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A novel type of myosin implicated in signalling by rho family GTPases

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A novel widely expressed type of myosin (fifth unconventional myosin from rat: myr 5) from rat tissues, defining a ninth class of myosins, was identified. The predicted amino acid sequence of myr 5 exhibits several features not found previously in myosins. The myosin head domain contains a unique N-terminal extension and an insertion of 120 amino acids at a postulated myosin–actin contact site. Nevertheless, myr 5 is able to bind actin filaments in an ATP-regulated manner. The head domain is followed by four putative light chain binding sites. The tail domain of myr 5 contains a region which coordinates two atoms of zinc followed by a region that stimulates GTP hydrolysis of members of the ras-related rho subfamily of small G-proteins. Myr 5 therefore provides the first direct link between rho GTPases which have been implicated in the regulation of actin organization and the actin cytoskeleton. It is also the first unconventional myosin for which a tail binding partner(s), namely members of the rho family, has been identified.

Key words: actin/GTPase-activating protein/myosin/rho/zinc binding

Introduction

Molecular motors of the myosin superfamily transduce chemical energy into mechanical force along actin filaments. The mechanism underlying this mechanochemical energy transduction remains unknown. However, the recent determination of the crystal structure of conventional myosin (Rayment *et al.*, 1993a) and the refinement of the *in vitro* motility assay (Finer *et al.*, 1994; Ishijima *et al.*, 1994) represent significant steps towards the elucidation of this mechanism. The identification and characterization of novel diverse (unconventional) myosin molecules with divergent sequences may also lead to an understanding of structure–function relationships. The fact that there may exist a large superfamily of myosin molecules is supported by the various actin-dependent motility phenomena observed in cells and the identification of unconventional myosins (Hammer, 1991; Pollard *et al.*, 1991; Cheney and Mooseker, 1992). All myosins share a characteristic motor domain that includes variable numbers of light chains which belong to the superfamily of calmodulin/EF-hand proteins. In addition to the motor domain, myosins contain different tail domains that are postulated to specify the respective function(s) of a given myosin. The tail domains of unconventional myosins are thought to interact with a 'receptor' and thereby determine the target for force production. However, no such receptor for any unconventional myosin has been identified yet.

The directed movement of myosin molecules depends on the organization and composition of actin filaments. The ras-related rho subfamily of small GTP binding proteins, which includes Rho, Rac and Cdc42Hs, is implicated in actomyosin-dependent processes and in growth factor-stimulated actin reorganization (Ridley and Hall, 1992; Ridley *et al.*, 1992; Kishi *et al.*, 1993; Jalink *et al.*, 1994). The cycling of these proteins between GTP-bound active and GDP-bound inactive states is partially controlled by GTPase-activating proteins (GAPs) which stimulate their intrinsic GTP hydrolysing activity. Such GAP proteins may also serve effector functions (Boguski and McCormick, 1993). No downstream components of rho proteins directly acting on the actin cytoskeleton have been identified.

To elucidate the diversity of myosin molecules and to determine their contribution to signal-mediated actin-based processes in mammalian cells, we initiated a search for novel unconventional myosin molecules. Here we report the identification and biochemical characterization of a novel type of myosin from rat that contains (i) a myosin head region with several structural features not found previously in other myosin head regions and (ii) a tail region with GAP activity for members of the rho family that might be regulated by a neighbouring zinc binding motif.

Results

Identification and sequence analysis of myr 5

To probe for novel myosins, we screened a rat brainstem/spinal cord cDNA library with a myr 2 cDNA fragment encoding a well-conserved region of the myosin head domain. This screen identified two novel myosins, myr 5 and myr 6. Myr 5 (fifth unconventional **my**osin from **r**at) was completely cloned and sequenced. The translated amino acid sequences of the isolated myr 5 cDNA clones predict a protein of 1980 amino acids with a relative molecular mass of 225 kDa (Figure 1A).

