
seq_3Hamst      LTYQAHLQGHPDLPRWLRYTQRSPYSPGFLYGTPTPEDRGRQVIEVTAYNRDSFDTTRQR
seq_4Rabbi      VTYHAHLQGHPDLPRWLRYTQRSPHHPGFLYGAATPEDRGRQVIEVTAYNRDSFDTAGQS
                .**.************..******  ******. ****** ***************. *

seq_1Mouse      LLLLIGDPEGPRLPYQAEFLVRSHDVEEVVSTTPANRFLTALGGLWEPGELQLLNITSAL 180
seq_2Human      LVLEIGDPEGPLLPYQAEFLVRSHDAEEVLPSTPASRFLSALGGLWEPGELQLLNVTSAL
seq_3Hamst      LLLLIEDPEGPRLPYQAEFLVRSHDVEEVLPSTPANRFLTALGGLWELGELQLLNITSAL
seq_4Rabbi      LVLLIRDPEGSPLPYQTEFLVRSHDVEEVLPPTPASHFLTALAGLWEPGELKLLNITSAL
                *.* * ****  ****.******** ***.  *** .**.** **** ***.***.****

seq_1Mouse      DRGGRVPLPIEGRKEGVYIKVGSATPFSTCLKMVASPDSYARCAQGQPPLLSCYDTLAPH 240
seq_2Human      DRGGRVPLPIEGRKEGVYIKVGSASPFSTCLKMVASPDSHARCAQGQPPLLSCYDTLAPH
seq_3Hamst      DRGGRVPLPIEGRKEGVYIKVGSATPFSTCLKMVASPDSYARCAQGQPPLLSCYDSLAPH
seq_4Rabbi      DRGGRVPLPIGGQKEGVYIKVGSASPFSTCLKMVASPDSHARCARGQPPLLSCYDTLAPH
                ********** *.***********.************** ****.**********.****

seq_1Mouse      FRVDWCNVSLVDKSVPEPLDEVPTPGDGILEHDPFFCPPTEATDRDFLTDALVTLLVPLL 300
seq_2Human      FRVDWCNVTLVDKSVPEPADEVPTPGDGILEHDPFFCPPTEAPDRDFLVDALVTLLVPLL
seq_3Hamst      FRVDWCNVSLVDKSVPEPLDEVPTPGDGILEHDPFFCPPTEATGRDFLADALVTLLVPLL
seq_4Rabbi      FRVDWCNVSLVDTSVPEPVDEVPTPGDGILEHDPFFCPPTEATARDFLADALVTLLVPLL
                ********.*** ***** ************************  **** **********

seq_1Mouse      VALLLTLLLAYIMCFRREGRLKRDMATSDIQMFHHCSIHGNTEELRQMAASREVPRPLST 360
seq_2Human      VALLLTLLLAYVMCCRREGRLKRDLATSDIQMVHHCTIHGNTEELRQMAASREVPRPLST
seq_3Hamst      VALLLTLLLAYIMCCRREGQLKRDMATSDIQMVHHCTIHGNTEELRQMAARREVPRPLST
seq_4Rabbi      VALLLALLLAYIMCCRREGRLKRDLATSDIQMVHHCTIHENTEELRQMAASREVPRPLFP
                *****.*****.** ****.****.******* ***.** ********** *******

                                                                        388
seq_1Mouse      LPMFNVRTGERLPPRVDSAQMPLILDQH
seq_2Human      LPMFNVHTGERLPPRVDSAQVPLILDQH
seq_3Hamst      LPMFNVRTGERLPPRVDSAQVPLILDQH
seq_4Rabbi      LPMFNVRTGERMPPRVDSAQVPLILDQH
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FIG. 2. Mouse adhalin deduced amino acid sequence; alignment with the sequences of human, hamster, and rabbit adhalins. Amino acids that are identical among all four species are indicated below by an asterisk; conservative substitutions are indicated by a dot. Two extracellular potential N-linked glycosylation sites are boxed and five extracellular cysteines are marked with a filled circle. The predicted hydrophobic signal sequence and the transmembrane domain are underlined.

by RT-PCR, it was again found exclusively in skeletal and heart muscle (data not shown).

