Journal of Cellular Biochemistry 77:418–431 (2000)

Identification and Characterisation of Transcript and Protein of a New Short N-Terminal Utrophin Isoform

Richard A. Zuellig, Beat C. Bornhauser, Irene Knuesel, Franziska Heller, Jean-Marc Fritschy, and Marcus C. Schaub*

Institute of Pharmacology and Toxicology, University of Zurich, CH-8057 Zurich, Switzerland

Abstract Dystrophin and utrophin are known to link the intracellular cytoskeleton to the extracellular matrix via a transmembraneous glycoprotein complex. Four short C-terminal isoforms (Dp71, Dp116, Dp140, and Dp260) are described for dystrophin and three for utrophin (Up71, Up113, and Up140). We describe here for the first time the existence of a 3.7-kb transcript and a 62-kDa protein in C6 glioma cells representing a short N-terminal isoform unique for utrophin (N-utrophin). More than 20 clones covering the entire coding region of utrophin were isolated from a rat C6 glioma cell cDNA library. Two clones were found to code for a protein with 539 amino acids. Its sequence is identical to that of the full-length utrophin, except for the last residue where Cys is replaced by Val. This isoform contains the actin binding domain (consisting of two calponin homology subdomains), followed by two spectrin-like repeats. A recombinant fragment corresponding to N-utrophin binds to F-actin in vitro with an equilibrium constant (affinity) K of 4.5×10^5 M^{-1} and a stoichiometry of one fragment per around five actin monomers. Immunocytochemical staining of C6 glioma cells with antisera specific for different utrophin regions localised full-length utrophin in the submembraneous cortical actin layer as revealed by confocal microscopy. A distinct staining pattern for the N-utrophin was not detectable, although it was expected to localise at the actin stress fibers. It is assumed that it co-localises via the two spectrin-like repeats with the full-length utrophin at the cell membrane. J. Cell. Biochem. 77:418–431, 2000.

Key words: actin binding; calponin homology domain; cytoskeleton; dystrophin; glioma cell line; muscular dystrophy; utrophin

Utrophin is an autosomal homologue of dystrophin whose gene localises to chromosome 6q24 [Pearce et al., 1993]. The two proteins share high sequence similarity and exhibit a homologous domain structure [Tinsley et al., 1992]. In both proteins, the N-terminus comprises an actin binding domain consisting of two calponin homology subdomains (CH1 and CH2) arranged in tandem [Carugo et al., 1997]. The central rod domain is built up of 22 (utrophin) or 24 (dystrophin) spectrin-like triple coiled-coil repeats. It is followed by a cysteine-rich and by a C-terminal domain, which are both involved in forming a transmembraneous protein complex [Tinsley et al., 1994; Winder, 1997]. This complex has been shown for utrophin as well as for dystrophin to interact with laminin and agrin of the extracellular matrix [Campanelli et al., 1994]. By contrast, from in vitro binding studies with protein fragments, it is known that utrophin and dystrophin can bind to actin with their N-terminal CH1-CH2 domain [Winder, 1997]. Hence utrophin and dystrophin constitute a continuous link between the intracellular actin cytoskeleton and the extracellular matrix, which is thought to

Abbreviations used: aa, amino acids; Ab, antibodies; ABS, actin binding site; BMD, Becker muscular dystrophy; CH, calponin homology domain; DMD, Duchenne muscular dystrophy; Dp (followed by number), C-terminal dystrophin isoform of specific molecular mass; G-utro, C-terminal utrophin isoform; H, hinge region; IPTG, isopropyl-β-D-1-thiogalactopyranoside; N-utro, N-terminal utrophin isoform; Ni-NTA, Ni-nitrilotriacetic acid agarose; NMJ, neuromuscular junction; nt, nucleotide; pI, isoelectric point; R, spectrin-like repeat; Up (followed by number), C-terminal utrophin isoform of specific molecular mass; UTR, untranslated region.

Grant sponsor: Roche Research Foundation; Grant number: 94-107; Grant sponsor: Swiss Foundation for Research on Muscle Diseases; Grant sponsor: Hartmann Mueller Foundation for Medical Research; Grant number: 557.

The first two authors contributed equally to this work.

*Correspondence to: M.C. Schaub, Institute of Pharmacology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. E-mail: schaub@pharma.unizh.ch

Received 19 July 1999; Accepted 2 December 1999

This article published online in Wiley InterScience, April 2000.

play a role in anchoring the cell in the surrounding architectural structures.

This anchorage becomes of particular importance in contractile tissues. Muscular diseases of various severity are known to be caused by defects either in dystrophin (DMD and BMD) or in other components of the associated transmembraneous protein complex. An increased mechanical fragility of muscle cells has indeed been demonstrated in vitro [Pasternak et al., 1995] and in situ [Brooks, 1998] of the mdx mouse. In addition to mechanical fragility, the membranes of mdx myotubes were shown to become leaky for Ca^{2+} entry under stress conditions [Leijendekker et al., 1996]. The overall pathophysiological syndrome is less severe in the mdx mouse as compared with human DMD. In both cases utrophin expression is compensatorily elevated, and the protein localises in part along the sarcolemma in regenerating myofibers [Karpati et al., 1993]. Only a utrophin-dystrophin deficient double knockout mouse develops a muscular dystrophy as severe as human DMD [Deconinck et al., 1997]. Together with the structural similarity, this may suggest some redundancy between the two proteins. Phenotypic rescue of the mdx mouse has indeed been achieved not only by muscle-specific transgenic expression of full-length dystrophin [Lee et al., 1993], but also of a utrophin minigene [Tinsley et al., 1996] or of the full-length utrophin [Tinsley et al., 1998].

Despite their structural and functional similarity, the two proteins exhibit distinct localisation in normal adult tissues. In fetal skeletal muscles, utrophin localises at the sarcolemma, from which it disappears and gets replaced by dystrophin during development after birth [Clerk et al., 1993]. In mature muscles, both are found in the neuromuscular junctions (NMJ) albeit with distinct subjunctional localisation [Berthier and Blaineau, 1997]. Both are also present in vascular smooth muscles [Rivier et al., 1997] and cardiomyocytes [Pons et al., 1994], where they display a distinct subcellular pattern.

In addition, different isoforms of both proteins have been described. Full-length dystrophin exists in four isoforms driven by different promoters expressed in a tissue-specific pattern [Sadoulet-Puccio and Kunkel, 1996; Winder, 1997]. At least four C-terminal short isoforms have been described whose expression is also tissue-specific and driven by additional promoters from within the gene. These four isoforms comprise the cysteine-rich and the C-terminal domains, as well as various numbers of spectrin-like repeats (Dp260, Dp140, and Dp116) or none, as in Dp71. Dp71 comprises a unique N-terminus of seven amino acid residues fused to the cysteine-rich domain, which enables it to bind to actin [Howard et al., 1998]. Besides the isoforms from different promoters, variation in mRNA processing has also been observed [reviewed in Sadoulet-Puccio and Kunkel, 1996]. The functional significance of this multitude of dystrophin isoforms remains unclear. The fact that they have been found in human, mouse, and chicken in a tissue-specific pattern, however, may point to their functional specialisation.

In contrast to dystrophin, only one short isoform has so far been well characterised from the utrophin locus. This 113-kDa protein (Up113 also called G-utrophin) is encoded by a 5.5-kb mRNA with a unique N-terminus comprising 48 amino acids fused to the truncated spectrin-like repeat-20, followed by two further repeats, the cysteine-rich and C-terminal domains [Blake et al., 1995]. G-utrophin diverges from utrophin at the same point as does Dp116 from dystrophin. Therefore, G-utrophin is considered to be an autosomal homolog of Dp116. Its expression is confined to sensory dorsal root and cranial nerve ganglia, as well as to several distinct brain regions. Both homologous isoforms are thus found exclusively in the nervous system. In a most recent report, two new utrophin transcripts for Up71 and Up140 analogous to Dp71 and Dp140 have been characterised from human and mouse tissues, including skeletal muscle [Wilson et al., 1999]. The corresponding proteins have been less well characterised [Lumeng et al., 1999].

