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Utrophin, the autosomal homologue of dystrophin, is widely-expressed and membrane-associated in cultured cell lines

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Utrophin, the autosomal dystrophin-related protein (DRP), is expressed in HeLa cells, smooth muscle-like BC_3H1 cells from mouse brain, COS monkey kidney cells, the $P388D_1$ monocyte-macrophage cell line and untransformed human skin fibroblasts, as well as in rat C6 glioma and Schwannoma cells. It was undetectable, however, in the Sp2/O mouse myeloma cell line and in hybridoma lines derived from it. Dystrophin was not detected in any of these cell lines. Although all utrophin-containing cells were capable of forming monolayers in culture, no major effects of either attachment to substratum or length of time in culture (2–17 days) on utrophin levels were observed. After subcellular fractionation of BC_3H1 or glioma cells, nearly all of the utrophin was found in the Triton-soluble fraction, suggesting an association with cell membranes.

Dystrophin; Dystrophin-related protein; Muscular dystrophy; DMDL gene; Membrane-associated protein

1. INTRODUCTION

Utrophin (dystrophin-related protein, DMDL protein; DRP) [1–4] is an autosomal homologue of dystrophin, the protein affected by mutation in Duchenne and Becker muscular dystrophies [5]. Unlike dystrophin which is mainly restricted to muscle, utrophin has been found in all tissues examined, particularly in nerve fibres, smooth muscle (especially that of the vascular system) and blood vessels. In adult skeletal muscle, it is restricted to neuromuscular junctions [4,6], but is more generally distributed in the sarcolemma in foetal muscle [2], in regenerating muscle fibres [7] and in the majority of fibres from patients with muscular dystrophies [4,8] or inflammatory myopathies [7]. The protein is also abundant in transformed brain cell lines, C6 glioma and SWA Schwannoma [4].

Although utrophin is found in tissues such as lung, liver, kidney and spleen [3], its localization has not been reported and the possibility remains that it could be restricted to the nerve and smooth muscle components of these tissues. We now show, however, that utrophin is present in many cell types in culture, including six tumour cell lines of different origins and untransformed human skin fibroblasts. The results raise the question of whether dystrophin can be reasonably regarded as a specialized muscle/nerve isoform of utrophin.

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2. MATERIALS AND METHODS

2.1. *Cell lines*

Human skin fibroblasts after several passages in culture were kindly provided by Dr. John Butterworth (Royal Hospital for Sick Children, Edinburgh). The Sp2/O mouse myeloma cell line is our standard cell line for hybridoma production and the MANDYS1 hybridoma producing antibody against dystrophin has been described previously [9]. The SWA rat Schwannoma cell line [10] was provided by Dr. George Dickson (Guy's Hospital Medical School, London). All other cell lines are available from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and the American Type Culture Collection (Rockville, MD, USA). COS-1 are SV40-transformed African Green monkey fibroblast-like kidney cells (ATCC CRL 1650), $P388D_1$ are monocyte-macrophage cells from a mouse lymphoid neoplasm (ATCC TIB 63), BC_3H1 are smooth muscle-like cells obtained from a mouse brain tumour (ATCC CRL 1443), HeLa S3 are epithelial-like cells from a human cervical carcinoma (ATCC CCL 2.2) and C6 are glial cells from a rat glial tumour (ATCC CCL 107). All cells were grown in DMEM + 10% selected horse serum, except that 0.1 mM $ZnCl_2$ was added to SWA cultures [10] and DMEM was replaced by Joklik's MEM for HeLa suspension cultures.

2.2. *Western blots*

Cultures cells were harvested in PBS and cell pellets were boiled in 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol and 5% sucrose (4 ml/ml of cell pellet) and centrifuged at 13,000 rpm in an Eppendorf centrifuge for 10 min. Extracts (10 μl) were loaded onto 4–12.5% gradient gels with a 4% stacking gel, together with 5 μl of prestained molecular weight markers (Sigma; 180, 116, 84, 58, 48.5, 36.5 and 26.6 kDa; 1 mg/ml of each). After electrophoresis, proteins were transferred electrophoretically (BioRad Transblot) to nitrocellulose sheets (Schleicher and Schuel, BA85) at 100 mA overnight in 25 mM Tris, 192 mM glycine, 0.003% SDS.