An antiserum to adhalin was generated by immunization of rabbits with a synthetic peptide based on the sequence of the cytoplasmic tail of adhalin. This antiserum, when used in immunoblotting, detected a 50 kDa polypeptide in extracts of skeletal muscle, but not in other tissues (Fig. 4A). When the antiserum was used in indirect immunofluorescence in tissues of adult mice, it stained the membrane of all striated muscle cells in skeletal muscle and heart (Figure 4B). No staining above background was seen in intestine (Fig. 4B) or in other tissues including smooth muscle, kidney, skin, testis, brain, nerve, spleen, lung, and thymus (not shown). The minor lower-molecular bands seen in intestine by immunoblotting (Fig. 4A) were considered nonspecific, since no adhalin message or protein were detected by RT-PCR or immunofluorescence in this organ. Hence, the expression of adhalin appears restricted to striated muscle tissue in the mouse.

To further investigate the expression of adhalin at the cellular level, RT-PCR was performed with RNA from several different cell lines representing different cell types. Adhalin mRNA was detected in cultures of the myogenic C2C12 mouse myoblasts and 10T1/2 cells (Fig. 6) as well as in L6 myoblasts and RD rhabdomyosarcoma cells (not shown). No adhalin mRNA was detected in cultures of P19 embryonal carcinoma cells, PF HR-9 endodermal cells, B16 melanoma cells, PC12 pheochromocytoma cells, or 3T3 fibroblasts. These data show that the expression of adhalin is not only tissue-specific but cell-type specific as well.

Adhalin Expression Correlates with Myogenic Differentiation

Since adhalin is specific for striated muscle cells in vivo as well as in vitro, we analyzed its expression in relation to myogenesis and the myogenic differentiation process.

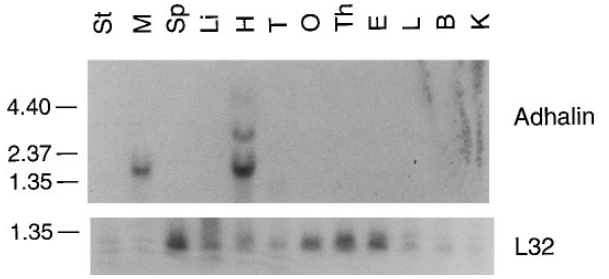

FIG. 3. Northern blot analysis of adhalin mRNA levels in various tissues from mouse. 25 μg of total RNA from stomach (St), leg muscle (M), spleen (Sp), liver (Li), heart (H), testicle (T), ovary (O), thymus (Th), midterm embryo (E), lung (L), brain (B), and kidney (K) were electrophoresed and hybridized with a full length adhalin cDNA. A probe for the ribosomal protein L32 was used as an internal RNA-loading control.

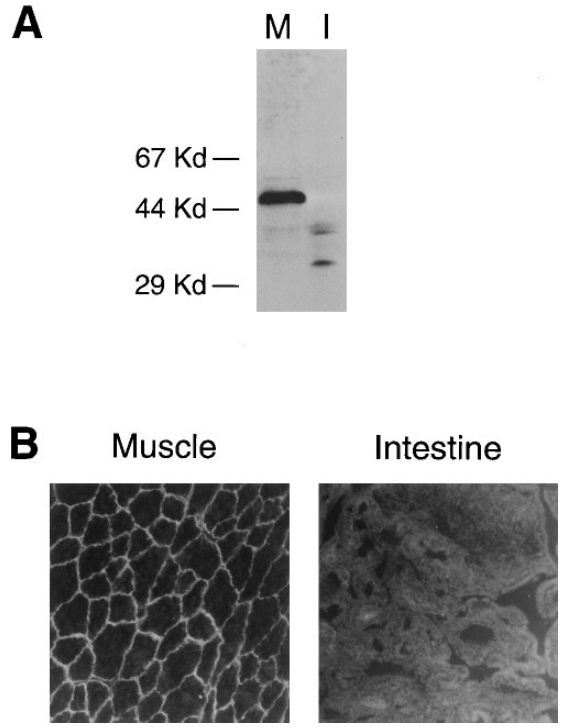

FIG. 4. Expression and localization of adhalin in mouse skeletal muscle (M) and intestine (I). *A.* Immunoblot analysis. Total proteins from tissues were extracted, electrophoresed (equal quatities loaded per well), transferred to nitrocellulose, and stained with an anti-adhalin antibody. Molecular weights in kDa are indicated on the left. *B.* Immunofluorescence analysis of adhalin on tissue sections of adult mouse muscle and intestine.