While no short N-terminal dystrophin isoform is known, such a utrophin protein has been postulated to exist in rat C6 glioma cells based on evidence of Western blots [Nguyen thi Man et al., 1995]. This article describes the cloning and characterisation of two transcripts from the utrophin locus in C6 glioma cells which code for this short N-terminal utrophin isoform (N-utro). N-utro contains 539 amino acids (aa), with the first 538 residues identical to the utrophin sequence. N-utro comprises the actin binding domain plus two spectrin-like repeats from the rod. We produced antisera

against the N-terminus, the rod domain, and the C-terminus of full-length utrophin that do not cross-react with dystrophin. They were used to detect utrophin and its isoforms in C6 glioma cells. In addition, we report on the actin binding properties in vitro of a recombinant utrophin fragment that corresponds to N-utro.

MATERIALS AND METHODS

Preparation of Clones and Probes

A cDNA library was prepared from mRNA isolated from the rat C6 glioma cell line using oligo(dT)$_{18-25}$ and random hexanucleotide primers [Blake et al., 1996; Vater et al., 1998]. For library screening, the following probes were used, 14-1 (nt 1′385-1′907), 220ES0.7 (*Eco*RI-*Sac*I fragment for nt 7′965-8′690) both from mouse utrophin cDNA and α-215EB0.9 (*Eco*RI-*Bam*HI fragment for nt 3′224-4′090) from rat clone α-215. All nucleotide numbers refer to rat utrophin cDNA sequence (EMBL accession No. AJ002967). Probes α-207EB0.9 (*Eco*RI-*Bam*HI fragment for nt 21-872), α-213H0.9 (*Hin*dIII-*Hin*dIII fragment for nt 1′813-2′693) and α-201S0.2 (*Sac*I-*Sac*I fragment for nt 1′902-2′112) were used for detection of utrophin mRNA from rat C6 glioma cells by Northern blots. The probe 14-1 was generated by polymerase chain reaction (PCR) using primers derived from the human utrophin sequence (EMBL accession No. X69086) after RNA isolation from mouse spleen and first-strand cDNA synthesis [Chomczynski and Sacchi, 1987; Blake et al., 1996]. Sense and antisense primers for the first PCR were 5′-TCAGGAACAGATGACCCTGCT-3′ (human utrophin nt 1′720-1′740) and 5′-CAATCTCAC-TCAGCTGATCCA-3′ (human utrophin nt 2′310-2′330), respectively. For a following nested PCR, the antisense primer was replaced by 5′-CAGACGTCGAACACTGACACT-3′ (human utrophin nt 2′249-2′269). All other probes were derived from isolated cDNA clones. After library screening, positive clones were purified and their ends sequenced. Sequences were compared to that of the human utrophin and the positions of the clones relative to each other determined. Overlapping clones were selected and digested with different restriction enzymes (Fig. 1). The fragments were subcloned in pBluescript II SK- and sequenced with T7-Sequenase 2.0 (Amersham) or by automated sequence analyzer (310 Genetic Analyzer, ABI Prism from Perkin-Elmer). Sequence analyses and assembling were done using the Genetics Computer Group Version 7.3 software package (GCG).

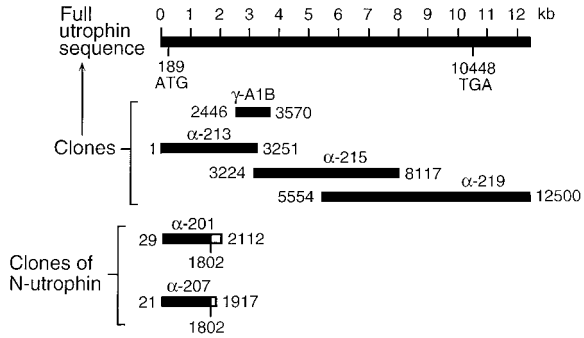

Fig. 1. Rat utrophin clones. Four rat utrophin clones, α213, γA1B, α215, and α219, cover the whole utrophin coding and parts of the 5′- and 3′-untranslated regions. The start codon ATG and the stop codon TGA are indicated in the full utrophin sequence above. The two clones α201 and α207 differ in their 3′-end (empty boxes) from the utrophin sequence. The alternative sequences have different lengths but are identical in the overlap. They start at nt 1803 and are followed by a stop codon at nt 1806. The two clones represent a new N-terminal utrophin isoform. Numbers of nucleotides are given at the start and end of the clones.

Northern Blots

RNA was isolated from rat C6 glioma cells or from rat tissues [Han et al., 1987] and passed twice over an oligo(dT) cellulose column (New England Biolabs) for preparation of poly(A)$^+$ RNA [Sambrook et al., 1989]. RNA (3 μg per lane) were loaded on 5-mm-thick 1% agarose formaldehyde gels. After electrophoresis, the gels were rinsed with water and photographed. Subsequently, the gels were either soaked in 20 × SSC for 40 min and blotted by capillary transfer to a nylon membrane (Positive Membrane from Appligene Oncor) or treated for 20 min with 0.05 M NaOH plus 0.15 M NaCl and neutralized in 0.15 M NaCl plus 0.1 M Tris-HCl, pH 7.5, for 30 min before pressure blotting in 20 × SSC (PosiBlot 30-30 Pressure Blotter; Stratagene). The membranes were then rinsed with 6 × SSC and heated for 1h at 80°C in vacuum. They were prehybridised with hybridisation solution (6 × SSPE, 5 × Denhardt's reagent, 0.1% sodium dodecyl sulfate (SDS), 50% formamide, 50 μg/ml salmon sperm DNA) for more than 3h at 42°C. Hybridisation was carried out overnight at 42°C with fresh hybridisation solution containing about 3 × 10^6 cpm/ml of the labeled probe α207EB0.9,

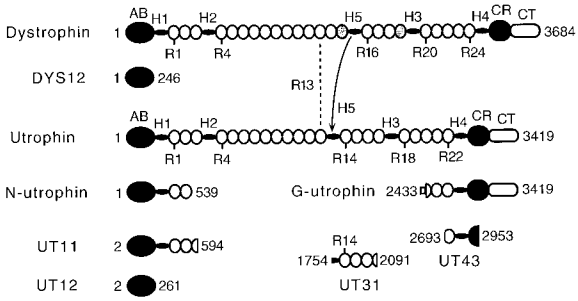

Fig. 2. Schematic protein domain representation. The recombinant utrophin fragments UT11, UT12, UT31, UT43 and the recombinant dystrophin fragment DYS12 are compared with the full-length species of utrophin and dystrophin. Also included is the new short N-terminal utrophin isoform described in the present work and the recently characterised C-terminal G-utrophin isoform. Numbers indicate amino acid residues; AB, actin binding domain; H1-H5, hinge regions; R1-R24, spectrin-like triple helical repeats; CR, cysteine rich domain; CT, C-terminal domain. The grey R15 and R19 in dystrophin are missing from utrophin. In utrophin H5 is displaced and appears between R13 and R14 (arrow). Dashed line, unchanged position of the homologous R13 in dystrophin and utrophin. The alignment of the subsequent homologous spectrin-like repeats in utrophin follows that in dystrophin despite the diverging numbering. The new short N-terminal isoform (N-utrophin) comprises the AB domain and two spectrin-like repeats. The C-terminal G-utrophin isoform contains 48 amino acids in front of the truncated R20 whose sequence differs from that of utrophin (empty box).

α213H0.9, or α201S0.2. The membranes were washed 2 × 3 min with (2 × SSC, 0.1% SDS) at 22°C, followed by 3 × 20 min with (0.1 × SSC, 0.05% SDS) at 50°C and exposed for 24 h or 144 h at −80°C to a BioMax MS film combined with a BioMax TranScreen-HE Intensifying Screen (Eastman Kodak).