Sequence comparisons revealed that myr 5 defines a novel type of unconventional myosin composed of an N-terminal head (motor) domain and a C-terminal tail domain. Both domains contain functional elements not found previously in myosins. A phylogenetic analysis of

A

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   1  MSAHEAGSSG RRRPATFHLH IYPQLPSAGS QTSCRVTATK DSTTSDVIRD
  51  VVASLHLDGS KHYVLVEVKE SGGEEWVLDA SDSPVHRVLL WPRRAQKEHP
 101  REDGYYFLLQ ERNADGSIQY LHVQLLAQPT AACRLVERGL LPRPQADFDD
 151  LCNLPELNEA NLLQSLKLRF VQQKIYTYAG SILVAINPFK FLPIYNPKYV
 201  KMYENQQLGK LEPHVFALAD VAYYTMLRKH VNQCIVISGE SGSGKTQSTN
 251  FLIHCLTALS QKGYASGVER TILGAGPVLE AFGNAKTAHN NNSSRFGKFI
 301  QVNYLESGIV RGAVVEKYLL EKSRLVSQEK DERNYHVFYY LLLGVSEEER
 351  QEFQLKQPQD YFYLNQHNLN IEDGEDLKHD FERLQQAMEM VGFLPATKQQ
 401  IFSVLSAILY LGNVTYKKRA TGRDEGLEVG PPEVLDTLSQ LLKVKRETLV
 451  EVLTKRKTIT VNDKLILPYS LSEAITARDS MAKSLYSALF DWIVLRINHA
 501  LLNKKDMEEA VSCLSIGVLD IFGFEDFERN SFEQFCINYA NEQLQYYFTQ
 551  HIFKLEQEEY QGEGISWHNI DYTDNVGCIH LISKKPTGLF YLLDEESNFP
 601  HATSHTLLAK FKQQHEDNKY FLGTPVLEPA FIIQHFAGRV KYQIKDFREK
 651  NMDYMRPDIV ALLRGSDSSY VRQLIGMDPV AVFRWAVLRA AIRAMAVLRE
 701  AGRLRAERAE KAEAGVSSPV TRSHVEELPR GANTPSEKLY RDLHNQIIKS
 751  LKGLPWQGED PRRLLQSLSR LQKPRTFFLK SKGIKQKQII PKNLLDSKSL
 801  RLIISMTLHD RTTKSLLHLH KKKKPPSISA QFQTSLNKLL EALGKAEPFF
 851  IRCIRSNAEK KELCFDDELV LQQLRYTGML ETVRIRRSGY SAKYTFQDFT
 901  EQFQVLLPKD VQPCREAIAA LLEKLQVDRQ NYQIGKTKVF LKETERQALQ
 951  ERLHGEVLRR ILLLQSWFRM VLERRHFVQM KHAALTIQAC WRSYRVRRTL
1001  ERTRAAVYLQ AAWRGYLQRQ AYHHQRHSII RLQSLCRGHL QRRSFSQMML
1051  EKQKAEQARE TAGAEMSEGE PSPVAAGEQP SEHPVEDPES LGVETETWMN
1101  SKSPNGLSPK KEIPSPEMET PAQKTVPAES HEKVPSSREK RESRRQRGLE
1151  HVERQNKHIQ SCREENSTLR EPSRKASLET GESFPEDTKE PREDGLETWT
1201  ETAAPSCPKQ VPIVGDPPRS PSPLQRPASL DLDSRVSPVL PSSSLESPQD
1251  EDKGENSTKV QDKPESPSGS TQIQRYQHPD TERLATAVEI WRGKKLASAM
1301  LSQSLDLSEK PRTAGAALTP TEERRISFST SDVSKLSPVK TSTEVDGDLS
1351  AKKPAGHKKK SEDPSAGPDA GLPTGSQGDS KSAFKRLFLH KAKDKKPSLE
1401  GVEETEGSGG QAAQEAPARK TLDVPSSQQH RHTTGEKPLK GKKNRNRKVG
1451  QITVSEKWRE SVFRKITNAN ELKFLDEFLL NKVNDLRSQK TPIESLFIEA
1501  TERFRSNIKT MYSVPNGKIH VGYKDLMENY QIVVSNLAAE RGEKDTNLVL
1551  NVFQSLLDEF TRSYNKTDFE PVKGKAQKKK RKQERAVQEH NGHVFASYQV
1601  NIPQSCEQCL SYIWLMDKAL LCSVCKMTCH KKCVHKIQSY CSYTGRRKSE
1651  LGAEPGHFGV CVDSLTSDKA SVPIVLEKLL EHVEMHGLYT EGLYRKSGAA
1701  NRTRELRQAL QTDPATVKLE DFPIHAITGV LKQWLRELPE PLMTFAQYGD
1751  FLRAVELPEK QEQLAAIYAV LDHLPEANHT SLERLIFHLV KVALLEDVNR
1801  MSPGALAIIF APCLLRCPDN SDPLTSMKDV LKITTCVEML IKEQMRKYKV
1851  KMEEINHLEA AESIAFRRLS LLRQNAPWPL KLGFSSPYEG VRTKSPRTPV
1901  VQDLEELGAL PEEAAGGDED REKEILMERI QSIKEEKEDI TYRLPELDPR
1951  GSDEENLDSE TSASTESLLE ERAVRGAAEE
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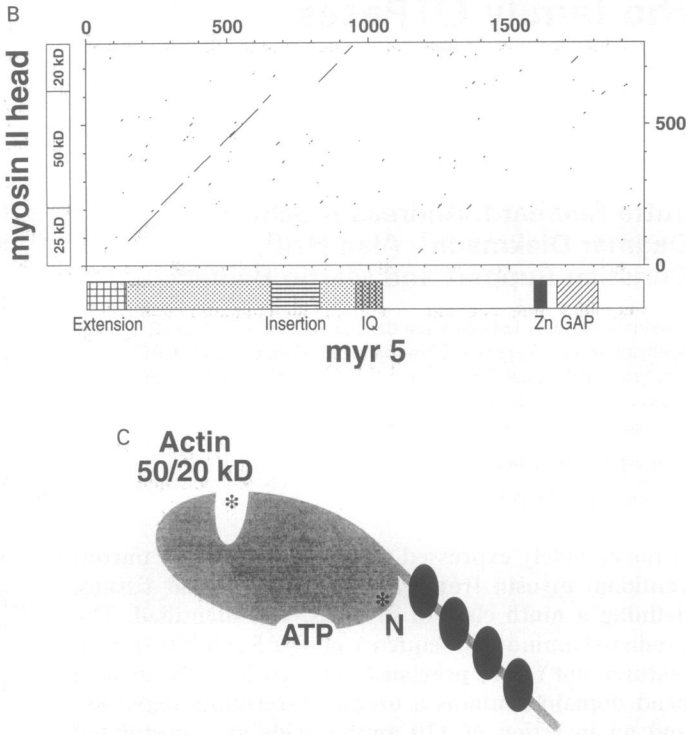