In the mouse, primary myogenesis occurs around days E14-15. RT-PCR (not shown) and immunofluorescence (Fig. 5) analyses showed that adhalin mRNA and protein are not detected prior to the onset of myogenesis (as shown here for day E12 by fluorescence detection; Fig. 5A), whereas it is expressed and membrane-localized in newly formed myofibers at day E14 (Fig. 5B). Thus, the expression of adhalin during mouse embryogenesis appears coincident with primary myogenesis.

In cultured mouse C2C12 cells, we found the levels of adhalin mRNA to be higher in cultures of differentiated multinucleated myotubes than in cultures of mononuclear myoblasts (Fig. 7A). Immunoblotting showed that this apparent up-regulation of expression with differentiation also occurs at the protein level (Fig. 7B). Immunofluorescence localization of adhalin in the differentiated C2C12 cultures revealed that adhalin is predominantly associated with myotubes (Fig. 8A). Therefore, the expression of adhalin correlates with myogenic differentiation.

The Membrane Localization of Adhalin Requires Myotube Maturation

Surprisingly, adhalin staining in 6-day postconfluent C2C12 myotubes appeared granular, perinuclear, and

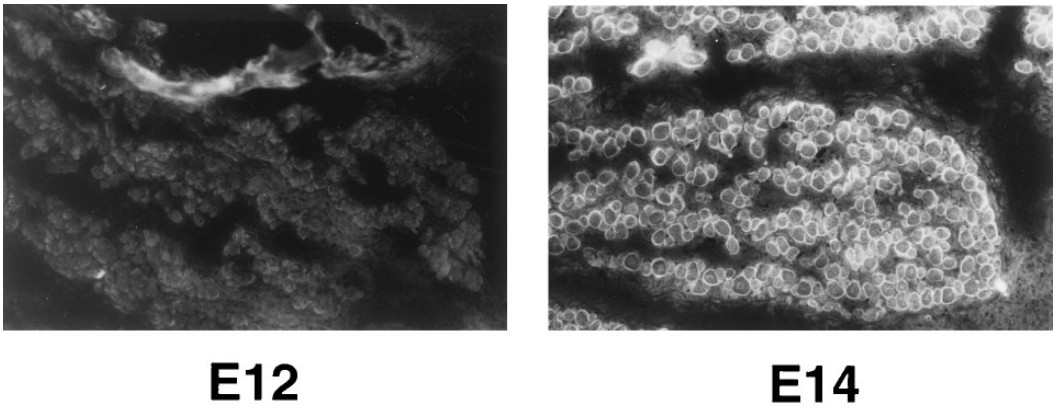

FIG. 5. The developmental expression of adhalin coincides with primary myogenesis in the mouse. Immunofluorescence analyses on sections of days E12 and E14 hindlimbs of mouse embryos. Adhalin is detected only once primary myogenesis has begun (day E14).

cytoplasmic (Fig. 8A) in contrast to the continuous sarcolemmal localization observed in vivo (Figure 4B). Since C2C12 myotubes do not contract spontaneously in medium containing 1% FCS and antibiotics, we hypothesized that the proper integration of adhalin into the membrane may be a consequence of myotube maturation and/or contraction. To verify this, we analyzed the localization of adhalin in myotubes at various times after initiation of differentiation under contraction-permissive culture conditions. For comparison, we also analyzed the localization of myosin heavy chain, sarcomeric α-actinin, utrophin, dystrophin, and caveolin-3. The expression of myosin heavy chain and sarcomeric α-actinin is a requirement for muscle cell contraction (37-38). Utrophin is expressed early in myotube formation and is gradually replaced by dystrophin in later stages (39-42). Caveolin-3 is a muscle specific caveolin that associates with the formation of the T-tubule system before becoming restricted to its final site of localization at the muscle cell membrane (24).