Recombinant Protein Expression

Four fragments from rat utrophin (UT11, UT12, UT31, and UT43) and one from human muscular dystrophin (DYS12) were cloned into pQE vectors (Qiagen) for expression in *Escherichia coli* M15[pREP4] (Fig. 2). This provides a $MRGSH_6$-tag at the N-terminus of the proteins. In order to maintain the correct reading frame, 1–10 additional amino acids (aa) appeared between the tag and the inframe proteins (see below). The UT11 plasmid coding for aa 2–594 is a blunted *Eae*I-*Eae*I-fragment of clone α-213 ligated into the blunted *Bam*HI-site of pQE-30; for production of the UT12 plasmid coding for aa 2–261, clone α-213 was digested with *Bsa*HI, blunted, digested again with *Stu*I; the resulting *Stu*I-*Bsa*HI-fragment was then ligated into the *Stu*I-*Hin*cII-site of the UT11 plasmid; for the UT31 plasmid coding for aa 1′754-2′091, the clone α-215 was first digested with *Bam*HI to produce a 2.7-kb fragment that was subcloned into a pBluescript II SK- vector from which a *Bam*HI-*Hin*cII-fragment was ligated into the *Bam*HI-*Hin*cII-site of pBluescript II SK- again from which a *Bam*HI-*Kpn*I fragment was ligated into the *Bam*HI-*Kpn*I site of pQE-31; for the UT43 plasmid coding for aa 2′693-2′953, the clone α-219 was digested with *Xho*I and religated to produce a 4.5-kb fragment from which a *Tha*I-*Stu*I-fragment was ligated into the *Eco*RV-site of pBluescript II SK-, from which a *Bam*HI-*Kpn*I-fragment was ligated into the *Bam*HI-*Kpn*I site of pQE-32. For DYS12, the DNA coding for aa 1–246 was cloned into the blunted *Bam*HI site of pQE-32 after PCR amplification from a human dystrophin minigene with the following sense and antisense primers: 5′-ATGCTTTGGTGGGAAGAAGTA-3′ and 5′-TATTCAATGCTCACTTGTTGAGGC-3′, respectively. The dystrophin minigene was kindly provided by Dr. S. J. Winder. All ligation junctions were sequenced for checking the correct reading frame. The following final expression constructs were obtained (numbers in brackets refer to the rat utrophin primary structure):

UT11: $MRGSH_6$GS-(2-594)-RSACELGTPGRPAAKLN

UT12: $MRGSH_6$GS-(2-261)-GPAAKLN

UT31: $MRGSH_6$T-(1′754-2′091)-SRGGPVPRVDLQPSLIS

UT43: $MRGSH_6$GIPRAAGIRS-(2′693–2′953)-SSLSIPSTSRGGPVPRVDLQPSLIS

DYS12: $MRGSH_6$GI-(1–246)

All five described plasmids could be expressed in *E. coli* by induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG) and purified by affinity chromatography on Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen). UT11, UT12, and DYS12 were purified under native conditions by elution with 200 mM imidazole (see example given in Fig. 3). UT31 and UT43 could only be extracted and purified under denaturing condition in 8 M urea with elution from the affinity column at pH 4.5. All protein preparations were tested by sodium dodecyl sulfate–polyacrylamide gel electro-phoresis (SDS-PAGE) [Laemmli, 1970]. The following molecular-weight markers

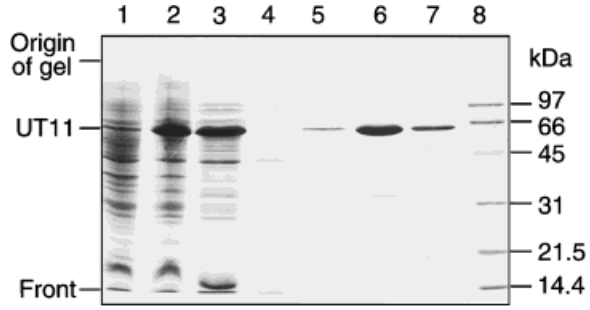

Fig. 3. Expression and purification of the recombinant fragment corresponding to N-utrophin (UT11). UT11 was expressed in *Escherichia coli* and purified by Ni-NTA affinity chromatography. SDS-PAGE stained for protein with Coomassie Blue R250. **Lane 1,** noninduced control; **lane 2,** IPTG-induced expression; **lane 3,** soluble extract; **lane 4,** flowthrough of affinity column; **lane 5,** wash with 20 mM imidazole; **lane 6,** first fraction eluted with 200 mM imidazole; **lane 7,** second fraction; **lane 8,** molecular-weight markers.

were used: phosphorylase-b, 97 kDa; bovine serum albumin (BSA), 66 kDa; ovalbumin, 45 kDa; carboanhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa (Bio-Rad, low-range markers). Protein concentrations were determined by the Bradford test [Bradford, 1976] with BSA as standard and UV absorption at E280 as derived from GCG software using an extinction coefficient of 87′580 M^{-1} cm^{-1} for UT11, 39′260 M^{-1} cm^{-1} for UT12 and 52′040 M^{-1} cm^{-1} for DYS12.

Immunostaining and Microscopy

New Zealand white rabbits and guinea pigs (GOHI-Ibm:GOHI-SPF) were immunized with UT11, UT31, and UT43 comprising Freund's adjuvant. Rabbit anti-UT11 and anti-UT31 were purified by affinity chromatography using the recombinant proteins coupled to Affi-Gel 15 (Bio-Rad).

For immunohistochemistry, rat diaphragm muscle was cut at 12 μm on a cryotome and fixed in 0.1% paraformaldehyde and 5% Kryofix (Merck) in a microwave oven for 45 s at 650 W. Cryosections were stained for epifluorescence microscopy with our polyclonal antibodies (Ab), monoclonal antibodies (mAb) NCL-DYS1 against the rod (between aa 1181 and 1388) domain of dystrophin (Novocastra Laboratories) and α-bungarotoxin-rhodamine (Molecular Probes). As secondary polyclonal Ab Cy-3-labeled goat anti-rabbit (Jackson ImmunoResearch Laboratories) and Alexa Green-labeled goat anti-mouse polyclonal Ab (Molecular Probes) were used.

For immunocytochemistry rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Sera-Tech), nonessential amino acids (NEAA from Sigma-Aldrich), and freshly added L-glutamine. After washing with phosphate-buffered saline (PBS) cells were fixed in 3.8% paraformaldehyde and permeabilised with 0.1% Triton-X-100 in PBS containing 10% normal goat serum for 10 min at 22°C. Affinity purified rabbit anti-UT11 and anti-UT31 were used for staining in combination with Cy-3 labeled goat anti-rabbit polyclonal Ab (Jackson ImmunoResearch). F-actin was visualized in double stainings with phalloidin-Oregon Green (Molecular Probes). Confocal microscopy was done with Leica objective lenses on a Leica confocal scanner TCS-NT and a Silicon Graphics Personal Iris 4D/25 workstation.

Western Blots

Lysates were prepared by boiling C6 glioma cells in 50 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, and 10 mM DTT, and run on 4–15% gradient SDS-PAGE [Laemmli, 1970]. Blotting was performed in 7 mM Tris, 87.5 mM glycine (pH 8.3) without methanol, using a Mini Trans-Blot Cell (Bio-Rad). After incubation with affinity-purified rabbit anti-UT11 or anti-UT31 combined with horseradish peroxidase (HRP)-labeled goat anti-rabbit polyclonal Ab (Pierce), detection was performed by SuperSignal chemiluminescence (Pierce) and exposure to Fuji Medical X-ray Film (Fuji Photo Film). Controls were done by omission of the first polyclonal Ab, by using preimmune serum and by competition with the appropriate antigen. A mAb (NCL-DRP1) against the C-terminus (last 11 aa) was used for identification of full-length utrophin. Molecular-weight markers were (Bio-Rad, broad-range markers): myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase-b, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carboanhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

Actin Binding Assay

Binding of the recombinant fragments UT11, UT12, and DYS12 to purified F-actin [Pardee and Spudich, 1982] from rabbit fast skeletal

muscle was determined by high-speed cosedimentation in a Beckman TLA-100 tabletop ultracentrifuge (30 min at 100′000*g* and 25°C) after incubation for 30 min in 50 mM Tris-maleate buffer, pH 7.4, 0.1 M NaCl, 1 mM ATP, 1 mM DTT, 0.5 mM $CaCl_2$ and 2 mM $MgCl_2$. The volume was 100 μl containing 6 μM F-actin and varying amounts of the ligand. Equal volumes of supernatant and pellet (resuspended to 100 μl) were run in an SDS sample buffer on 10% PAGE [Laemmli, 1970]. Gels stained with Coomassie Blue R250 were analysed with the Image Analysis System MCID/M2 (Imaging Research) by integrating the area of the protein bands. Staining overnight and destaining in 30% methanol, 10% acetic acid, and 60% H_2O was rigorously controlled. Calibration for staining was performed with concentration rows of actin and BSA. The measurements yielded a linear relationship from 0.2 to 10 μg of protein. Evaluation of binding data was done using nonlinear regression analyses with GraphPad Prism version 2.0 (GraphPad Software). For each electrophoretic run, the actin concentration was held constant and thus served as internal loading standard. BSA and tropomyosin were used for negative and positive controls of the binding assay. The recombinant fragments were always subjected to the same centrifugation procedure in the absence of actin. Actin concentration was determined by the Bradford test [Bradford, 1976] and by ultraviolet (UV) absorption at E290 with an absorption coefficient of 26′400 M^{-1} cm^{-1} [Winder et al., 1995]. Protein concentration of the recombinant fragments was assessed as mentioned above.