Fig. 1. Sequence analysis of myr 5. (**A**) The predicted amino acid sequence of myr 5 is shown in single-letter code (amino acids 1–1980). The N-terminal extension preceding conserved myosin head sequences is double underlined; the ATP binding P-loop is shown in italics and consensus residues are marked by points above each residue; an insertion of residues not found in other myosin head sequences is underlined with a dashed line; the four putative light chain binding motifs (IQ motifs) are boxed; residues proposed to coordinate zinc are shown in bold type; the region homologous to GTPase-activating proteins of the rho subfamily of G-proteins is underlined. (**B**) Dot-matrix comparison of chicken pectoralis myosin head domain (myosin II head; Maita *et al.*, 1991) with myr 5. Tryptic cleavage sites (25/50 and 50/20 kDa junctions) in the myosin II head domain are indicated, as well as sequence motifs present in myr 5. The myr 5 domain sharing significant homology to the myosin II head domain is indicated by the diagonals in the dot plot and the lightly shaded area of the schematic myr 5 representation. The N-terminal extension (Extension, square-hatched), the insertion at the 50/20 kDa junction (Insertion, horizontal lines), the four IQ motifs (IQ, dark shaded) and the tail domain (white) with the zinc binding domain (Zn, black) and the GAP domain (GAP, cross hatched) are indicated. For the dot-matrix comparison, the amino acid sequences were compared using the GCG software package with a stringency of 14 amino acids in a window of 25 amino acids. (**C**) Schematic representation of the location of the myr 5 N-terminal extension and the 50/20 kDa insertion on the myosin head structure (indicated by asterisks). The location of the four putative light chains is also indicated.

the myr 5 myosin head domain and the head domains of other known myosin classes revealed that myr 5 forms a distinct, ninth class of myosins (data not shown; Bement *et al.*, 1994). In comparison with conventional class II myosins, the myr 5 head domain exhibits a unique N-terminal extension of ~140 amino acids (Figure 1B). A 3-D structure determination of skeletal muscle myosin heads (S1) places this extension close to the first light chain at the head–neck junction (Rayment *et al.*, 1993a; Figure 1C). Limited tryptic digestion cuts vertebrate skeletal myosin S1 at two flexible loops, giving rise to three major fragments: a 25 kDa N-terminal nucleotide binding fragment, a central 50 kDa fragment and a C-terminal 20 kDa fragment. At the position of the first flexible loop (25/50 kDa junction) the myr 5 sequence exhibits a 10 amino acid deletion, as found in some other unconventional myosins. At the position of the second flexible loop (50/20 kDa junction) the myr 5 sequence contains an insertion of 120 amino acids (Figure 1B). This insertion is especially worth noting because the 50/20 kDa junction has been proposed to represent an actin contact site (Rayment *et al.*, 1993b; Schröder *et al.*, 1993).

