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Muskelin, a novel intracellular mediator of cell adhesive and cytoskeletal responses to thrombospondin-1

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We have used an expression cloning strategy based on a cell-attachment assay screen to seek identification of molecules required in cellular responses to thrombospondin-1, a regulated macromolecular component of extracellular matrix. We report the identification and functional characterization of a novel, widely expressed, intracellular protein, named muskelin, which contains dispersed motifs with homology to the tandem repeats first identified in the *Drosophila* kelch ORF1 protein. In adherent C2C12 cells, muskelin localizes in the cytoplasm and at cell margins. Overexpression of muskelin in C2C12 cells promotes cell attachment to the thrombospondin-1 C-terminal domain, alters the mechanisms of attachment to intact thrombospondin-1 and correlates with decreased formation of fascin microspikes and increased assembly of focal contacts by cells adherent on thrombospondin-1. Reciprocally, cell attachment, spreading and cytoskeletal organization are specifically reduced in TSP-1-adherent cells after antisense depletion of muskelin. These results establish a requirement for muskelin in cell responses to thrombospondin-1 and demonstrate that such responses involve a novel process which is integrated into the regulation of cell-adhesive behaviour and cytoskeletal organization.

Keywords: cell adhesion/extracellular matrix/fascin microspike/kelch motif

Introduction

The extracellular matrix (ECM) plays a central role in the development and maintenance of tissue organization in metazoan organisms. ECM is composed of secreted, multifunctional, multidomain macromolecules which are assembled extracellularly into organized arrays. In addition to providing structural support within tissues, ECM components coorganize with acutely regulated soluble mediators many aspects of cell behaviour, including adhesion, migration, proliferation, differentiation and survival (reviewed by Hynes and Lander, 1992; Gumbiner, 1996). It is therefore possible that the exposure of cells to different matrix macromolecules offers one mechanism by which particular cell behaviours are coordinated. A knowledge of the molecular events involved in cell interactions with, and response to, diverse ECM components is thus of great interest in determining how specific physiological networks are built up within tissues.

To date, attention has focused on ubiquitous ECM components including fibronectin, laminins and collagens. Other macromolecules, exemplified by thrombospondins and tenascins, are transient within matrix and display temporally and spatially restricted expression patterns in embryonic and adult tissues. These glycoproteins have distinctive adhesive and adhesion-modulating functional properties in cell biological assays and are therefore considered to have generic roles in modulating cell migration and proliferation during inductive events or in situations of tissue remodelling (reviewed by Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991). Within this functional group, thrombospondin-1 (TSP-1) has been studied widely. Whereas cells attach to fibronectin, laminins and collagens through integrin-mediated interactions (reviewed by Hynes, 1987; Ruoslahti, 1988; Akiyama *et al*., 1990), cell attachment to TSP-1 occurs by complex mechanisms which involve cooperative interactions between multiple domains of TSP-1 and various cell-surface molecules which include in certain cell types, integrins, proteoglycans such as syndecan-1, CD36, CD47 or integrin-associated protein (Gao *et al*., 1996) and uncloned molecules (reviewed by Frazier, 1991; Adams *et al*., 1995; Bornstein, 1995). Adhesion modulation by TSP-1 N-terminal domain involves a heparin-sensitive process (Murphy-Ullrich *et al*., 1993). Cell spreading on a TSP-1 substratum is a cell-type-dependent process which involves organization of the actin cytoskeleton and correlates with the formation of microspikes containing the 55 kDa actin-binding protein, fascin (Adams, 1995, 1997). It thus appears possible that analysis of the molecular mechanisms by which cells interact with, and respond to, TSP-1 will yield novel insights into the modulation of cell-adhesive behaviour by ECM.

Towards this purpose, we have used an expression cloning strategy in COS cells in which an expression library was screened by cell adhesion assay. C2C12 murine skeletal myoblasts were chosen as an mRNA source because these cells undergo extensive spreading on a TSP-1 substratum (Adams and Lawler, 1994). Given the complexity of adhesive sites presented by the intact TSP-1 molecule, a fusion protein corresponding to the C-terminal globular domain of TSP-1 was used as a substratum for the adhesion assays (hTSP-1C; Adams and Lawler, 1994). hTSP-1C appears structurally similar to TSP-1 in that it is recognized by antibodies reactive with native TSP-1 (Adams and Lawler 1993, 1994).

Here we report the functional characterization of a

novel intracellular protein, designated muskelin, identified by using this approach. The predicted open reading frame (ORF) contains dispersed motifs which have homology with the tandem repeats identified in the actin-associated kelch ORF1 protein (Xue and Cooley, 1993). Over-expression or antisense depletion of muskelin in C2C12 myoblasts causes alterations in the adhesive behaviour and cytoskeletal organization of TSP-1-adherent cells and has subtle effects on the focal contacts of fibronectin-adherent cells. These functional properties define a requirement for muskelin in intracellular responses to TSP-1 which serve to modulate cell-adhesive behaviour.

Results

Isolation of muskelin cDNA by cell-attachment assay

To identify cDNA(s) encoding molecules capable of promoting cell adhesion to TSP-1, COS-7 cells were transfected transiently with C2C12 myoblast library DNA and 48 h later screened for acquisition of the ability to adhere to hTSP-1C substratum. Plasmids recovered from attached cells were put through two further rounds of screening. After the third round, insert mapping confirmed that the same 1.3 kb cDNA insert was present in 80% of the plasmids recovered from attached cells (data not shown). Transient transfection of plasmids containing the 1.3 kb insert promoted COS-7 cell adhesion to hTSP-1C by 5-fold, relative to untransfected COS-7 cells or to COS-7 cells transfected with a CD8 expression plasmid (Figure 1a). Adhesion-promoting activity appeared substratum specific, in that transient transfection of COS-7 cells did not promote adhesion to a glutathione *S*-transferase (GST) substratum, or to a GST fusion protein substratum consisting of the C-terminal domain of human TSP-4, hTSP-4C, which has 57% amino acid identity with the TSP-1 fusion protein (Adams and Lawler, 1994; Figure 1b). COS-7 cells adhere strongly to laminin, collagen IV and fibronectin, and increased adhesion of transfected cells to these matrix proteins was not detectable by phase contrast microscopy (data not shown).

Muskelin transcripts are expressed widely in murine tissues

The 1.3 kb cDNA detected an RNA of ~2.5 kb present in C2C12 and G8 skeletal myoblasts and C2C12 myotubes. This transcript was not detected in COS-7 cells (Figure 1c and data not shown). RNA species of ~2.5 kb, 7.5 kb and a minor species of 11 kb, were detected in adult mouse skeletal muscle, the 2.5 kb transcript being the most abundant (Figure 1d, lane 6). The 2.5 kb transcript was present at similar abundance in liver, kidney, testis and heart and at lower abundance in brain, spleen and lung, relative to actin expression in these tissues (Figure 1d). The relative abundance of the two larger transcripts also varied between different tissues (for example, compare Figure 1d, lanes 4, 5 and 6). Thus, the RNA transcripts are expressed widely in murine tissues.