RESULTS

Cloning and Sequencing of Rat Utrophin

Some 20 cDNA clones were isolated from a rat C6 glioma cell cDNA library, using two different mouse utrophin probes (14-1 and 220ES0.7). By sequencing the ends of all, three clones were found to overlap and to cover the entire rat utrophin coding region including parts of the 5′- and 3′-untranslated regions (UTR) (Fig. 1). Since the overlap between α-213 and α-215 extends only over 28 nt, an additional probe α-215EB0.9 (nt 3224–4090) was generated for further screening. With this probe the clone γ-A1B was detected which overlaps upstream by 806 nt with α-213 and downstream by 347 nt with α-215. The full-length nucleotide sequence has been deposited under EMBL: AJ002967. In addition, two clones, α-201 and α-207, of around 2 kb from the 5′-end were identified that differ at their 3′-end from the utrophin sequence. These two additional clones are identical except for one nucleotide difference in the 5′-UTR and for having different lengths. α-201 extends from nt 29 to 2112 and α-207 from 21 to 1917; thus, α-201 is 187 nt longer than α-207. Both sequences, except for the one mutation in the 5′-UTR in α-201 (G36A), are identical to that of utrophin up to nt 1802. The deviation begins there with GTA, immediately followed by the stop codon TGA (Fig. 1). The rest of the sequence is completely different from utrophin. The comparison with the utrophin sequence suggests that the one mutation in the 5′-UTR of α-201 may have resulted from an error either of the reverse transcriptase or of the *E. coli* polymerase. The GT motif in α-201 and α-207 at the beginning of the sequence differences could represent an unused splice donor site, followed by intron material. Should the message for these clones exist in vivo, it would code for a short N-terminal utrophin isoform (N-utro). This protein would comprise 539 amino acid residues with a calculated molecular mass of 61′744 Da (around 62 kDa) and identical sequence to utrophin, except for the last residue, where Cys is replaced by Val. It spans from the N-terminus over the actin binding domain, followed by the first hinge region to the end of the second spectrin-like repeat (Fig. 2).

From protein sequence alignments between several species (human, mouse, and rat) we concluded that the spectrin-like repeats R15 and R19 of dystrophin are missing in utrophin. A short linker of 18 aa residues containing one Pro that is sensitive to proteolysis has been located between R15 and R16 in human dystrophin [Hori et al., 1995]. We identified a short homologous linker with 12–20 residues comprising one or two Pro between R13 and R14 in mouse, rat and human utrophin (Fig. 2). In contrast with these short linkers, the hinge regions H1–H4 are longer (47–75 aa) and contain several Pro both in utrophin and dystrophin. The deletion of two spectrin-like repeats together with the shift of the short linker preserves the same domain arrangement in the C-terminal half of utrophin as in dystrophin.

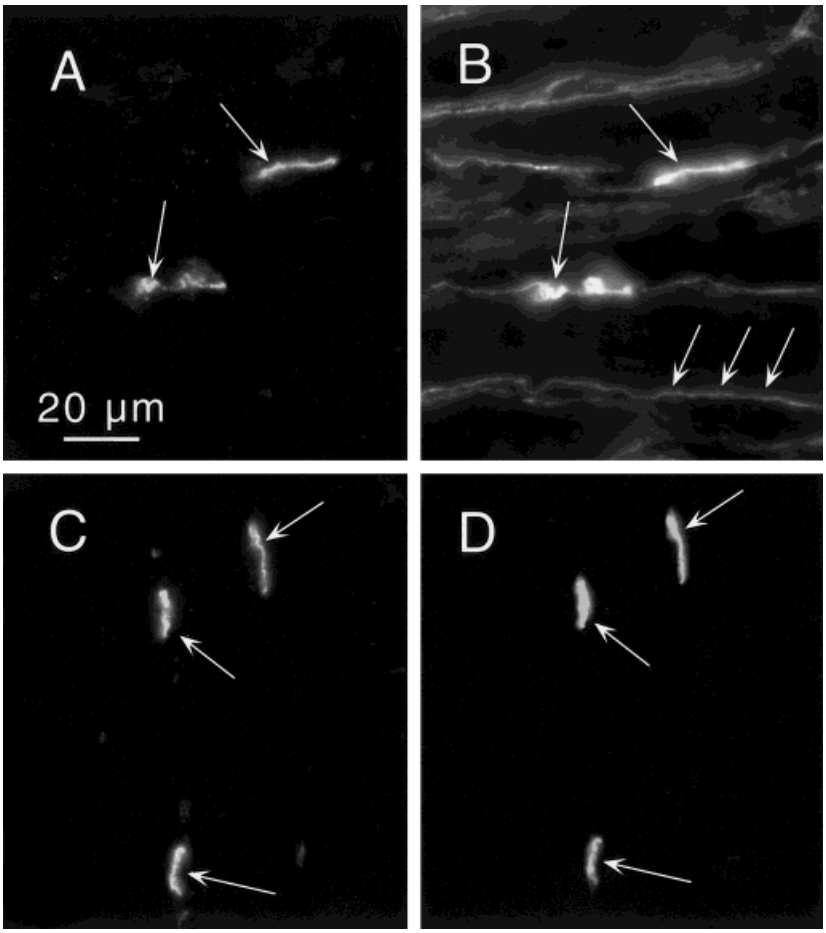

Fig. 4. Fluorescent micrographs of immunohistochemistry on rat diaphragm muscle. Sections were stained with affinity column-purified antibodies raised in rabbit against UT11. Double immunofluorescence labeling with anti-UT11 (**A**) and with a monoclonal antibody (mAb) NCL-DYS1 against the rod domain of dystrophin (**B**). **A:** Anti-UT11 only stains the neuromuscular junction (NMJ). **B:** The mAb NCL-DYS1 stains the NMJ and along the sarcolemma (triple arrow). Double immunofluorescence labeling with anti-UT11 (**C**) and with α-bungarotoxin-rhodamine (**D**). anti-UT11 (**C**) and α-bungarotoxin-rhodamine (**D**) stain only the NMJ. anti-UT11 colocalises with both the monoclonal Ab NCL-DYS1 and α-bungarotoxin at the NMJ as indicated by directionally paired arrows.

Identification of the N-Utrophin Message and Protein in C6 Glioma Cells

The recombinant fragments (UT11, UT31, and UT43) are schematically represented in Figure 2 and were used for antisera production. As an example, stimulation of expression of UT11 in *E. coli* and its purification is shown in Figure 3. All antisera raised both in rabbit and guinea pig against UT11 (anti-UT11), UT31 (anti-UT31) and UT43 (anti-UT43) proved specific for utrophin in immunohistochemical stainings of rat diaphragm muscle sections (Fig. 4). The antisera were found to stain the NMJ, but not the sarcolemma. In double immunofluorescent labeling, the utrophin staining co-localises in the NMJ both with that of a mAb against the dystrophin rod domain (NCL-DYS1) and with that of α-bungarotoxin. We thus have available antisera specific for the N-terminus, the rod domain, and the C-terminal region of utrophin that do not cross-react with dystrophin.