Whether under normal or contraction-permissive culture conditions, we found that myosin heavy chain and sarcomeric α-actinin were expressed into assembled myofibrils and striations of C2C12 myotubes after 6 days of postconfluent culture (not shown), indicating the presence of a basic contracting apparatus (38). This was confirmed by the observation that some of the myotubes at this early stage contracted under the contraction-permissive conditions (not shown). Nevertheless, adhalin was found still localized in the cytoplasm of

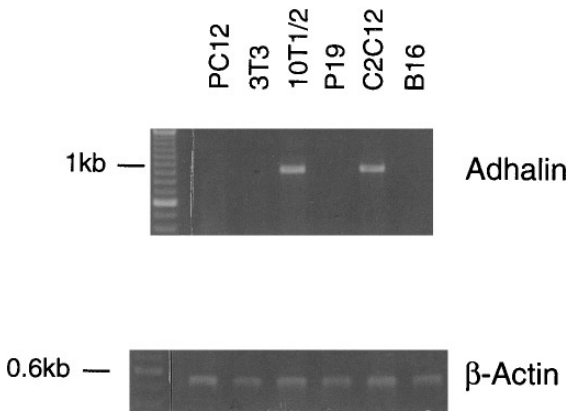

FIG. 6. RT-PCR analyses for the presence of adhalin mRNA in cell lines representing different cell types. Amplification of β-actin mRNA was performed as control for RNA quantities used. The mRNA of adhalin is detected only in cells with a potential for myogenic differentiation.

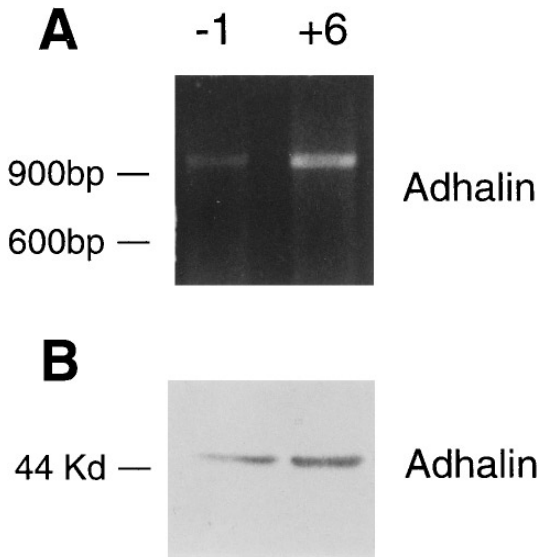

FIG. 7. Expression of adhalin during the myogenic differentiation of mouse C2C12 cells. *A.* RT-PCR analysis from total RNA of undifferentiated cells (−1 day; before confluence) and differentiated cells (+6 days; after confluence) for adhalin mRNA. *B.* Immunoblot analysis of extracted proteins from same-stage cultures. The mRNA and protein levels of adhalin undergo up-regulation as a function of the myogenic differentiation program of the cells.

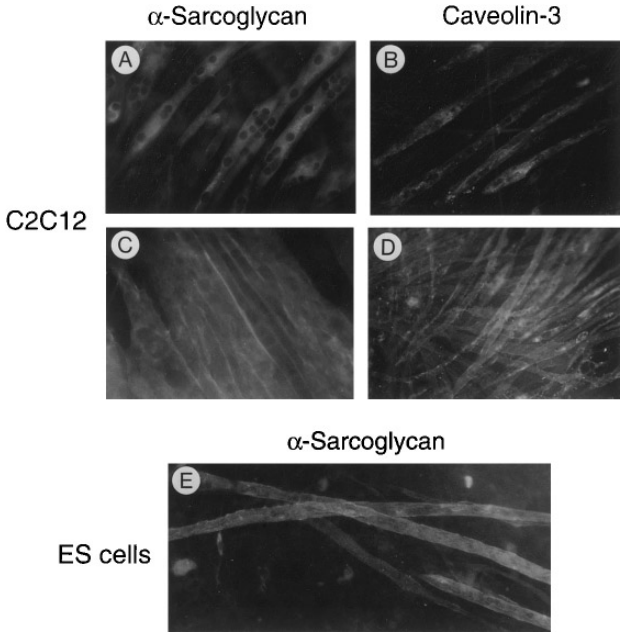

FIG. 8. Immunofluorescence analysis of differentiated C2C12 cells and ES cells. C2C12 cells were stained for adhalin (A, C) and for caveolin-3 (B, D) at 6 days (A, B) and 22 days (C, D) postconfluence. ES cells were stained for adhalin (E) at 30 days after differentiation. Adhalin staining is associated with the perinuclear regions of 6-day myotubes (A). At the later stages, many myotubes show adhalin localized predominantly to the sarcolemma (D, E).