Muskelin is a novel protein which contains kelch motifs

Since the cDNA size of 1.3 kb was substantially less than the smallest RNA detected on Northern blots, it appeared

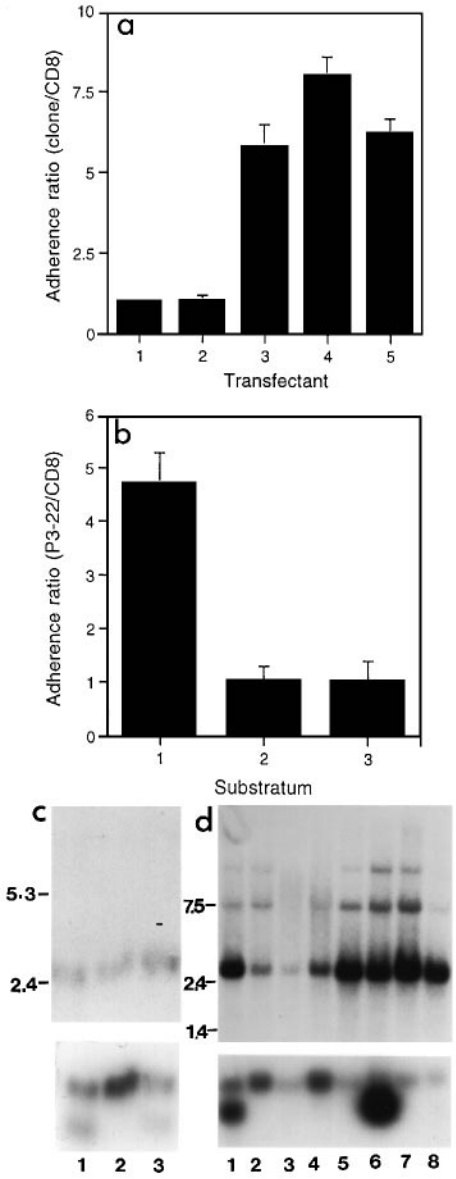

Fig. 1. Activity of the 1.3 kb cDNA in cell attachment assays and Northern blot analysis. (**a**) Ability of COS-7 transfectants to adhere to substrata coated with 10 μM hTSP-1C fusion protein. 1, untransfected COS-7 cells; 2, COS-7 transfected with CD8 expression plasmid ; 3, 4 and 5, COS-7 cells transfected with three clonal isolates of the 1.3 kb cDNA expression plasmid. (**b**) Ability of COS-7 cells transfected with 1.3 kb cDNA expression plasmid to adhere to substrata coated with : 1, 10 μM hTSP-1C fusion protein; 2, 10 μM hTSP-4C fusion protein; 3, 10 μM unfused GST. (c, d) Expression of muskelin transcript in cell lines and murine tissues. (**c**) Northern blot of cell lines probed with 1.3 kb cDNA (upper panel) or β-actin cDNA (lower panel). Lane 1, G8 myoblasts; lane 2, C2C12 myoblasts; lane 3, C2C12 myotubes. (**d**) Northern blot of adult mouse tissues probed with 1.3 kb cDNA (upper panel) or β-actin cDNA (lower panel). Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. RNA size markers are indicated in kb.

that a truncated cDNA had been isolated. Filter hybrization screening of an E11.5 mouse embryo cDNA library yielded additional cDNAs, designated 2.4 kb and clone 5, which yielded an identical pattern of hybridization to the 1.3 kb probe on the mouse tissue RNA blot (data not shown). The 2.4 kb cDNA contained an ORF of 2205 nucleotides, of which the 5′ portion matched the nucleotide sequence

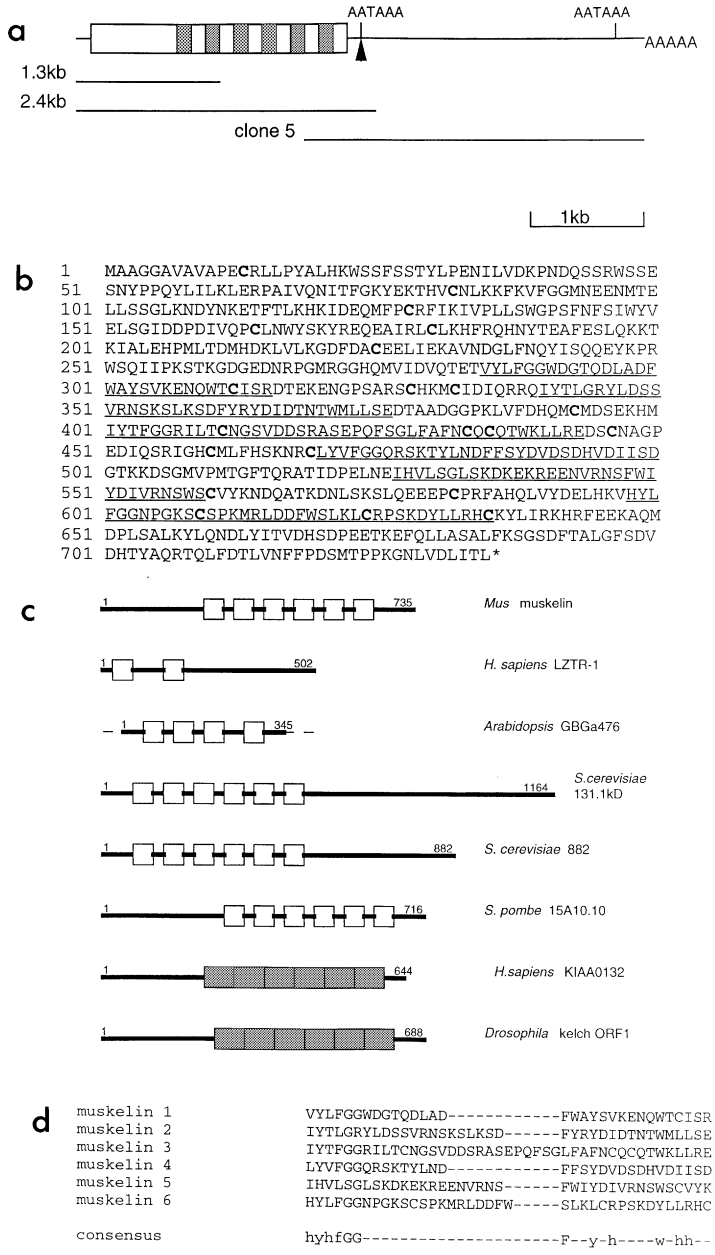

Fig. 2. Structure of muskelin cDNA, deduced amino acid sequence and kelch motif homologies. (**a**) Schematic diagram of muskelin transcript. Single lines indicate untranslated regions and the open box the ORF. Regions with kelch motif homology are indicated as stippled boxes within the ORF. The original cDNA isolate and further murine cDNAs are indicated as lines below the composite model. (**b**) Inferred polypeptide sequence of murine muskelin. Kelch motifs are underlined; cysteine residues are in bold type. (**c**) Schematic diagrams of muskelin and examples of other proteins containing dispersed kelch motifs. White boxes indicate the versions of the kelch motif; stippled boxes indicate the 50-amino-acid tandem repeats. Black lines indicate non-kelch portions of sequence. (**d**) Sequence alignment of kelch motifs from muskelin. In the consensus sequence, upper case indicates invariant residues; lower case indicates residues present in at least four of the motifs; dashes indicate non-conserved positions; h, indicates hydrophobic residues.