Blots were blocked in 3% skimmed milk powder in incubation buffer (0.05% Triton X-100 in phosphate-buffered saline (PBS: 25 mM sodium phosphate, pH 7.2, 0.9% NaCl). After two 5-min washes in PBS, blots were incubated with monoclonal antibody culture supernatant (1/100 dilution in incubation buffer/1% horse serum/1% foetal calf serum/0.3% bovine serum albumin) for 1 h at 20°C. After three 5-min

washes with PBS, blots were incubated with biotinylated anti-(mouse Ig) and a peroxidase-avidin detection reagent (Vectastain ABC kit), according to the manufacturer's instructions (Vector Laboratories). After four 5-min washes with PBS, substrate was added (0.4 mg/ml diaminobenzidine (Sigma) in 25 mM phosphate-citrate buffer, pH 5.0, with 0.012% H_2O_2.

2.3. *Cell fractionation*

Monolayer cultures of BC_3H1 cells (five 90 mm Petri dishes) were rinsed with PBS and thoroughly drained before harvesting sequentially with the same 0.4 ml of 10 mM Tris-HCl, pH 6.7, using a rubber policeman. The cell suspension was placed in a 1.5 ml microcentrifuge tube and cooled to 4°C before sonicating with a microprobe (2 mm tip) at 22 μm amplitude. Centrifugation was performed in a Beckmann 50Ti rotor at 40,000 rpm and 4°C. The pellet was resuspended in 0.4 ml of 1% Triton X-100 in 10 mM Tris-HCl, pH 6.7, and centrifuged again. Fractions were adjusted to 50 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol and 5% sucrose in the same final volume (ca. 0.3 ml recovered) before boiling for 2 min.

3. RESULTS

In Fig. 1a, utrophin from rat glioma cells is shown co-migrating with mouse muscle dystrophin at 400 kDa as previously described [4], using a monoclonal antibody which reacts with both proteins (MANCHO11). The HeLa cell extract also shows a band of utrophin at 400 kDa (H: Fig. 1a) which is detected by mAbs against several different epitopes on utrophin but is not detected by dystrophin-specific antibodies ([4] and data not shown). Fig. 1b shows that utrophin is present in HeLa cells grown both in suspension culture and on tissue culture plastic. In the latter case, utrophin levels were similar in firmly-attached cells (Ha4) and floating cells (Hs4) from the same 4-day culture and in confluent 17-day attached cultures (Fig. 1b). Similarly, no clear change in utrophin levels could be seen as C6 glioma cells grew to confluency between 2 and 4 days in culture, though this method would not reliably detect small changes (Fig. 1c).

Fig. 2 shows that utrophin-specific mAbs detect the 400 kDa band in COS monkey kidney cells, $P388D_1$ macrophage-like cells and human skin fibroblasts as well as in glioma and Schwannoma cells, but not in either Sp2/O myeloma cells or a hybridoma cell line derived from a fusion between Sp2/O and mouse spleen cells. This last extract contains large amounts of mouse Ig (mAb MANDYS1) which explains the major bands of 50 kDa protein (H-chain) and aggregated material on the Western blot. The band at about 52 kDa present in most extracts is a non-specific cross-reaction of the second antibody detection system. The large number of bands between 60 kDa and 180 kDa is striking in the glioma and Schwannoma extracts (Fig. 2 and 1c). These appear to be utrophin-related bands rather than cross-reactive bands, since they are recognized by mAbs against several different utrophin epitopes (data not shown). Degradation cannot be ruled out at this stage, though there is no apparent reason why degradation should be greater in these two cell lines than in others.

Fig. 3 shows that utrophin is also found in the BC_3H1 smooth muscle-like cell line and that it is not present in the soluble cytoplasmic fraction of these cells, but is almost completely extracted from the insoluble pellet by the non-ionic detergent, Triton X-100. Dystrophin is known to be membrane-associated and is extracted from muscle in the same way [11]. This suggests that utrophin is also associated in some way with membranes, especially since two integral membrane proteins we have examined, N-CAM and the 43 kDa dystroglycan component [12], are also found exclusively in the