All three antisera (anti-UT11, anti-UT31 and anti-UT43) recognise the full-length utrophin in Western blots of rat C6 glioma cell lysates. In addition, anti-UT11 recognises a protein of 62 kDa that may represent N-utro (Fig. 5). UT11 has a somewhat higher calculated molecular mass of 71′084 Da than N-utro and, therefore, migrates slowlier in SDS-PAGE. By contrast, anti-UT31 specific for the rod domain recognises only the full-length utrophin, but not the putative N-utro isoform. The UT31 protein fragment with a calculated molecular mass of 41′684 Da migrates in SDS-PAGE at around 45 kDa. The recognition of the full-length utrophin by anti-UT11 and anti-UT31 is confirmed by the monoclonal Ab (NCL-DRP1) against the C-terminus of utrophin (Fig. 5, lane b). A C-terminal peptide corresponding to the short G-utrophin isoform could not be detected in rat C6 glioma cells with anti-UT43 raised in guinea pig (Fig. 5, lane a).

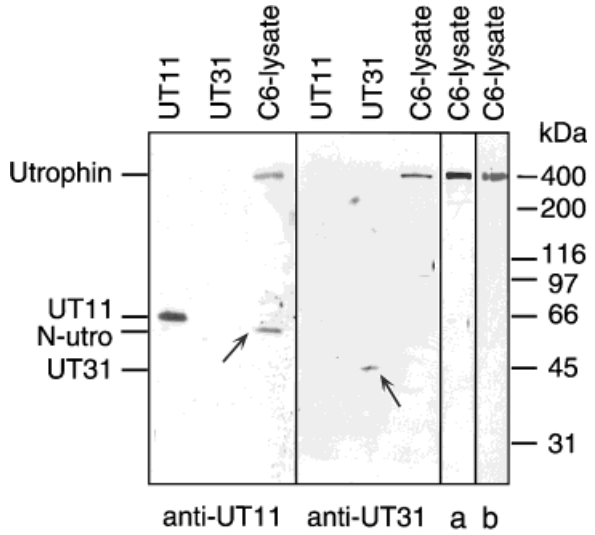

Fig. 5. Western blots of recombinant utrophin fragments and C6 glioma cell lysates. Western blots of recombinant UT11 (fragment corresponding to the N-utrophin isoform), UT31 (from the rod domain), and rat C6 glioma cell lysate stained with anti-UT11 and anti-UT31 both raised in rabbit and affinity column purified. The last two lanes show staining of the full-length utrophin with (**a**) anti-UT43 raised in guinea-pig and (**b**) a monoclonal Ab (NCL-DRP1) against the last 11 residues of utrophin. Chemiluminescent detection after blotting from 4–15% gradient SDS-PAGE. Anti-UT11 recognises its antigen, both full-length utrophin, and the N-utrophin isoform (N-utro) in the C6 cell lysate (arrow, slightly faster migrating than UT11), but not UT31. Anti-UT31 recognises its antigen (arrow) and full-length utrophin in the C6 cell lysate, but neither UT11 nor N-utro.

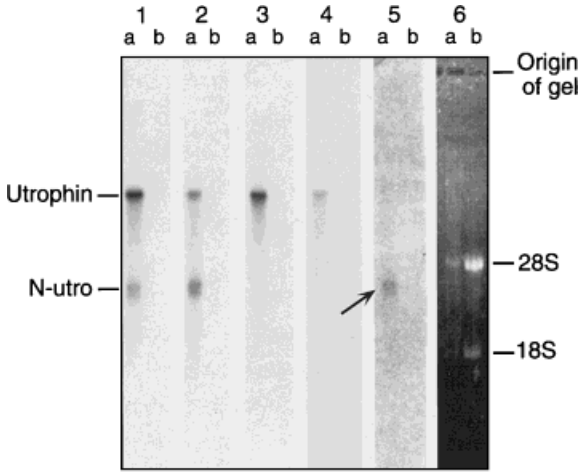

Fig. 6. Northern blots of RNA from rat C6 glioma cells. Indicated are the signals for the full-length utrophin message of 13,000 nt, the postulated N-terminal utrophin isoform (N-utro) of 3700 nt, the 28S rRNA and the 18S rRNA. **a:** 3 μg poly(A)$^+$ RNA. **b:** 3 μg poly(A)$^-$ RNA. **Lanes 1–5,** autoradiograms after hybridisation. **Lanes 1,3,5,** capillary blots; **lanes 2,4,** pressure blots; **lanes 1,2,** RNA probed with α207EB0.9 specific for the N-terminal domain; **lanes 3,4,** RNA probed with α213H0.9 specific for the rod domain; **lane 5,** RNA probed with α201S0.2 specific for the 3′-UTR of N-utro (arrow) exposed for 144 h instead of only 24 h as in **lanes 1–4; lane 6,** photograph of ethidium bromide-stained RNA gel before blotting for hybridisation (**lane 1**).

Northern blots of C6 cell RNA probed with α-207EB0.9 (nt 21–872) from the 5′-end of utrophin (Fig. 1) demonstrate the signal of the full-length species with around 13′000 nt as well as the signal for the smaller N-utro of around 3700 nt (Fig. 6, lanes 1 and 2). Only the full-length utrophin RNA is detected by the probe α-213H0.9 (nt 1813–2693) (Fig. 6, lanes 3 and 4). This latter probe covers a region in the utrophin RNA coding for the rod domain, immediately downstream of the homology breakdown to N-utro (Fig. 1). The signal for the short N-utro isoform exhibits similar intensity in the two blotting methods employed. By contrast, the signal for the full-length utrophin is significantly reduced in the pressure blotting, as compared with the capillary blotting system (Fig. 6, lanes 2 and 4). In both methods, the N-utro signal appears to contain an additional, slightly faster migrating band of around 3400 nt. Most likely, this may indicate the occurrence of two transcripts for N-utro with different length in the 3′-UTR region. The probe α-201S0.2 (nt 1902–2112) specific for a stretch located within the 3′-UTR of the N-utro clone reveals only the short transcript (Fig. 6, arrow). As mentioned above, the entire 3′-UTR of the short transcript shares no homology with the full-length sequence. As this latter probe is about four times shorter than α-207EB0.9 from the 5′-end, it binds less efficiently to its target and contains less radioactively labeled nucleotides. Therefore, blots for detection of the short N-utro transcript needed longer exposure times. The fact that both probes, one from the 5′-end and the other from the 3′-end, recognise the same short N-utro transcript confirms the existence of this message in C6 glioma cells. It is therefore highly unlikely that the α-201 clone resulted from ligation of two different cDNA fragments into one vector. Taken together, the existence of a short N-utrophin isoform in C6 glioma cells is evidenced on the protein and message level.

Localisation of Utrophin in C6 Glioma Cells

No data are available on subcellular localisation of utrophin in rat C6 glioma cells. We

examined cells grown for 24 h on polystyrene dishes and stained before forming a confluent layer by confocal laser scanning microscopy after double staining for F-actin and for utrophin, with either anti-UT11 against the N-terminus or anti-UT31 against the rod domain (Fig. 7). No staining was obtained with anti-UT43 against the C-terminal fragment. Positive immunoreactivity for dystrophin was never seen in these cells.

Most cells exhibit a spindle-like shape with few pseudopodia. At this stage, their length varies around 40–80 μm with a height of 6–8 μm. Optical sectioning by confocal microscopy indicated that the actin staining follows the membrane around the entire cell. Utrophin staining follows rather closely the membrane lining. Some cells spread and grow out radially on the substratum. In such cases, the staining for utrophin follows the entire cortical actin layer (Fig. 7C). Staining for utrophin with either anti-UT11 or anti-UT31 displays very similar patterns, marking the membranes around the cell preferentially besides some diffuse staining throughout the cytoplasm (Fig. 7F,J). Some stress fibers can usually be found at the bottom of the cells near the substratum. The stress fibers were never stained, however. It was not possible at this level to discern a distinct localisation of the staining with either anti-UT11 or anti-UT31. A distinct staining pattern would indicate a differential localisation of the N-utro isoform from that of the full-length protein. Specific localisation of N-utro could thus not be shown by immunocytochemistry.