of the 1.3 kb cDNA. The 2.4 kb cDNA encoded a polypeptide of 735 amino acid residues (Figure 2a). At the 5′ end of both cDNAs, 12 nucleotides preceded an ATG codon within a sequence of nucleotides which fulfilled the criteria for a translational initiation consensus sequence (Kozak, 1987). The predicted 3′ untranslated region of the 2.4 kb cDNA contained an AAUAAA transcription termination signal, but did not terminate in a poly(A) tail. The nucleotide sequence of clone 5 matched that of the 2.4 kb cDNA at its 5′ terminus, contained two AAUAAA transcription termination signals within the putative 3′ untranslated region and included a poly(A) tail at it 3′ terminus (Figure 2a). A probe corresponding to the unique 3′ portion of clone 5 hybridized with the larger RNAs and not with the 2.5 kb cDNA on the mouse tissue RNA blot, indicating that these transcripts arise by alternative splicing within the 3′ untranslated region. To verify that the 5′ end of the 1.3 kb and 2.4 kb cDNAs corresponded to the 5′ end of the transcript, 5′ RACE was carried out on cDNA prepared from mouse heart. Products of the predicted size were obtained using either of two internal PCR primers. When sequenced, these products were found to match the 5′ ends of the cDNAs isolated previously (data not shown).

The 735 amino acid polypeptide had a predicted molecular mass of 84 888 and predicted *p*I of 6.1. Noteworthy features of the primary sequence were its low content of alanine residues: 3.9% compared with 7.2% in an average vertebrate protein, and its elevated content of aromatic residues: 11.3% as compared with the 8.6% average of vertebrate proteins (Doolittle, 1987) (Figure 2b). BLAST database sequence comparisons indicated that this was a novel protein, however, three regions of 30 amino acids exhibited 36% identity and 53% homology to the 50 amino acid repeats of the kelch ORF1 protein of *Drosophila melanogaster* (Xue and Cooley, 1993) and to similar repeats in the predicted human gene product KIAA0132 (Nagase *et al*., 1995), (Figure 2a and b; motifs 1, 4 and 5). Further analysis by pattern-searching approaches including gapped BLAST identified three additional kelch motifs within the carboxyl portion of the polypeptide (Figure 2b and c; motifs 2, 3 and 6). The novel protein identified herein has been named muskelin, to indicate its identification in a mouse muscle cell line and the presence of kelch-like motifs within the amino acid sequence.

The kelch ORF1 protein contains six 50-amino-acid kelch repeats in tandem array (Figure 2c). The motifs in muskelin also exhibited homology to a number of polypeptides in which individual kelch motifs are separated by unrelated portions of amino acid sequence ranging from 20 to >60 residues in length. These polypeptides include human LZTR-1 (Kurahashi *et al*., 1995); *Arabidopsis thaliana* hypothetical protein GBGa476; *Saccharomyces cerevisiae* hypothetical 131.1 kDa protein (YHR158c; Johnston *et al*., 1994) and hypothetical protein 882, (YGR238c) and *Schizosaccharomyces pombe* hypothetical protein 15A10.10 (Figure 2c). Alignment of the six motifs in muskelin yielded a consensus sequence with high identity to the kelch motif consensus derived by a broadly based pattern-searching approach (Figure 2d; Bork and Doolittle, 1994).

Localization of muskelin protein in C2C12 myoblasts

In vitro transcription/translation of the muskelin ORF resulted in the production of a single labelled product with an apparent molecular mass of 82 kDa (Figure 3a). Similarly, muskelin N-terminal synthetic peptide antiserum specifically detected a protein of apparent molecular mass 82 kDa in C2C12 whole cell lysates (Figure 3b, lane 1). A smaller band of apparent molecular mass 37 kDa, presumably a proteolytic fragment, was variably detected (Figure 3b, lane 2). Antiserum to a synthetic peptide corresponding to the muskelin C-terminus also recognized the 82 kDa protein (data not shown). These findings confirm the isolation of cDNA encoding full-length muskelin polypeptide.

The subcellular distribution of muskelin was examined in two types of extract of C2C12 cells. After lysis in 1% Triton X-100 buffer, the majority of muskelin was detected in the detergent-soluble fraction (Figure 3c). By comparison, vinculin was present in both fractions (Figure 3c). In membrane and cytosolic fractions, the majority of muskelin was detected in the cytosol and a small proportion was membrane associated. In contrast, vinculin localization was almost entirely cytosolic (Figure 3d).

Indirect immunofluorescent staining of C2C12 cells

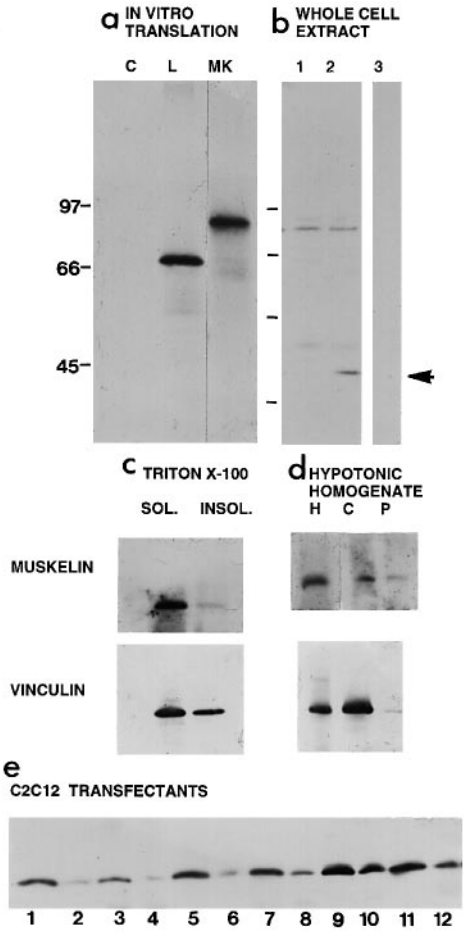

Fig. 3. Analysis of muskelin protein. (**a**) *In vitro* translation of muskelin cDNA. *In vitro* transcription/translation reaction products were analysed on a 12.5% polyacrylamide gel under reducing conditions. (C), no plasmid; (L), plasmid encoding firefly luciferase; (MK), plasmid encoding muskelin. Molecular mass markers in kDa. (**b**) Reactivity of N-terminal anti-peptide serum with muskelin protein in C2C12 cells. Western blots of two separate preparations of C2C12 whole cell extracts were probed with immune serum (lanes 1 and 2) or preimmune serum (lane 3). Arrow indicates presumed proteolytic fragment in lane 2. (**c**) Distribution of muskelin in subcellular extracts. Western blot of Triton X-100 soluble (SOL) or insoluble (INSOL) fractions of C2C12 myoblasts were probed for muskelin or vinculin. (**d**) Distribution of muskelin in subcellular fractions. Western blots of hypotonic homogenates of C2C12 cells (H); cytosol (C) or membrane pellet (P) were probed for muskelin or vinculin. (**e**) Over-expression of muskelin in C2C12 stable transfectants. Western blot of Triton X-100 soluble (lanes 1, 3, 5, 7, 9 and 11) and insoluble (lanes 2, 4, 6, 8, 10 and 12) fractions of neo-1 (1 and 2); neo-2 (3 and 4); neo-3 (5 and 6); OE-1 (7 and 8); OE-2 (9 and 10) and OE-3 (11 and 12) cells lines were probed for muskelin.