In the neck region, between the myosin head region and the tail region, myr 5 contains four imperfect 23 amino acid repeats which conform to the 'IQ motif' (Cheney and Mooseker, 1992; Figure 2A). IQ motifs are thought to represent binding sites for light chains of the calmodulin/EF-hand superfamily (Mercer *et al.*, 1991). These four putative light chains of myr 5 might play a role in the regulation of motor activity and step size, as inferred from data from class I, II and V myosins (Xie *et al.*, 1994).

The deduced myr 5 tail amino acid sequence exhibits no similarity to other myosin tail sequences and is not predicted to form an α-helical coiled-coil structure. The tail region is composed of four arbitrarily defined regions: (i) a long relatively proline-rich region, (ii) a cysteine-rich region, (iii) a region with homology to GAPs of the rho subfamily of small GTP binding proteins and (iv) an acidic C-terminal region (Figure 1). The cysteine-rich region comprises a C_6H_2 motif found in several signalling molecules such as protein kinase C, raf kinase, diacylglycerol kinase, the oncogene vav and others (Figure 2B).

Directly following the C_6H_2 motif, as in the proteins chimaerin (Hall *et al.*, 1990; Ahmed *et al.*, 1994) and

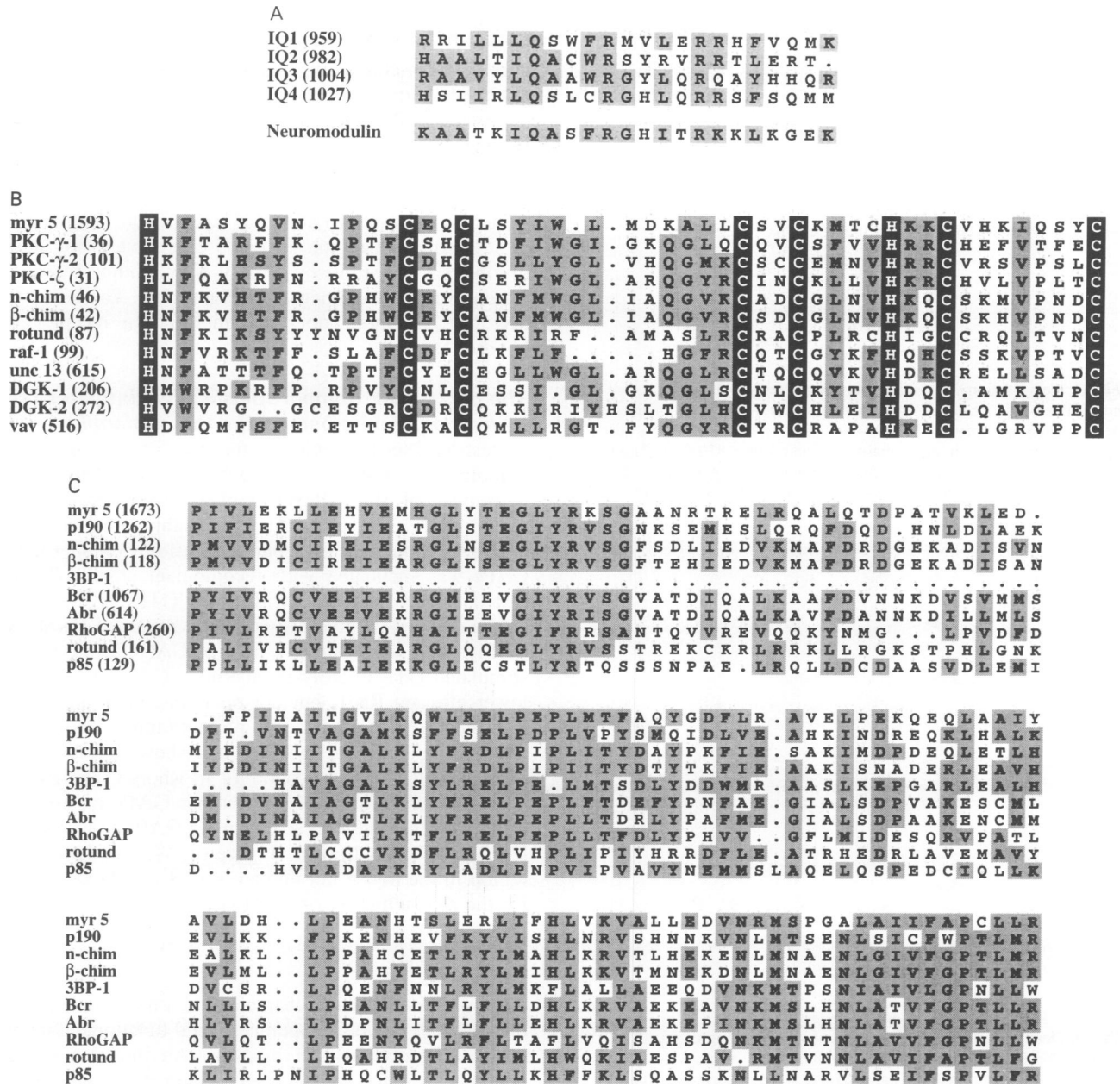

Fig. 2. Sequence comparison of myr 5. (**A**) Alignment of the four putative light chain binding motifs (IQ motifs) found in myr 5 with each other and the calmodulin binding motif of neuromodulin (GAP-43; Chapman *et al.*, 1991). Identical or similar residues are stippled. Numbers of first amino acid residues are given in parentheses. (**B**) An alignment of sequences exhibiting a C_6H_2 motif. The myr 5 cysteine-rich domain was aligned with the two repeats in the C1 region of the γ-isoform of rat protein kinase C, the single motif in the rat protein kinase Cζ isoform (Azzi *et al.