Actin Binding

Actin binding was assessed by high-speed cosedimentation assays for the recombinant UT11 (2–594), corresponding to N-utro. It carries a poly-His-tag ($MRGSH_6$) at its N-terminus and is 55 residues longer than N-utro, reaching into the third spectrin-like repeat (Fig. 2). Binding data were obtained by densitometric evaluation of SDS-PAGE gels and plotted against the concentration of free UT11 (Fig. 8). A small amount of nonpolymerised actin, never exceeding 2% of the total, was usually found in the supernatant. As a control for a protein that is not expected to bind to F-actin, BSA with a molecular mass of about 66 kDa was carried along with all sedimentation tests in the same molar concentration as the recombinant protein. Binding to F-actin was never observed. The binding data indicate that the UT11 construct binds to rabbit skeletal muscle F-actin with an overall equilibrium constant (affinity) K value of $4.5 \times 10^5\ M^{-1}$ with a correlation coefficient $r = 0.94$ and a saturation of one per 5 actin monomers. For comparison the same type of binding tests was performed with UT12 (2–261) and DYS12 (1–246). UT12 and DYS12 bind with affinities of $3.2 \times 10^5\ M^{-1}$ ($r = 0.97$) and $9.2 \times 10^4\ M^{-1}$ ($r = 0.98$), respectively, as determined from three independent experiments each. The corresponding stoichiometries are one UT12 per 1.3 actin and 1.9 DYS12 per one actin monomer.

DISCUSSION

We provide evidence for the existence of the message and protein of a new short N-terminal utrophin isoform (N-utro) in C6 glioma cells. This isoform comprises the actin binding domain and the first two spectrin-like repeats. In addition, we show that the recombinant utrophin fragment (UT11) that corresponds to N-utro binds to actin in a saturable manner.

Two independent clones encoding N-utro were found. They start at different positions in the 5′-UTR and have different lengths in the 3′-UTR. The two clones are otherwise identical in sequence, except for one nucleotide exchange. This may either have arisen from an error during cDNA construction, as mentioned under Results, or it may indicate an allelic difference. If the latter were the case, it would still have no effect on the protein sequence, as it resides in the 5′-UTR. Both the different lengths of the two clones, as well as the single mutation, indicate that they must have arisen from two different mRNA molecules. They cannot result from ligation of two different cDNA into the same vector.

The divergent sequence at the 3′-end of the clones appears to originate from an intron. It starts with GT, which marks the beginning of introns [Padgett et al., 1986]. Furthermore, this GT is indeed embedded in a sequence (AGGTATGA) that resembles closely the consensus sequence (AGGTAAGT) for a splice-donor site. So the last residue of N-utro (Val-539) and the following stop signal are encoded by the first two triplets at the beginning of the nonspliced intron. It is worth noting that the exon-intron boundary described here corresponds by ho-

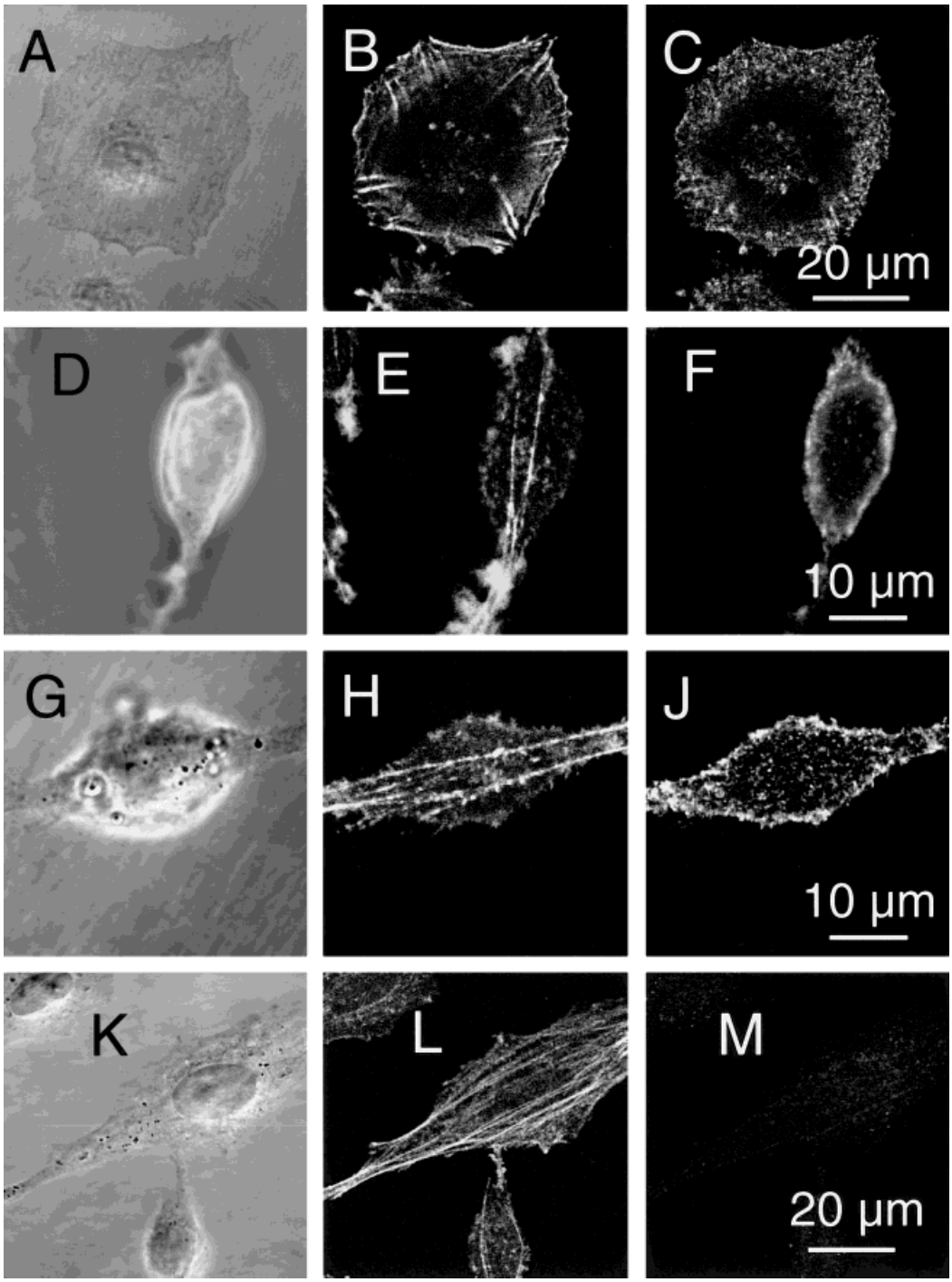

Fig. 7. Confocal micrographs of rat C6 glioma cells in culture. Double immunofluorescent labeling with phalloidin-Oregon Green for F-actin (**B,E,H**) and polyclonal Ab against UT11 (**C,F**) or anti-UT31 (**J**) for utrophin. **A,D,G,K:** Corresponding phase-contrast pictures. For control, in one case of double stainings for F-actin (**L**) and utrophin, labeling of the anti-UT11 polyclonal antibodies was competed by the antigen (**M**). Confocal layer was at 2.0 μm from the bottom of the cells. In all three cases (**C,F,J**), the actin stress fibers are not stained by the polyclonal Ab against utrophin. Scale bar = 10 μm (**D–J**); Scale bar = 20 μm (**A–C,K–M**).

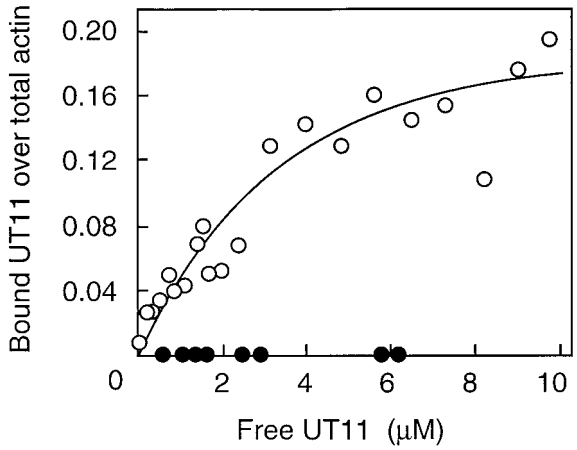

Fig. 8. Binding of the recombinant fragment corresponding to N-utrophin (UT11) to actin. The binding data are compiled from four high-speed cosedimentation experiments and show saturation at around 0.2 ligands per actin monomer with an affinity K value of 4.5×10^5 M^{-1} and a correlation coefficient r of 0.94 (open symbols). For control (closed symbols) bovine serum albumin does not bind to actin under identical assay conditions.

mology to that at the end of exon-14 in human dystrophin [Roberts et al., 1993]. Although the utrophin gene structure is not known in detail, it is believed to share similarity to that of dystrophin [Pearce et al., 1993; Blake et al., 1995].