was undertaken to relate muskelin protein distribution to subcellular structures. In long-term adherent cells, muskelin had a predominantly diffuse distribution with localized concentrations in membrane ruffles (Figure 4a and a'). Staining was abolished by peptide preincubation (Figure 4b). In C2C12 cells adherent on TSP-1, muskelin was present throughout cell bodies including cell margins in regions of microspike formation (Figure 4c). Double labelling for F-actin revealed zones of overlap between muskelin and F-actin within cortical regions in 83% of the cells (examples shown by arrows in Figure 4c and e). Muskelin in cells adherent on fibronectin was also diffuse

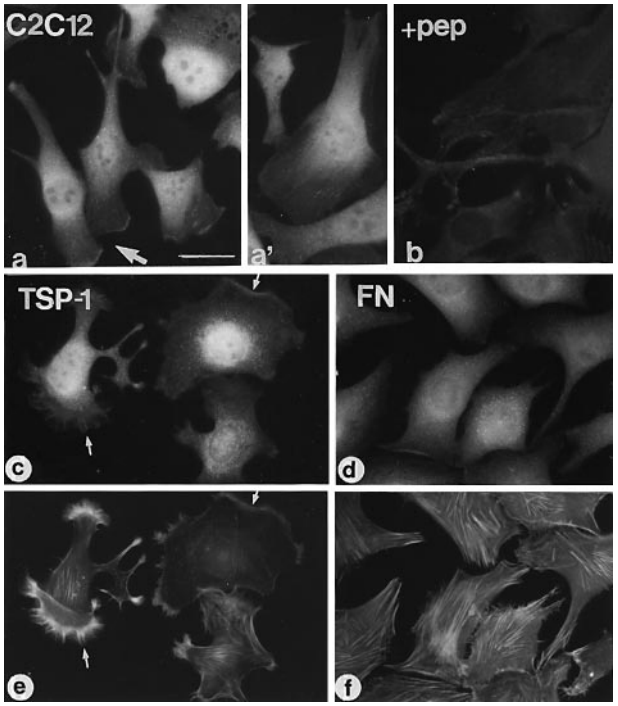

Fig. 4. Localization of muskelin in adherent C2C12 cells. (**a, a'**) Distribution of muskelin in cultured C2C12 cells. Arrow in (a) indicates concentration of staining in marginal ruffle. (**b**) Competition of staining by peptide antiserum. (**c**) Distribution in C2C12 cells adherent on TSP-1 for 1 h, (**e**) Same field stained with phalloidin. Arrows indicate cortical F-actin structures. (**d**) Distribution in C2C12 adherent on fibronectin for 1 h, (**f**) same field stained with phalloidin. Bar = 10 μm.

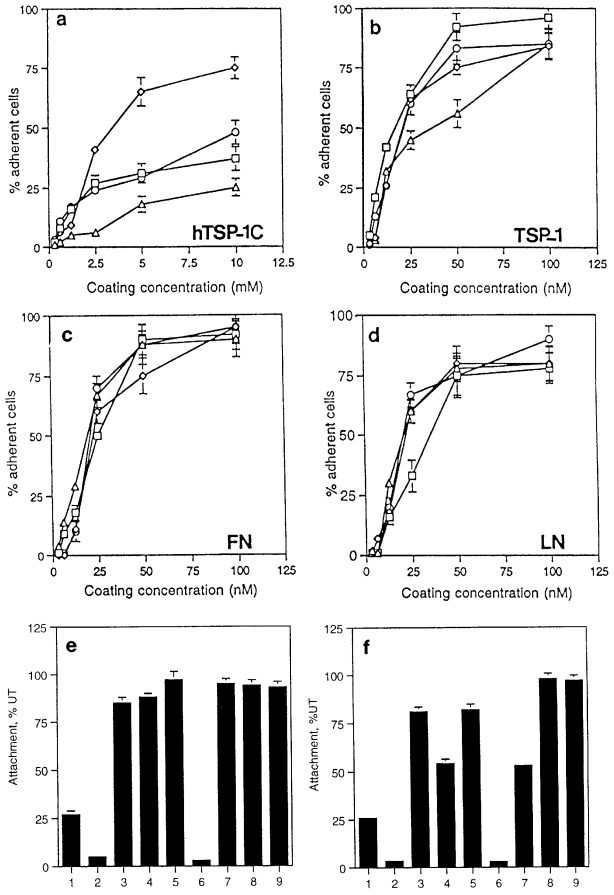

Fig. 5. Effects of muskelin over-expression on substratum adhesion properties of C2C12 cells. (**a**–**d**) Quantitation of attachment to ECM substrata. Equivalent numbers of C2C12/neo1 (△), OE-1 (□), OE-2 (○) or OE-3 (◇) were exposed to substrata coated with the indicated concentrations of (a) hTSP-1C; (b) TSP-1; (c) fibronectin; or (d) laminin, for 1 h at 37°C. Each point is the mean of triplicate experiments, bars indicate SEM. (**e**–**f**) Mechanism of attachment to TSP-1. C2C12-neo-1 cells (e), or muskelin over-expressor clone OE-2 (f) were exposed to substrata coated with 50 nM TSP-1 for 1 h, in the presence of (1) 10 μg/ml Mab ESTs 10 IgG; (2) 10 μg/ml Mab 4.1 IgG; (3) 50 μg/ml C6.7 IgG; (4) 500 μg/ml heparin; (5) 1 mM GRGDSP peptide; (6) 500 μg/ml chondroitin sulfate A; (7) antiserum to human fibronectin receptor; (8) antiserum to human vitronectin receptor; (9) 1 mM VTCG peptide. Data are plotted as the percentage of adherent cells relative to the adherence of untreated (UT) control cells, which corresponded to 100–120 cells/mm^2, and are the mean of quintuplicate determinations. Bars indicate SEM.

in cell bodies and colocalization of muskelin with marginal F-actin was observed in 9% of the cells (Figure 4d and f).

Muskelin over-expression alters mechanisms of cell attachment to TSP-1 in C2C12 cells

To obtain insight into the mechanisms underlying the adhesion-promoting activity of muskelin, C2C12 myoblasts were used as a vehicle to generate stable transfectant cell lines over-expressing muskelin. Compared with vector-transfected lines (Figure 3e, lanes 1–6), three clonal over-expressor lines exhibited increased levels of muskelin in both the Triton X-100 soluble and insoluble fractions (Figure 3e, lanes 7–12). None of the cell lines attached to GST or bovine serum albumin (BSA) substrata (not shown) and as reported previously, ~25% of the input C2C12/neo-1 cells attached to the hTSP-1C fusion protein at the maximum (Figure 5a; Adams and Lawler, 1994). In contrast, attachment of over-expressor lines OE-2 and OE-3 to hTSP-1C was increased by 2–3-fold at the maximum (Figure 5a). Full-length muskelin thus displays similar activity to the N-terminal moiety (Figure 1 and data not shown). On TSP-1, the over-expressor lines showed little alteration in the maximal number of adherent cells (Figure 5b). Attachment to fibronectin or laminin was not altered in the over-expressor lines (Figure 5c and d). Furthermore, no alterations in cell adhesion to fibronectin or laminin were apparent under conditions of weakened integrin-ligand binding capacity, achieved by divalent cation removal or exchange (data not shown). The results reported here are representative of those obtained from a total of 10 muskelin over-expressor cell lines and six vector control lines derived from two independent transfection experiments.