*, 1992), human n-chimaerin (Hall *et al.*, 1990), rat β-chimaerin (Leung *et al.*, 1993), *Drosophila* rotund (Agnel *et al.*, 1992), human raf-1 (Beck *et al.*, 1987), *Caenorhabditis elegans* unc-13 (Maruyama and Brenner, 1991), human diacylglycerol kinase (Schaap *et al.*, 1990) and the mouse vav proto-oncogene (Adams *et al.*, 1992). The conserved C_6H_2 motif is stippled in black; other identical or similar residues are stippled in grey. Numbers of the first residue are given in parentheses. (**C**) Amino acid sequence comparison of myr 5 with proteins containing a GAP rho family homology domain. The sequence of myr 5 is compared with p190 (Settleman *et al.*, 1992b), n-chimaerin (n-chim; Hall *et al.*, 1990), β-chimaerin (β-chim; Leung *et al.*, 1993), 3BP-1 (Cicchetti *et al.*, 1992), Bcr (Lifshitz *et al.*, 1988), Abr (Heisterkamp *et al.*, 1993), RhoGAP (Lancaster *et al.*, 1994), rotund (Agnel *et al.*, 1992) and p85 (Escobedo *et al.*, 1991). Identical or similar residues are stippled. Numbers of first residues are given in parentheses.

rotund (Agnel *et al.*, 1992), the myr 5 tail domain exhibited a region with significant homology to GAPs specific for ras-related G-proteins of the rho subfamily (31–37% sequence identity; Figure 2C).

Expression of myr 5 protein

Antibodies directed against the N-terminal region, the myosin head insertion and the C-terminal region were raised in rabbits and affinity purified. They recognized a protein of the appropriate size of 225 kDa in several rat tissues such as testis, lung, thymus, brain, liver and spleen (Figure 3 and data not shown). These data demonstrate that the novel features found in the predicted amino acid sequence of myr 5 cannot be ascribed to cloning artefacts. Furthermore, the results are indicative of a widespread tissue distribution.