these early contracting myotubes as in the case of non-contracting ones (Fig. 8A). However, adhalin gradually re-localized from the cytoplasm to the cell membrane after prolonged periods of contraction-permissive culture and was properly integrated in myotubes of 22-day cultures (Fig. 8C). At this late stage of maturation, many myotubes were contracting. We observed a similar, progressive re-localization of caveolin-3 between 6 and 22 days of postconfluent contraction-permissive culture (compare Fig. 8B and 8D) as expected (24). Furthermore, dystrophin -but not utrophin- was also localized to the cell membrane at these late stages (not shown). Adhalin was likewise localized exclusively to the cell surface of myotubes in 30-day embryonic ES cell cultures (Fig. 8E). These myotubes contract vigorously presumably due to stimulation by neurons also present in these differentiated cultures (29,43). Thus, these data indicate that adhalin gradually re-localizes from the cytoplasm to the cell membrane in correlation to the terminal maturation of myotubes.

DISCUSSION

Adhalin and other sarcoglycans appear to be crucial components in skeletal and heart muscle judging from the diseases caused by their absence. Previous studies, undertaken primarily in humans, have addressed some aspects of the genetics and biochemistry of adhalin (21,39,44-45), but essential knowledge of the structure and function of adhalin and other components of the sarcoglycan complex is lacking. Since numerous structure-function questions about adhalin and related proteins are best addressed in the mouse and in vitro, we cloned and sequenced the mouse adhalin. We show here that mouse adhalin is a cell surface transmembrane protein, which is highly conserved in mammals, and which is characteristically present in striated muscle but not in other tissues or cell types. In this respect, we observed that the expression of adhalin is coincident with primary myogenesis during mouse development. We further show that the expression of adhalin is up-regulated during the myogenic differentiation program in which mononucleated, proliferative myoblasts fuse into postmitotic, multinucleated myotubes. Finally, we find that adhalin undergoes re-localization from the cytoplasm to the sarcolemma when myotubes reach their final maturation stage.

Adhalin Is Highly Conserved among Mammals

Cloning and sequencing of mouse adhalin cDNA showed that it is very similar to the human, rabbit, and hamster molecules. Many of the primary structural features of the protein are conserved, including the positions of the transmembrane domain, the extracellular domain cysteines, and the potential glycosylation sites. Our sequencing of three partially overlapping cDNA clones and numerous RT-PCR assays provided no evidence for the existence of splice variants of mouse adhalin as was reported for its human counterpart (21). However, additional clones would need to be analyzed in order to clearly establish whether mouse adhalin mRNA is subjected to differential splicing or not. In this respect, we did find an additional 2.8 kb mRNA species in the heart tissue. The relationship of this novel transcript to the main mRNA species will be investigated in the future.

Searching the data bank for sequences homologous to adhalin revealed no sequences other than those of its three other known mammalian counterparts. It is noteworthy that a sequence homologous to a portion of both γ and δ sarcoglycans is present in the genome of the nematode *C. elegans* (46). Considering that expression of all four sarcoglycans appears necessary for their presence and function in muscle (47), it is likely that adhalin homologues will be found in invertebrates in the future.

Adhalin Is a Marker of Myogenic Differentiation

The expression and localization of adhalin in mouse tissues was found similar to that already described in human tissues (21,48-49). In both species, adhalin is restricted to striated muscle and can not be detected in any other somatic tissue types. Previous data on the

expression of adhalin during human fetal development have showed that adhalin can be detected at a rather late stage of muscle development (49). We found that adhalin expression coincides with primary myogenesis in mouse development. Our in vitro analyses now define further the expression and cellular localization of adhalin as a function of differentiation. Of the cell lines analyzed, only myocytes and immediate myogenic precursor cells were found positive for both the mRNA and the protein. Such cells included the mouse C2C12 myoblasts and the mouse 10T1/2 cells. The C2C12 cell line is well characterized in terms of its myogenic differentiation properties (30, 38), and these cells spontaneously fuse to form myotubes in conditions of low serum concentrations. The 10T1/2 cells can also form myotubes but only do so when treated with 5-azacytidine (31). The human rhabdomyosarcoma RD cells and the rat L6 myoblasts, which undergo myogenic differentiation in the same manner as the normal C2C12 (30), were also found to express adhalin mRNA. By contrast, the 3T3 fibroblasts, which can differentiate into fat and cartilage cells but not into muscle (31), did not express adhalin before or after differentiation. Likewise, the embryonal carcinoma P19 cells, which preferentially differentiate into neurons and glia (50), were negative for adhalin, as were a number of other non-muscle cell lines, thus indicating that adhalin expression is specific for the striated muscle cell type. In this respect, the apparent muscle-specific expression of adhalin was also seen in cultures of differentiated ES cells, which contain numerous other cell types (29,38). These data, together with our observation that adhalin expression undergoes up-regulation both at the mRNA and protein levels during myogenic differentiation, identify adhalin as a marker of myogenic differentiation.