Next, the effect of muskelin over-expression on mechanisms of C2C12 attachment to TSP-1 was examined. C2C12 myoblast attachment to TSP-1 involves interactions with the type 1 repeats and C-terminal domain and is inhibited by chondroitin sulfate A and xylosides. Attachment does not involve the heparin-binding domain or the RGD site and is not blocked by heparin, VTCG peptide, GRGDSP peptide or antisera to β1 or αv-containing integrins (Adams and Lawler, 1994). These mechanisms of attachment were unaltered in vector transfectant cell lines (shown for C2C12/neo-1 cells, Figure 5e). Attachment of the three over-expressor clonal lines was also inhibited by antibodies reactive with TSP-1 C-terminus or type 1 repeats (data shown for clone OE-2 only; Figure 5f, columns 1, 2 and

3). These clones showed increased sensitivity to heparin (shown for OE-2, Figure 5f, column 4) and as expected attachment was inhibited by chondroiton sulfate A (Figure 5f, column 6). Attachment of OE-2 was also inhibited 25% by GRGDSP peptide (Figure 5f, column 7) and by 50% by a polyclonal antiserum to human fibronectin receptor, reactive with all β1 integrins (Figure 5f, column 7). These reagents were also partially inhibitory for OE-3 cells (not shown). Polyclonal serum to α_v integrins had no inhibitory effect on any of the cell lines (Figure 5f, column 8) and attachment was not inhibited in the presence of 1 mM VTCG peptide (Figure 5f, column 9).

Muskelin over-expression alters cytoskeletal organization in ECM-adherent C2C12 cells

Cell spreading on TSP-1 is F-actin dependent and correlates with the formation of microspikes which contain the protein fascin (Adams, 1995), an actin-bundling protein not present in focal contacts (reviewed by Edwards and Bryan, 1995). To explore whether over-expression of muskelin affects cytoskeletal organization, C2C12 over-expressor cell lines adherent on TSP-1 or on fibronectin were stained for fascin or for vinculin as a structural marker of focal contacts. In C2C12/neo-1 cells adherent on TSP-1 substrata, large arrays of fascin microspikes were present at the margins of the cells (Figure 6a). Vinculin staining appeared diffuse, was absent from the microspikes, and focal contacts were not detected (Figure 6b). Thus, these cells behave as wild-type C2C12 cells (Adams, 1997). OE-2 cells displayed a low number of ruffle-like, cortical fascin-containing structures (Figure 6c). Unexpectedly, and in marked contrast to C2C12/neo-1 cells, OE-2 cells assembled focal contacts when adherent on TSP-1 (Figure 6d).

In C2C12/neo-1 cells adherent on fibronectin, fascin was distributed diffusely (Figure 6e). These cells assembled many focal contacts. Diffuse perinuclear vinculin staining was also present (Figure 6f). OE-2 cells on fibronectin also displayed a diffuse distribution of fascin (Figure 6g) and assembled large numbers of focal contacts (Figure 6h). However, whereas uniform phosphotyrosine staining was present in focal contacts over the entire ventral surfaces of C2C12/neo-1 cells (Figure 6j), staining was concentrated most intensely in the cortical focal contacts of C2C12/OE-2 cells (Figure 6k). These results suggest alterations in the dynamics of focal contact assembly in muskelin over-expressor cells.

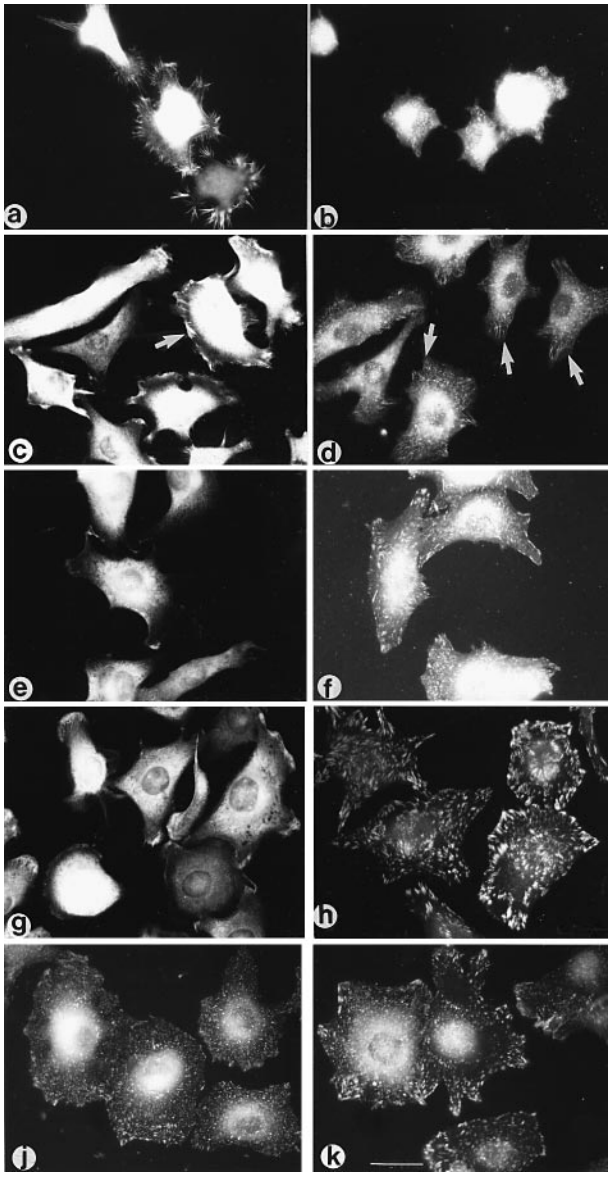

Fig. 6. Effects of muskelin over-expression on cytoskeletal organization in C2C12 cells adherent on ECM substrata. C2C12-neo1 cells (**a**, **b**, **e**, **f** and **j**) or OE-2 cells (**c**, **d**, **g**, **h** and **k**) were exposed for 1 h to substrata coated with 50 nM TSP-1 (a, b, c and d), or 50 nM fibronectin (e, f, g, h, j and k), then fixed and stained with antibody to fascin (a, c, e and g); antibody to vinculin (b, d, f and h) or antibody to phosphotyrosine (j and k). Arrow in (c) indicates an area of small fascin-positive ruffles; arrows in (d) indicate focal contacts. Bar = 12.5 μm.