Our results indicate the existence of two transcripts with different lengths (around 3400 and 3700 nt) coding for N-utro. The probe α-213H0.9 does not detect these two transcripts. This probe covers the utrophin coding sequence homologous to exons 15–20 of dystrophin. The probe α-201S0.2 from the 3′-UTR of N-utro recognises only the two short transcripts that code for N-utro comprising the dystrophin homolog exons 1–14. Thus their 3′-UTR sequences of 1600 and 1900 nt are derived from the subsequent intron and do not contain further coding regions for utrophin. They must therefore originate from different adenylation sites within the dystrophin homologue intron-14. The generation of N-terminal short isoforms by use of intronic polyadenylation and termination signals has been described for several other proteins, such as rat luteinizing hormone/chorionic gonadotropin receptor [Koo et al., 1994], neurofibromatosis type 1 protein [Suzuki et al., 1992], immunoglobulin-ϵ [Anand et al., 1997], and the calcitonin/calcitonin gene-related peptide [Lou and Gagel, 1998].

Both two transcripts code for the same N-utro protein. This short N-terminal isoform comprises the actin binding domain, the first two spectrin-like repeats, and 13 residues of repeat-3. It is identical in sequence to utrophin, except for the last residue-539, where Val replaces Cys. The proper actin binding region (aa 1–261) consists of two calponin homology domains (CH1 and CH2) in tandem [Carugo et al., 1997]. In rat utrophin, CH1 and CH2 extend over residues 31–140 and 150–258, respectively. This region is conserved in the protein family, which, in addition to utrophin includes dystrophin, β-spectrin, α-actinin, plectin, cortexillin, filamin, and fimbrin [Stradal et al., 1998].

We show that UT11 comprising $2\frac{1}{2}$ spectrin-like repeats (corresponding to N-utro) and UT12 with the actin binding domain alone bind with very similar equilibrium constant K values of around 4×10^5 M^{-1}. The difference in the binding stoichiometry suggests that the additional two and a half spectrin-like repeats in UT11 could be responsible for the more widely spaced distribution along the actin filament. Our DYS12 fragment (1–246), which is homologous to UT12, again, binds to F-actin with an affinity similar to UT11 and UT12. The binding affinity of our DYS12 with a His-tag at its N-terminus is almost identical to that of a corresponding dystrophin fragment (1–246) with a K value of 7.3×10^4 M^{-1} carrying the His-tag at the C-terminus [Renley et al., 1998]. The affinity of 2.3×10^4 M^{-1} of an untagged recombinant dystrophin fragment (1–246) is in the same range [Way et al., 1992]. A somewhat lower affinity of 5.3×10^4 M^{-1} [Winder et al., 1995] has been reported for an untagged utrophin fragment (1–261) than the value reported in this article (3.2×10^5 M^{-1}). Taken together, the affinities for UT12 and DYS12 we report here and those from the literature for corresponding fragments all fall within a rather narrow range. We therefore conclude that the His-tag on the recombinant fragment does not influence the binding to F-actin whether attached to the N-terminus or the C-terminus. By contrast, binding affinities of at least two orders of magnitude higher were obtained for similar dystrophin fragments, using solid-phase immunoassays [Corrado et al., 1994; Jarrett and Foster, 1995]. This latter discrepancy appears to originate from the different

methodological approaches [Rybakova et al., 1996].

Subcellular localisation of utrophin in C6 glioma cells has not been reported thus far. Its association with the cell membrane has only been inferred from subcellular fractionation studies using detergents [Nguyen thi Man et al., 1992]. Anti-UT11 against the N-terminus and anti-UT31 against the rod stain utrophin along the membranes, as well as the submembraneous cortical actin layer in C6 glioma cells. The stress fibers that stain for actin do not show utrophin immunolabeling. It is surprising that anti-UT43 against the C-terminal region does not stain the full-length utrophin in C6 cells while in muscle utrophin is detected at the NMJ with all three polyclonal Ab. This suggests that the epitopes of the C-terminal domains are not accessible in the C6 cells. Since anti-UT43 did neither detect any bands smaller than 400 kDa in Western blots, we conclude that no C-terminal short isoforms are present in C6 cells. Immunoreactive bands smaller than 400 kDa have, however, been described in Western blots with Ab against the C-terminal utrophin domain in C6 cells [Nguyen thi Man et al., 1992] as well as in several animal tissues [Lumeng et al., 1999]. In the first case it was suggested that these bands may represent degradation products of the full-length species, and in the second case, that they may have arisen from multiple start codons within the 5.5 kb transcript for G-utrophin.

In the C6 cells no different staining pattern was visible between anti-UT11 (against the N-terminus) and anti-UT31 (against the rod). This means that a differential staining for N-utro is not detectable. So in C6 cells it must colocalise with full-length utrophin or its level is too low for detection. In the Northern blots, the N-utro mRNA seems to be around 10 times less abundant than the full-length message. Nevertheless, almost equal amounts of protein of N-utro and full-length utrophin are apparent in Western blots (Fig. 5) on detection with anti-UT11. A firm estimate as to their relative amounts cannot be given, however, since the larger of the two proteins may not blot as efficiently as the smaller one. The recombinant utrophin fragment aa 1–261 (comprising the actin binding domain alone) fused to glutathione S-transferase associated with actin stress fibers when microinjected into fibroblasts [Winder et al., 1995]. On the other hand, the N-terminal dystrophin fragment aa 1–713 containing the first three spectrin-like repeats sorted to the sarcolemma after expression in cultured mdx myotubes or in mdx muscle fibers in vivo [Dunckley et al., 1994]. This latter finding agrees with in vitro studies showing that the recombinant spectrin-like repeat-2 of dystrophin interacts tightly with membrane phosphatidylserine provided it is properly folded [Dewolf et al., 1997]. The second spectrin-like repeat of utrophin is highly homologous to that of dystrophin both having a pI value of 4.5 and 9 (rat utrophin) or 10 (human dystrophin) negative charges. It may thus be speculated that the corresponding spectrin-like repeat of utrophin binds also to the cell membrane as that in dystrophin.

Taken together, these findings indicate that the truncated N-terminal species of utrophin comprising the actin binding domain plus two or more spectrin-like repeats may be able to interact simultaneously with the cytoskeletal actin and the cell membrane moiety. The N-utro isoform whose transcript and actin binding properties are described here for the first time seems to fulfill this requirement and may thus serve a specific function. Preliminary results with RT-PCR indicate the existence of such a transcript in adult rat kidney and lung tissues. In addition, we detected a protein of corresponding size with N-terminal utrophin antibodies in Western blots of rat kidneys. A comparable dystrophin isoform has not been detected so far. We are currently searching for the N-terminal utrophin isoform in various other vertebrate tissues as well as during development. Its expression pattern could be indicative of its putative function.

ACKNOWLEDGMENTS

The authors thank Dr. D.J. Blake for making available the C6 glioma cell cDNA library and Professor K.E. Davies for initial help in this work as well as Frédérique Rebetez for skilled technical assistance. The work was supported in part by grants to M.C. Schaub from the Roche Research Foundation (94-107), the Swiss Foundation for Research on Muscle Diseases (1995), and the Hartmann Mueller Foundation for Medical Research (557).

REFERENCES

Anand S, Batista FD, Tkach T, Efremov DG, Burrone OR. 1997. Multiple transcripts of the murine immunoglobulin epsilon membrane locus are generated by alternative splicing and differential usage of two polyadenylation splicing and differential usage of two polyadenylation sites. Mol Immunol 34:175–183.

Berthier C, Blaineau S. 1997. Supramolecular organization of the subsarcolemmal cytoskeleton of adult skeletal muscle fibers—a review. Biol Cell 89:413–434.