Adhalin Localizes Properly at the Sarcolemma in Fully Mature Myotubes

The sequence of adhalin, as well as biochemical and immunolocalization data, have established adhalin as a transmembrane protein (this study; 2-3,20-22). Nonetheless, we found a cytoplasmic localization of adhalin in 6-day postconfluent C2C12 myotubes, despite these cells having a basic contracting apparatus containing myofibrills and striations positive for myosin heavy chain and sarcomeric α-actinin. This inappropriate localization of adhalin was also observed under contraction-permissive culture conditions. However, in this latter situation, adhalin underwent a gradual re-localization to the membrane over prolonged periods of postconfluent culture in a similar fashion to that observed in the vigorously contracting myotubes formed in differentiated ES cell cultures. Furthermore, this re-localization of adhalin appeared coincident with the localization of caveolin-3 and dystrophin to the cell membrane. Caveolin-3 is transiently associated with forming T-tubules in developing myotubes, but is associated predominantly with cell surface caveoli in fully mature myotubes (24,51). The re-distribution of caveolin-3 strikingly illustrates the maturation of myofibers (24,51). Thus, our finding that adhalin is present primarily within the cytoplasm of myotubes with a functional but basic contracting apparatus but then becomes localized at the sarcolemma of myotubes with a fully established cytoarchitecture, suggests a linkage between the appropriate membrane integration of adhalin and the terminal maturation of myofibers.

What then is the process which determines the membrane localization of adhalin? It may be that adhalin requires the association with β-, γ and/or δ-sarcoglycan, and possibly other muscle components, for its sarcolemma localization. These components may not be expressed at optimal levels or may themselves be inappropriately localized in early-stage myotubes. The cytoplasmic localization of adhalin in C2C12 myotubes may also be caused by the absence or inappropriate expression of all required cytoarchitectural and myofibrillar components. Proteins such as α-actinin, actin, myosin, and utrophin are expressed, localized, and organized properly in myotubes formed by these cells (this study; 38). However, dystrophin is expressed and organized later (this study; 40-41), and it is possible that dystrophin-associated proteins including adhalin are organized in syncrony with dystrophin at the membrane (40-42). The process of adhalin reorganization during myotube maturation is also reminiscent of receptor relocalization and clustering upon ligation and may therefore represent an unknown aspect of the regulation of this protein. In this respect, the nature of putative extracellular ligand(s) for the sarcoglycan complex remains to be elucidated, but a direct or indirect linkage to the basement membrane has been proposed (42).

CONCLUSIONS

In conclusion, we have determined the primary structure of adhalin in the mouse, extended previous data as to the tissue- and cell-type specificity of adhalin expression, and shown a novel association between its proper sarcolemmal targeting and the terminal maturation of myofibers. These findings will be useful in future experimental analyses to address fully the biological roles of adhalin. Furthermore, the cloning and sequencing of mouse adhalin will greatly facilitate the cellular and molecular dissection of its functions through the use of gene-targeted mutant mice and various cell culture systems.

ACKNOWLEDGMENTS

We thank Solveig Sundberg for technical support, the Shriners Hospital for Children for the use of their Core Facility, and our colleagues Drs. R. Albrechtsen and W. Stallcup for reading the manu-

script and for valuable advice. This work was supported in part by grants from the Swedish Natural Science Research Council and from the National Institutes of Health (NIH), U.S.A., to E.E., and from the Danish Cancer Society to U.M.W. P.H.V. was supported by a fellowship of the Medical Research Council of Canada. H.X. was supported by a postdoctoral training grant from the NIH.

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