Muskelin is required in C2C12 spreading and cytoskeletal organization on TSP-1

To define more completely a functional requirement for muskelin in the responses of C2C12 cells to TSP-1, clonal cell lines depleted in muskelin protein were generated by expression of antisense RNA. Fifty cell lines were derived in two independent transfections and three lines exhibiting around 20% endogenous muskelin levels used for functional assays (Figure 7a). Attachment of lines AS1, AS2 and AS3 to hTSP-1C was reduced 4-fold compared with the control vector transfected C2C12 cells, C2-V (Figure 7b). In assays involving intact TSP-1, muskelin-depleted cells attached in similar numbers to C2-V cells, yet cell spreading was reduced markedly and organization of F-actin into microfilament bundles was not apparent. Muskelin-depleted cells displayed fewer F-actin cortical spikes than control cells (compare C2-V cells in Figure 7c i with lines AS1 or AS-2 in Figure 7c, panels ii and iii). These projections contained fascin (arrow in Figure 7c v), although the cells did not display the typical large arrays of microspikes (compare C2-V in Figure 7c iv with AS1 in Figure 7c v). The muskelin depleted lines did not form focal contacts when adherent on TSP-1 (shown for AS1, Figure 7c vi). These alterations in spreading and cytoskeletal organization were not due to a reduced rate of spreading, because even after 140 min on TSP-1, muskelin-depleted cells remained less spread than control cells (data not shown). On fibronectin, muskelin-depleted cells spread extensively and assembled microfilament bundles (com-

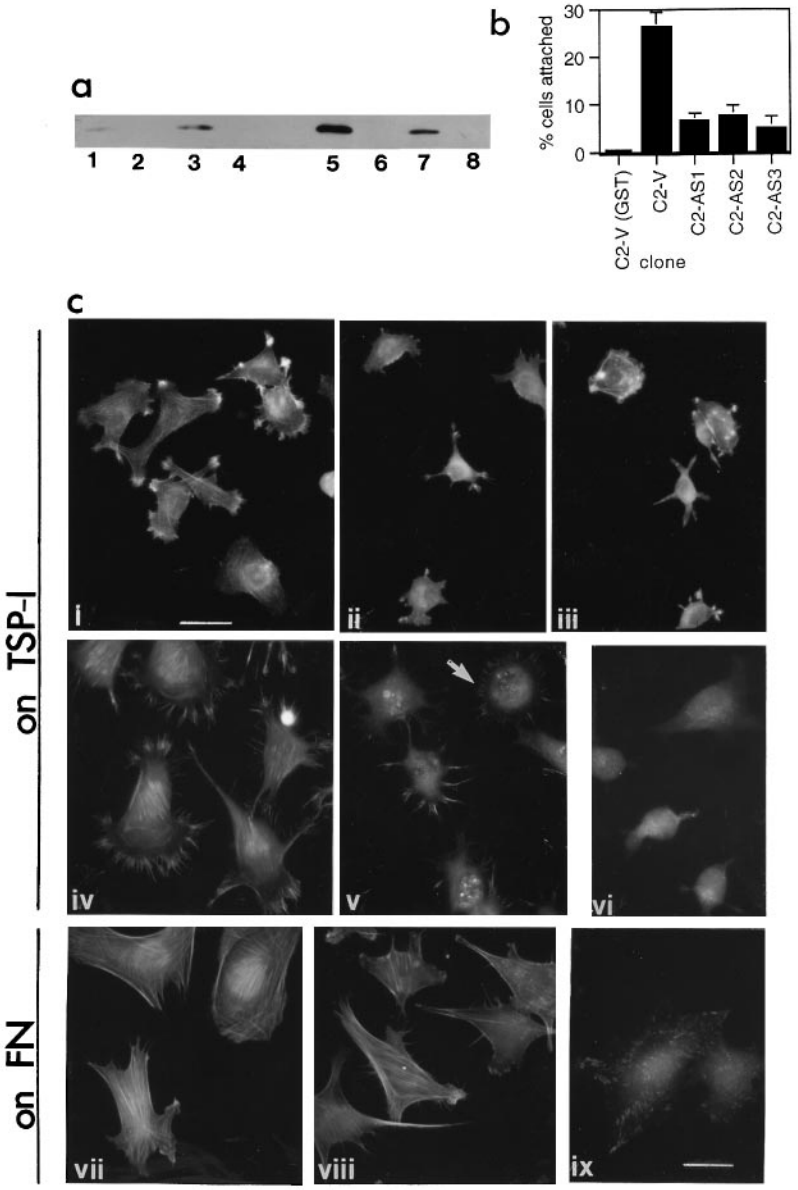

Fig. 7. Adhesive and cytoskeletal behaviour of muskelin-depleted C2C12 cells. (**a**) Western blot analysis of muskelin in Triton-X100 soluble (lanes 1, 3, 5 and 7) and insoluble (lanes 2, 4, 6 and 8) fractions of C2C12 vector transfectant (lanes 5 and 6) or C2C12 muskelin antisense transfectant clones AS1 (lanes 1 and 2); AS2 (lanes 3 and 4); AS3 (lanes 7 and 8). (**b**) Attachment of C2C12-vector control (C2-V) or clones AS1-3 on substrata coated with 10 μm GST (first column) or 10 μM hTSP-1C (other columns). Each column is the mean of triplicate measurements, bars = SEM. (**c**) Cytoskeletal organization on TSP-1 or fibronectin. Examined in C2-V cells (i, iv and vii); AS1 cells (ii, v, vi, viii and ix); AS2 cells (iii), stained with phalloidin (i, ii, iii, vii and viii), antibody to fascin (iv and v) or antibody to vinculin (vi and ix). i–iii, bar = 15 μm; iv–ix, bar = 10 μm.

pare C2-V cells in Figure 7c vii with AS1 cells in Figure 7c viii). Fibronectin-adherent AS1 cells assembled focal contacts, yet vinculin staining typically appeared to be of lower intensity than in control C2C12 cells (compare Figure 7c ix with Figure 6f). These results demonstrate a requirement for muskelin in the normal cell-spreading responses of C2C12 cells to thrombospondin-1.

Discussion

The use of an expression cloning approach based on a cell-attachment assay screen has resulted in the identification of a novel intracellular protein, muskelin. Our functional assays define a requirement for muskelin in cell-adhesive responses to TSP-1. It is of interest that the cell-adhesion assay screening method resulted in the isolation of an intracellular protein. Because COS-7 cells do not attach to hTSP-1C protein, it might have been expected that expression of a cell-surface molecule would be required to confer adhesive activity. However, cell adhesion is a multistep process, involving not only initial ligand/receptor recognition at the cell surface, but also receptor clustering, cytoskeletal organization and cell spreading (reviewed by Clark and Brugge, 1995; Schwartz *et al*., 1995; Yamada and Miyamoto, 1995). Since the screening method did not discriminate between these various aspects of cell adhesion, the screen has the potential to pick up molecules active at any step of the process. The identification of an intracellular molecule implies both that COS-7 cells carry cell-surface binding proteins for the C-terminal domain

of TSP-1, and that these molecules need to be activated to permit cell attachment. In attachment assays using intact TSP-1, COS-7 cells attach in low numbers and do not spread. This interaction involves the type 1 repeats and is sensitive to heparin and chondroitin sulfate A (J.C.Adams, unpublished observation).