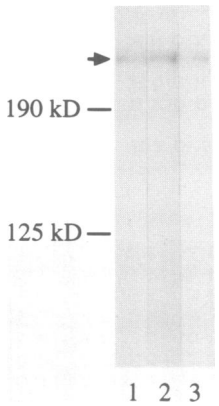

Fig. 3. Identification of myr 5 protein in rat testis. Rat testis homogenate was immunoblotted with antibody Tü 35 raised against a peptide encompassing residues 91–105 (lane 1), antibody Tü 55 raised against a fusion protein encompassing residues 664–830 (lane 2) and antibody Tü 66 raised against a fusion protein encompassing residues 1851–1980 (lane 3). Blots were developed using the Proto-Blot reagents (Promega).

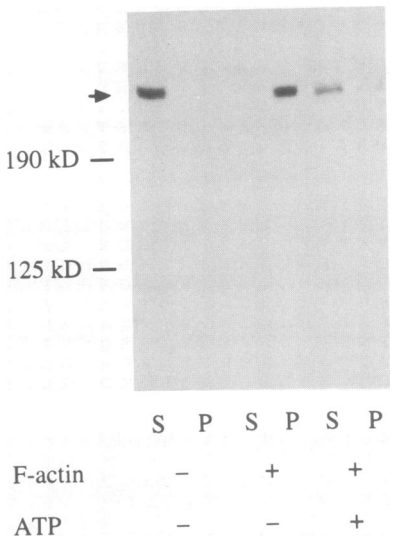

Fig. 4. Demonstration of nucleotide-dependent binding of myr 5 to F-actin. Partially purified myr 5 was incubated in the absence (−) or presence (+) of actin (5 mM) and ATP (2 mM), respectively. The F-actin binding was assayed by co-sedimentation with F-actin. Supernatants (s) and pellets (p) were analysed for myr 5 protein content by immunoblotting with affinity-purified Tü 55 antibody. The myr 5 protein band is indicated.

Myr 5 binds F-actin in an ATP-regulated manner

The myr 5 myosin head domain contains an insertion at a postulated actin contact site (see above). Such an insertion of 120 amino acids (13 kDa) could sterically inhibit or alter the interaction of myr 5 with actin filaments. However, actin binding assays demonstrated that myr 5 interacts with actin filaments in an ATP-regulated manner, as is characteristic for all myosins (Figure 4). Because no purified myr 5 was available, we cannot formally exclude the possibility that myr 5 interacted indirectly with actin filaments, although we consider this possibility unlikely.

Myr 5 C_6H_2 motif binds two atoms of zinc, but not phorbol ester

The homologous regions in protein kinase C βI have been demonstrated to coordinate with high affinity two atoms of zinc (Hubbard *et al.*, 1991). In isoforms of protein kinase C (with the exception of the PKCζ isoform), in unc-13 and n-chimaerin, this region has also been shown to bind the tumour-promoting phorbol ester phorbol 12,13-dibutyrate (PDBu; Ahmed *et al.*, 1990; Maruyama and Brenner, 1991; Quest *et al.*, 1994b).

Purified glutathione S-transferase (GST) fusion proteins encoding the cysteine-rich region of myr 5 were demonstrated to contain two atoms of zinc (Figure 5B). Hence, the cysteine-rich region in myr 5 coordinates two atoms of zinc. Examinations of phorbol ester binding by myr 5 fusion proteins were negative (Figure 5C). Therefore, the myr 5 C_6H_2 motif does not represent a receptor for phorbol ester.

The myr 5 GAP domain specifically stimulates GTP hydrolysis of members of the rho subfamily

To test whether the region in the myr 5 tail domain with homology to GAPs was capable of stimulating GTP hydrolysis of rho family members, we expressed this region as a fusion protein with glutathione S-transferase in *Escherichia coli*. The purified fusion protein (myr 5 GAP) was incubated with recombinant small GTPases that had been preloaded with [γ-^{32}P]GTP and the effect on the rate of GTP hydrolysis was investigated. As shown in Figure 6, the purified myr 5 GAP fusion protein stimulated GTP hydrolysis on RhoA, Cdc42Hs and with lower efficacy Rac1, but not on H-ras. GST alone or a truncated GAP domain fusion protein (amino acids 1711–1857) were both inactive (data not shown). Substitution of glycine to valine at codon 14 in RhoA (Val14RhoA), which abolished the ability of other GAPs to stimulate GTP hydrolysis, also abolished myr 5 GAP-induced stimulation of GTP hydrolysis (Figure 6). We conclude that a region in the myr 5 tail domain serves as a GAP specific for the rho subfamily of small GTP binding proteins.