Blake DJ, Schofield JN, Zuellig RA, Gorecki DC, Phelps SR, Barnard EA, Edwards YH, Davies KE. 1995. G-utrophin, the autosomal homologue of dystrophin Dp116, is expressed in sensory ganglia and brain. Proc Natl Acad Sci USA 92:3697–3701.

Blake DJ, Nawrotzki R, Peters MF, Froehner SC, Davies KE. 1996. Isoform diversity of dystrobrevin, the murine 87-kDa postsynaptic protein. J Biol Chem 271:7802–7810.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brooks SV. 1998. Rapid recovery following contraction-induced injury to in situ skeletal muscles in mdx mice. J Muscle Res Cell Motil 19:179–187.

Campanelli JT, Roberds SL, Campbell KP, Scheller RH. 1994. A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. Cell 77:663–674.

Carugo KD, Banuelos S, Saraste M. 1997. Crystal structure of a calponin homology domain. Nature Struct Biol 4:175–179.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Clerk A, Morris GE, Dubowitz V, Davies KE, Sewry CA. 1993. Dystrophin-related protein, utrophin, in normal and dystrophic human fetal skeletal muscle. Histochem J 25:554–561.

Corrado K, Mills PL, Chamberlain JS. 1994. Deletion analysis of the dystrophin actin binding domain. FEBS Lett 344:255–260.

Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, Watt DJ, Dickson JG, Tinsley JM, Davies KE. 1997. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell 90:717–727.

Dewolf C, McCauley P, Sikorski AF, Winlove CP, Bailey AI, Kahana E, Pinder JC, Gratzer WB. 1997. Interaction of dystrophin fragments with model membranes. Biophys J 72:2599–2604.

Dunckley MG, Wells KE, Piper TA, Wells DJ, Dickson G. 1994. Independent localization of dystrophin N- and C-terminal regions to the sarcolemma of mdx mouse myofibres in vivo. J Cell Sci 107:1469–1475.

Han JH, Stratowa C, Rutter WJ. 1987. Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA cloning. Biochemistry 26:1617–1625.

Hori S, Ohtani S, Nguyen thi Man, Morris GE. 1995. The N-terminal half of dystrophin is protected from proteolysis in situ. Biochem Biophys Res Commun 209:1062–1067.

Howard PL, Klamut HJ, Ray PN. 1998. Identification of a novel actin binding site within the Dp71 dystrophin isoform. FEBS Lett 441:337–341.

Jarrett HW, Foster JL. 1995. Alternate binding of actin and calmodulin to multiple sites on dystrophin. J Biol Chem 270:5578–5586.

Karpati G, Carpenter S, Morris GE, Davies KE, Guerin C, Holland P. 1993. Localization and quantitation of the chromosome 6-encoded dystrophin-related protein in normal and pathological human muscle. J Neuropathol Exp Neurol 52:119–128.

Koo YB, Ji I, Ji TH. 1994. Characterization of different sizes of rat luteinizing hormone/chorionic gonadotropin receptor messenger ribonucleic acids. Endocrinology 134:19–26.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee CC, Pons F, Jones PG, Bies RD, Schlang AM, Leger JJ, Caskey CT. 1993. Mdx transgenic mouse: restoration of recombinant dystrophin to the dystrophic muscle. Hum Gene Ther 4:273–281.

Leijendekker WJ, Passaquin AC, Metzinger L, Ruegg UT. 1996. Regulation of cytosolic Calcium in skeletal muscle cells of the mdx mouse under conditions of stress. Br J Pharmacol 118:611–616.

Lou H, Gagel RF. 1998. Alternative RNA processing—its role in regulating expression of calcitonin/calcitonin gene-related peptide. J Endocrinol 156:401–405.

Lumeng CN, Phelps SF, Rafael JA, Cox GA, Hutchinson TL, Begy CR, Adkins E, Wiltshire R, Chamberlain JS. 1999. Characterization of dystrophin and utrophin diversity in the mouse. Hum Mol Genet 8:593–599.

Nguyen thi Man, Le TT, Blake DJ, Davies KE, Morris GE. 1992. Utrophin, the autosomal homologue of dystrophin, is widely-expressed and membrane-associated in cultured cell lines. FEBS Lett 313:19–22.

Nguyen thi Man, Helliwell TR, Simmons C, Winder SJ, Kendrick-Jones J, Davies KE, Morris GE. 1995. Full-length and short forms of utrophin, the dystrophin-related protein. FEBS Lett 358:262–266.

Padgett RA, Grabowski PJ, Konarska MM, Seiler S, Sharp PA. 1986. Splicing of messenger RNA precursors. Annu Rev Biochem 55:1119–1150.

Pardee JD, Spudich JA. 1982. Purification of muscle actin. Methods Enzymol 85:164–181.

Pasternak C, Wong S, Elson EL. 1995. Mechanical function of dystrophin in muscle cells. J Cell Biol 128:355–361.

Pearce M, Blake DJ, Tinsley JM, Byth BC, Campbell L, Monaco AP, Davies KE. 1993. The utrophin and dystrophin genes share similarities in genomic structure. Hum Mol Genet 2:1765–1772.

Pons F, Robert A, Fabbrizio E, Hugon G, Califano JC, Fehrentz JA, Martinez J, Mornet D. 1994. Utrophin localization in normal and dystrophin-deficient heart. Circulation 90:369–374.

Renley BA, Rybakova IN, Amann KJ, Ervasti JM. 1998. Dystrophin binding to nonmuscle actin. Cell Motil Cytoskeleton 41:264–270.

Rivier F, Robert A, Hugon G, Mornet D. 1997. Different utrophin and dystrophin properties related to their vascular smooth muscle distributions. FEBS Lett 408:94–98.

Roberts RG, Coffey AJ, Bobrow M, Bentley DR. 1993. Exon structure of the human dystrophin gene. Genomics 16: 536–538.

Rybakova IN, Amann KJ, Ervasti JM. 1996. A new model for the interaction of dystrophin with F-actin. J Cell Biol 135:661–672.

Sadoulet-Puccio HM, Kunkel LM. 1996. Dystrophin and its isoforms. Brain Pathol 6:25–35.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Stradal T, Kranewitter W, Winder SJ, Gimona M. 1998. CH domains revisited. FEBS Lett 431:134–137.

Suzuki A, Yoshida M, Yamamoto H, Ozawa E. 1992. Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain. FEBS Lett 308:154–160.

Tinsley JM, Blake DJ, Roche A, Fairbrother U, Riss J, Byth BC, Knight AE, Kendrick-Jones J, Suthers GK, Love DR, Edwards YH, Davies KE. 1992. Primary structure of dystrophin-related protein. Nature 360:591–593.

Tinsley JM, Blake DJ, Zuellig RA, Davies KE. 1994. Increasing complexity of the dystrophin-associated protein complex. Proc Natl Acad Sci USA 91:8307–8313.

Tinsley JM, Potter AC, Phelps SR, Fisher R, Trickett JI, Davies KE. 1996. Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature 384:349–353.

Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, Gillis JM, Davies KE. 1998. Expression of full-length utrophin prevents muscular dystrophy in mdx mice. Nature Med 4:1441–1444.

Vater R, Young C, Anderson LVB, Lindsay S, Blake DJ, Davies KE, Zuellig R, Slater CR. 1998. Utrophin mRNA expression in muscle is not restricted to the neuromuscular junction. Mol Cell Neurosci 10:229–242.

Way M, Pope B, Cross RA, Kendrick-Jones J, Weeds AG. 1992. Expression of the N-terminal domain of dystrophin in *E. coli* and demonstration of binding to F-actin. FEBS Lett 301:243–245.

Wilson J, Putt W, Jimenez C, Edwards YH. 1999. Up71 and Up140, two novel transcripts of utrophin that are homologues of short forms of dystrophin. Hum Mol Genet 8:1271–1278.

Winder SJ. 1997. The membrane-cytoskeleton interface—the role of dystrophin and utrophin. J Muscle Res Cell Motil 18:617–629.

Winder SJ, Hemmings L, Maciver SK, Bolton SJ, Tinsley JM, Davies KE, Critchley DR, Kendrick-Jones J. 1995. Utrophin actin binding domain: analysis of actin binding and cellular targeting. J Cell Sci 108:63–71.