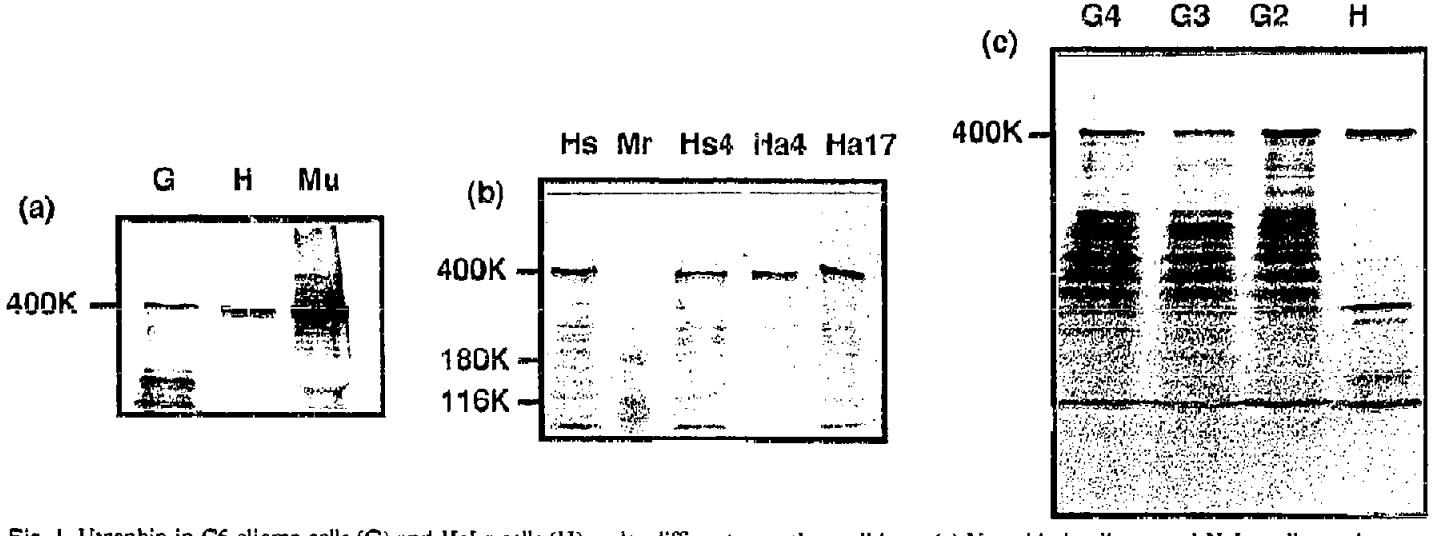

Fig. 1. Utrophin in C6 glioma cells (G) and HeLa cells (H) under different growth conditions. (a) Utrophin in glioma and HeLa cells comigrates with dystrophin from mouse muscle (Mu). The mAb used, MANCHO11, reacts with both proteins. (b) Utrophin levels are similar in HeLa cells maintained in suspension culture in Joklik's MEM (Hs), HeLa cells attached to tissue culture flasks after 4 days (Ha4) or 17 days (Ha17) growth in Dulbecco's MEM and cells from the same 4 day culture which were unattached to the substratum ('floaters', Hs4). The M_r markers visible are 116 kDa and 180 kDa and the mAb was MANCHO11. (c) No detectable change in utrophin levels in C6 glioma cells cultured for 2 days (G2), 3 days (G3) or 4 days (G4). The mAb was utrophin-specific, MANCHO3, and HeLa cell extract is shown as a control (H).

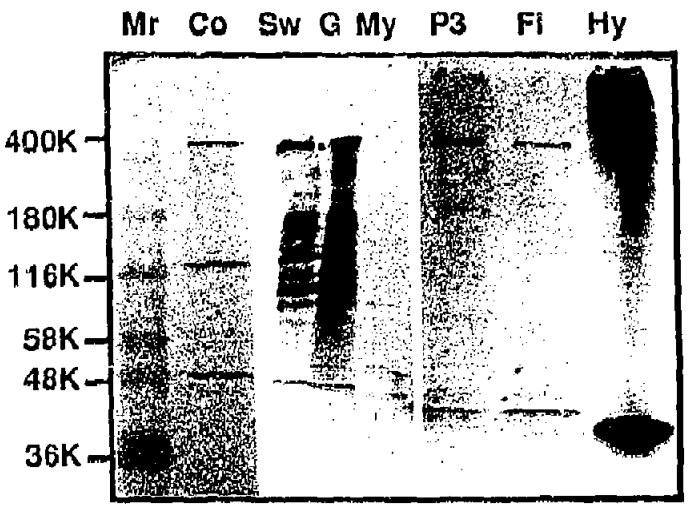

Fig. 2. Utrophin is present in three other cell lines and in human skin fibroblasts, but not in myeloma or hybridoma cells. The cell lines shown are COS-1 monkey kidney (CO), SWA rat Schwannoma (SWA), C6 glioma (G), Sp2/O mouse myeloma (My), P388D_1 mouse monocyte-macrophage (P3), human skin fibroblasts (Fi) and the MANDYS1 mouse hybridoma (Hy). The heavy staining in (Hy) reflects the production of large amounts of mouse Ig which reacts with the second antibody detection system. The utrophin-specific mAbs used were MANCHO7 (Co, P3, Hy) and MANCHO3 (Sw, G, My).