A number of polypeptides which contain tandem kelch repeats have been identified. These include viral ORFs, MIPP, scruin, SPE-26, actin-fragmin kinase, tea1 protein and host cell factor (Chang-Yeh *et al*., 1991; Senkevich *et al*., 1993; Xue and Cooley, 1993; Varkey *et al*., 1995; Way *et al*., 1995; Eichinger *et al*., 1996; Mata and Nurse, 1997; Wilson *et al*., 1997). Individual examples of this motif typically show low sequence identity, in the range of 11–50%. Seven residues are invariant in the six repeats of kelch ORF1 (Xue and Cooley, 1993) and only the double glycine motif is invariant in the 12 repeats of scruin (Way *et al*., 1995). Database searches using the individual motifs from muskelin identified over 45 entries with homology to these motifs. These sequences originate from human, mouse, *Caenorhabditis elegans*, *S.cerevisiae*, *S.pombe* and *Arabidopsis thaliana* and include additional proteins and ORFs which contain dispersed kelch motifs (J.C.Adams, unpublished observation). Tandem arrays of kelch motifs correspond to a common structural fold in which each motif forms a four-stranded β-sheet, with six to eight such motifs arranged circularly to form a β-propellor structure (Bork and Doolittle, 1994). It is conceivable that arrays of dispersed kelch motifs may form similar structures with larger intervening loops between individual β-sheets. These might be important for protein–protein interactions.

Muskelin is the first example of a protein containing dispersed kelch motifs for which functional information is available. The kelch ORF1 protein colocalizes with pre-assembled actin microfilaments in the ring canals of *Drosophila* egg chambers (Robinson *et al*., 1994). It has been proposed that kelch repeats are a novel type of actin-binding domain (Knowles and Cooley, 1994) and indeed the kelch repeat domain of kelch ORF1 mediates ring canal localization (Robinson and Cooley, 1997). However, recombinant proteins corresponding to the C-terminal six repeats of scruin self-aggregate into stable filaments, suggesting that repeats may participate in inter-molecular reactions with other kelch repeats (Way *et al*., 1995). Furthermore, the tea1 protein localizes at the ends of microtubules (Mata and Nurse, 1997) and the six kelch repeats of HCF mediate its association with VP16 (Wilson *et al*., 1997). Given that both the 50-amino-acid and 30-amino-acid versions of the motif are found in a large number of otherwise unrelated proteins from diverse organisms, the prospect that they serve some general role in protein–protein interactions is attractive.

Over-expression of muskelin in COS-7 or C2C12 cells results in increased cell attachment to the hTSP-1 C-terminal globular domain. The N-terminal portion of muskelin, including the first kelch motif (amino acid residues 1–327), is sufficient for this activity. Over-expression of muskelin also leads to qualitative changes in the mechanism of attachment to intact TSP-1, such that heparin-inhibitable interactions become involved. In terms of TSP-1 binding proteins, C2C12 cells express both heparan sulfate and chondroitin sulfate proteoglycans including syndecan-1, and also IAP/CD47 and αvβ3, but not CD36 (Miller *et al*., 1991; J.C.Adams, unpublished). Whereas the C-terminal globular domain of TSP-1, which contains the IAP/CD47 binding site, is the smallest domain with independent adhesive activity for myoblasts (Adams and Lawler, 1994), the contrasting effects of xylosides, glycosaminoglycans or GRGDSP peptide on attachment and cytoskeletal organization implicate proteoglycans in these processes (Adams, 1997). The C-terminal globular domain contains low-affinity heparin-binding sites (Haverstick *et al*., 1984; Kosfeld and Frazier, 1992; Lawler *et al*., 1992) and is implicated in binding an unknown 80 kDa–105 kDa heterodimer (Yabkowitz and Dixit, 1991). In the absence of a measurable effect of muskelin over-expression on integrin ligand-binding capacity, it is probable that the β1 integrin-mediated component of attachment detected in over-expressor cell attachment arises by recruitment of β1 integrins into pre-existing adhesion complexes, thus leading to focal contact assembly in TSP-1-adherent cells.

Although muskelin does not quantitatively increase cell attachment to fibronectin or laminin or grossly alter microfilament organization, subtle alterations in focal contact organization are apparent in over-expressor or antisense-depleted cell lines. Functional interplay between adhesion-modulating and adhesive matrix components with endpoint effects on cell spreading and/or cytoskeletal organization have been described in several cell systems (Chiquet-Ehrismann *et al*., 1988; Lightner and Erickson, 1990; Murphy-Ullrich *et al*., 1993; Gao *et al*., 1996; Williamson *et al*., 1996; Adams, 1997; Fischer *et al*., 1997a, b). To date, the intracellular processes involved have remained largely unexamined. The required role for muskelin in cellular responses to TSP-1 suggests a model in which muskelin functions at a nodal point in substratum contact formation and cytoskeletal organization. Notably, the majority of endogenous muskelin protein is detergent extractable and the marked functional consequences of over-expression or antisense depletion of muskelin on cytoskeletal organization are context dependent, being detectable in TSP-1-adherent cells. These results imply a physiological function of muskelin as an adjunct, rather than a structural component of the cytoskeleton. The identification of binding partners for muskelin is now an interesting and important goal.

In conclusion, the identification of muskelin's functional role in cellular responses to TSP-1 offers the first example of a molecular component not previously identified in integrin signalling pathways (reviewed by Clark and Brugge, 1995; Schwartz *et al*., 1995) and furthermore provides a new entry point for determination of the molecular mechanisms by which cells integrate informational input from their microenvironment into fine regulation of cytoskeletal organization and cell behaviour.

Materials and methods

cDNA library construction

Poly(A)$^+$RNA was prepared from subconfluent cultures of C2C12 murine skeletal myoblasts grown in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS) (Blau *et al*., 1985) using Pharmacia Quick-prep oligo(dT) columns according to the manufacturer's instructions. cDNA was sythesized from 4 μg of this material using a procedure based on the method of Gubler and Hoffman as described by

Aruffo and Seed (1987), using AMV reverse transcriptase for first-strand synthesis. The double-stranded cDNA was prepared for the addition of non-compatible phosphorylated *Bst*XI adaptors by treatment with Klenow DNA polymerase. After ligation of the adaptors, the cDNA was size-fractionated through a 5–20% potassium acetate gradient. Fractions containing cDNAs longer than 1 kb were pooled, concentrated by ethanol precipitation and the cDNA ligated into the CDM8 mammalian expression plasmid (Seed, 1987), which had also been prepared by digestion with *Bst*XI and fractionation on a 5–20% potassium acetate gradient (Seed and Aruffo, 1987). The ligated DNA was transfomed by electroporation into *Escherichia coli* MC1016/p3. Ampicillin-resistant bacteria were selected on solid medium and the resultant colonies pooled and used to prepare plasmid DNA using the alkaline lysis method and caesium chloride density gradient centrifugation. This DNA constituted the primary library which was used in screening experiment.