Discussion

We have identified and characterized a novel mammalian myosin, myr 5, which contains several functional domains not found previously in myosins. An insertion of 120 amino acids in the myosin head domain challenges current structural acto−myosin models; a zinc binding domain followed by a GAP domain implicates myr 5 in signal transduction.

Myosins have been subdivided into classes according to phylogenetic analysis of the head domains (Cheney *et al.*, 1993; Goodson and Spudich, 1993). Such an analysis demonstrated that myr 5 represents the first completely cloned member of a new, ninth class of myosins. With the exception of the *Drosophila* ninaC myosin (myosin III), myr 5 is the phylogenetically most distant relative of conventional myosin (myosin II). Recently, Bement *et al.* (1994) reported the sequences of two short PCR fragments of human and porcine origin, referred to as myosins IXA and IXB, respectively. Comparison of these sequences with the myr 5 sequence suggests that human and porcine myosin IXB are homologues of myr 5. The identification of a myosin IXA fragment suggests that there might exist additional myosins closely related to myr 5. Further complexity might be added by differential splicing of myr 5 as found for other myosins, e.g. for conventional myosin

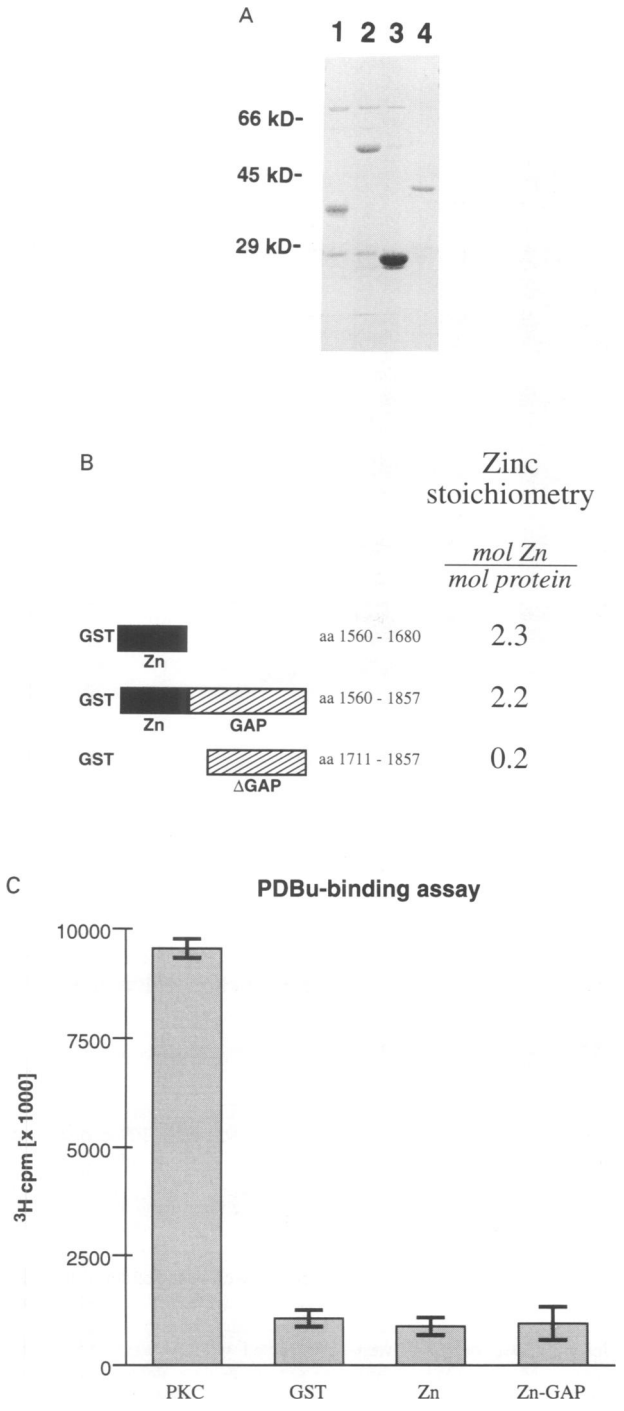

Fig. 5. Expression of GST−myr 5 fusion proteins and determination of zinc stoichiometry and phorbol ester binding. (**A**) Purified recombinant GST−myr 5 fusion proteins. Coomassie blue-stained SDS−polyacrylamide gel of affinity-purified GST−myr 5 C_6H_2 motif, residues 1560–1680 (lane 1), GST−myr 5 C_6H_2 motif GAP homology region, residues 1560–1857 (lane 2), GST (lane 3) and GST−myr 5 truncated GAP homology region, residues 1711–1857 (lane 4). Molecular weight markers are indicated. (**B**) Schematic diagram of GST−myr 5 fusion proteins and stoichiometry of zinc binding (mol of zinc/mol of fusion protein). Values of a typical experiment are shown. (**C**) Binding of [^{3}H]PDBu to fusion proteins of GST−PKCγ N-terminal C_6H_2 motif (1 μg), GST (1 μg), GST−myr 5 C_6H_2 motif (3 μg) and GST−myr 5 C_6H_2 motif GAP homology region (4 μg) is shown. The purified fusion proteins were incubated with 20 nM [^{3}H]PDBu for 30 min in the presence of 100 μg/ml phosphatidylserine. Values represent the mean ± SEM (bars) of three determinations.

at the 50/20 kDa junction (Takahashi *et al.*, 1992). Indeed, antibodies directed against myr 5 always stained on immunoblots a relatively broad band which sometimes could be resolved into two or more bands. This heterogeneity might also be due to post-translational modifications or partial degradation.