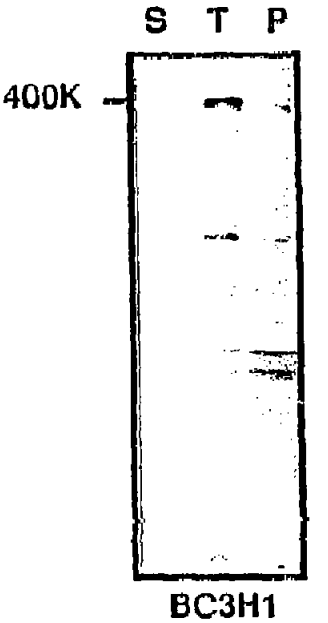

Fig. 3. Utrophin in BC_3H1 smooth muscle-like cells is insoluble in low-salt extraction buffers, but soluble in Triton X-100. The extraction procedure is described in Materials and Methods. The fractions are a low-salt 'cytoplasmic' fraction (S), the Triton-soluble fraction of the low-salt pellet (T) and the Triton-insoluble pellet (P). The mAb used was utrophin-specific MANCHO3.

Triton X-100 fraction (manuscript submitted). A similar result was obtained using C6 glioma cells (not shown).

4. DISCUSSION

Utrophin cDNA was originally isolated from a fetal muscle library [1] and subsequent studies on utrophin itself have been consistent with a primary role for this protein in muscle and nerve tissues, like dystrophin itself. Thus, it was shown that utrophin was present at adult skeletal neuromuscular junctions [4,6,13], in vascular and myometrial smooth muscles [4] and in nerve fibres [2]. We have also shown that it is present in proliferating brain cell lines (glioma and Schwannoma) [4] and have suggested that its high levels in lung might be due to the high proportion of vascular and other smooth muscles in this tissue [4].

We have now shown, however, that utrophin is expressed in most transformed continuous cell lines with a variety of developmental origins, though none of these express detectable amounts of dystrophin. It is also present in significant amounts in untransformed human skin fibroblast cultures, potentially useful for studies of normal and pathological variation in utrophin in human populations, since they are one of the easiest human cell cultures to establish.

Since utrophin is present at higher levels in foetal and regenerating muscle, its presence in cultured cells might seem consistent with a relationship with cell proliferation. Utrophin was absent, however, from the mouse myeloma cell line tested and from hybridomas resulting from its fusion with mouse spleen lymphocytes. This shows that utrophin is not essential for cell growth and division. The myeloma cell line is the only one which grows in suspension and does not attach readily to tissue culture plastic. Although HeLa cells grow in suspension, they can also attach to plastic, but no difference in utrophin content was observed when attached and 'floating' cells were compared in a culture containing both (Fig. 1b). This does not rule out the possibility that utrophin is required for cell attachment and spreading, though utrophin levels do not show any marked change in response to changes in these factors. The results clearly show the need to look beyond any specialized utrophin function in muscle and nerve to a more general function, certainly in transformed cells and perhaps in most normal cells in culture.

The evidence that utrophin is associated with membranes is significant since this has so far only been clearly demonstrated in muscle. This may be partly due to the fact that the plasmalemma is easier to differentiate immunohistochemically from cytoplasm in large muscle fibres than in tissues made up of much smaller cells. Extraction with Triton X-100 does not, of course, distinguish between the plasma membrane fraction and any internal microsomal membranes and there is evidence that even dystrophin itself can associate with internal membranes such as transverse tubules under some circumstances [14,15]. The possibility that Triton releases utrophin by a mechanism other than its solubilization of membranes, though unlikely, cannot be formally ruled out. Dystrophin, however, is also extracted

from muscle by Triton X-100 [11] and, by analogy, any utrophin interaction with membrane lipids is likely to be indirect. Dystrophin interacts with membranes via the dystrophin-associated glycoprotein complex (DAG) which is also present in non-muscle tissues [12], but it has not yet been established whether utrophin can interact with trans-membrane DAG (or a similar protein) in the same way as dystrophin. It is of interest, however, that DAG interacts extracellularly with the basement membrane protein, laminin. [12] and that some cultured cell monolayers can also express laminin, a feature shared with utrophin-containing tissue components, such as vascular smooth muscle and nerve [16].

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