Screening of cDNA clones by cell adhesion assay

For the first round of screening, 6×10^5 COS-7 cells were plated per 90 mm dish and 48 h later, 10 dishes were each transfected with 1 μg of library DNA, using the DEAE-dextran method as described previously (Seed and Aruffo, 1987). After 4 h at 37°C, the cells were shocked with 10% DMSO in PBS for 2 min, then left to recover in DMEM containing 10% FCS overnight. Cells were harvested for the adhesion assays 48 h post-transfection.

Pilot experiments established that COS-7 cells did not adhere to hTSP-1C (Adams and Lawler, 1994) substratum. Adhesion assays were carried using 90 mm dishes coated with 50 μg/ml hTSP-1C and 1.6×10^6 transfected COS-7 cells per dish, at a concentration of 2×10^5 cells/ml in serum-free DMEM. After 1 h at 37°C non-adherent or weakly adherent cells were removed by aspirating dishes three times with TBS containing 2 mM $CaCl_2$. Residual adherent cells, mostly well spread, were lysed in 10 mM EDTA/0.6% SDS and plasmid DNA isolated according to the method of Hirt (1967). This DNA was transformed into MC1016/p3 cells by electroporation. The colonies recovered were pooled and the plasmids re-introduced into COS-7 cells by spheroplast fusion (Sandri-Goldin *et al*., 1981) for two further rounds of screening by adhesion assay.

cDNA cloning and sequence analysis

The muskelin cDNA was ligated into the pBluescript II SK vector (Stratagene) and sequenced by the dideoxy chain termination method, using Sequenase 2.0 T7 polymerase sequencing kits (Amersham). Full sequence was obtained on both strands using Bluescript T7 and T3 primers against a series of truncated clones and a series of internal primers against the original cDNA and the truncated clones. To obtain additional clones and confirm the 5′ and 3′ ends of the coding sequence, the C2C12 cDNA library and commercial mouse brain or mouse heart cDNA libraries (Clonotech) were screened by PCR, using primer pairs such that 5′ or 3′ ends of muskelin cDNAs were specifically amplified. 5′ RACE was also carried out on single-stranded cDNA prepared from mouse heart (Clonotech). PCR products were blunt-ligated into pBluescript SK and sequenced on both strands, using Bluescript T7 and T3 primers. An E11.5 mouse embryo library (Clontech) was screened by filter replica hybridization, using the 1.3 kb cDNA isolated by adhesion assay. Databases searches of DDBJ/EMBL/GenBank and dbest were carried out using BLAST programmes (Altschul *et al*., 1990, 1997) at default parameters. The DDBJ/EMBL/Genbank accession number is U72194.

RNA and DNA blot hybridization

RNA and DNA blot hybridizations were carried out according to standard procedures using either a 0.9 kb *Hin*dIII–*Xba*I fragment corresponding to the central portion of the muskelin ORF, or a 2.4 kb *Eco*R1 fragment. The adult mouse tissue RNA blot was obtained from Clontech. Northern blots were hybridized at 42°C in the presence of 50% formamide and the final wash was at 60°C in 0.1× SSC, 0.1% SDS.

Protein analysis

A rabbit polyclonal antiserum was raised against a 15-mer synthetic peptide corresponding to the predicted N-terminal portion of muskelin (rabbits immunized and bled according to standard in house procedures by Zenaca/CRB, Northwich, Cheshire, UK). Affinity-purified IgG was prepared by passing the serum over a column containing the immunizing peptide coupled to CNBr-Sepharose 6B (Pharmacia). Bound IgG was eluted with 100 mM glycine at pH 2.8. Reactivity of the antiserum with muskelin protein was determined by immunoprecipitation of *in vitro* translated polypeptide (TNT kit; Promega). C2C12 extracts were prepared by lysis in boiling SDS–PAGE sample buffer (whole cell extract) or by extraction of 5×10^5 cells for 15 min on ice in buffer containing 50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl and Complete protease inhibitor cocktail (Boehringer Mannheim), with lysis of the residue in hot sample buffer. Cell homogenates were prepared by washing 2×10^6 cells cells in TBS, scraping into 1 ml of ice-cold 10 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$ containing protease inhibitor cocktail and homogenizing with 10 strokes of a ball-bearing homogenizer, clearance 12 μm (EMBL Workshop, Heidelberg, Germany). After centrifugation at 14 000 r.p.m. for 5 min in an Eppendorf microfuge to remove nuclei, membranes were pelleted by ultracentrifugation at 100 000 *g* for 1 h. Membrane and cytosolic fractions were brought to equal volumes and equal volumes thereof resolved on 12.5% polyacrylamide gels under reducing conditions and transferred to nitrocellulose. Blots were probed with muskelin antiserum or VIN 11.5 monoclonal antibody to vinculin using the ECL detection method (reagents from Clontech and Perkin-Elemer; exposure onto Hyperfilm ECL, Amersham).

Generation of stable transfectant C2C12 cell lines

Subconfluent cultures of C2C12 myoblasts were transfected using Lipofectin (Life Technologies) with the mammalian expression plasmid, pCDNA3, (InVitrogen, San Diego, USA) or pCDNA3 into which 2.4 kb muskelin cDNA had been ligated in the sense or antisense orientations. After 48 h, cells were treated with 0.5 mg/ml of geneticin (Sigma) and antibiotic-resistant colonies of cells isolated 1 week later by ring cloning. Expression levels of muskelin in the different clones were compared by Western blot. For over-expression, clonal lines were derived from two independent transfection experiments and a total of 10 muskelin over-expressor and six vector control lines were examined in functional assays, between three and 10 passages post-transfection. For antisense experiments, 50 clonal cell lines were derived in two independent transfections and analysed by Western blot for muskelin protein levels. Three antisense-depleted and three vector-control lines were used in functional assays.

Adhesion assays

Platelet thrombospondin-1 and human TSP-1C fusion protein were prepared as described previously (Adams and Lawler, 1994). Some experiments used recombinant human TSP-1 expressed by baculovirus infection of HIGH 5 cells (InVitrogen) according to standard procedures. This protein is secreted in trimeric form and was purified from serum-free conditioned medium by heparin–agarose chromatography with elution at 0.55 M NaCl. Rat plasma fibronectin, GRGDSP peptide, antiserum to human fibronectin receptor, antiserum to human vitronectin receptor, antibody A4.1 (Prater *et al*., 1991) and antibody C6.7 (Dixit *et al*., 1985) were obtained from GIBCO-BRL; mouse EHS laminin, heparin and chondroitin sulfate A were from Sigma Chem. Co. Cell adhesion assays were carried out as previously described (Adams, 1995).

Immunofluorescence microscopy

Immunofluorescent staining using rhodamine-phalloidin (TRITC-phalloidin, Sigma Chemical Company), mouse monoclonal antibody against vinculin (VIN 11.5, ICN Immunobiologicals), mouse monoclonal 349 to phosphotyrosine (Transduction Laboratories) or mouse monoclonal antibody to fascin (Yamashiro-Matsumura and Matsumura, 1986), was carried out as described previously (Adams 1995, 1997). For staining with IgG fraction of muskelin serum, fixed and permeabilized cells were blocked for 1 h with 10 mg/ml BSA, 1 mM EGTA in Tris-buffered saline (TBS) before staining. Samples were examined by epifluorescence using a Zeiss Axioplan microscope and photographs taken using Kodak T-MAX 100 film.

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