Myr 5 exhibits in the head domain at the 50/20 kDa junction a unique insertion of ~120 amino acids. In myosin II from skeletal muscle the 50/20 kDa junction comprises a flexible loop sensitive to trypsin cleavage (Balint *et al.*, 1978). Sensitivity to trypsin cleavage is lost upon binding of myosin II to F-actin (Mornet *et al.*, 1981a). Cross-linking studies (Mornet *et al.*, 1981b) and an atomic model of the acto−myosin complex (Schröder *et al.*, 1993) suggest that this region interacts directly with actin. Chimeras of this region constructed by substituting the nine amino acid *Dictyostelium* myosin junction region with those from myosins from other species demonstrated that this region is important in determining the enzymatic activity of myosin and that this region affects the speed of movement (Uyeda *et al.*, 1994). An insertion of 13 kDa in this region, as found in myr 5, is therefore expected to have pronounced effects on the mechanochemical properties. Such an insertion might interact with F-actin and serve as a regulatory domain or sterically block the interaction with F-actin. We ruled out the latter possibility because we demonstrated that myr 5 is able to interact with F-actin in an ATP-regulated manner. It remains to be determined whether the insertion interacts with F-actin or whether it might influence myr 5 function in some other way. Myr 5 contains in addition a unique N-terminal extension that in analogy to the head structure of myosin II (Rayment *et al.*, 1993a) is proposed to be located close to the first light chain. Myr 5 is predicted to bind four light chains. It exhibits four IQ motifs which represent putative calmodulin/EF-hand type protein light chain binding sites (Mercer *et al.*, 1991; Bähler *et al.*, 1994). Preliminary evidence suggests that calmodulin is present in myr 5 immunoprecipitates (R.Müller and M.Bähler, unpublished observations).

The tail domain of myr 5 is not predicted to form an α-helical coiled-coil structure. We therefore assume that myr 5 is a single-headed myosin. The C-terminal half of the myr 5 tail sequence includes a C_6H_2 motif followed by a domain with homology to GAPs for members of the rho subfamily of small G-proteins. The C_6H_2 motifs found in protein kinase C and in raf kinase have been demonstrated to coordinate two atoms of zinc (Hubbard *et al.*, 1991; Ghosh *et al.*, 1994). We showed that the myr 5 C_6H_2 motif is also able to coordinate two atoms of zinc. The zinc atoms are likely to stabilize a specific structure acting as a recognition site for binding partners. In protein kinase C isoforms (with the exception of protein kinase Cζ), unc-13 and n-chimaerin, this motif serves as a receptor for phorbol esters (Ahmed *et al.*, 1990; Maruyama and Brenner, 1991; Quest *et al.*, 1994a,b). However, the myr 5 C_6H_2 motif did not bind phorbol ester, like the C_6H_2 motifs present in raf kinase and vav (Ghosh *et al.*, 1994; Kazanietz *et al.*, 1994). The raf kinase C_6H_2 motif does bind phosphatidylserine (Ghosh *et al.*, 1994) and may be involved in binding 14-3-3 proteins (Freed *et al.*, 1994; Irie *et al.*, 1994). Therefore, the myr 5 C_6H_2 motif probably represents a lipid or protein binding motif.

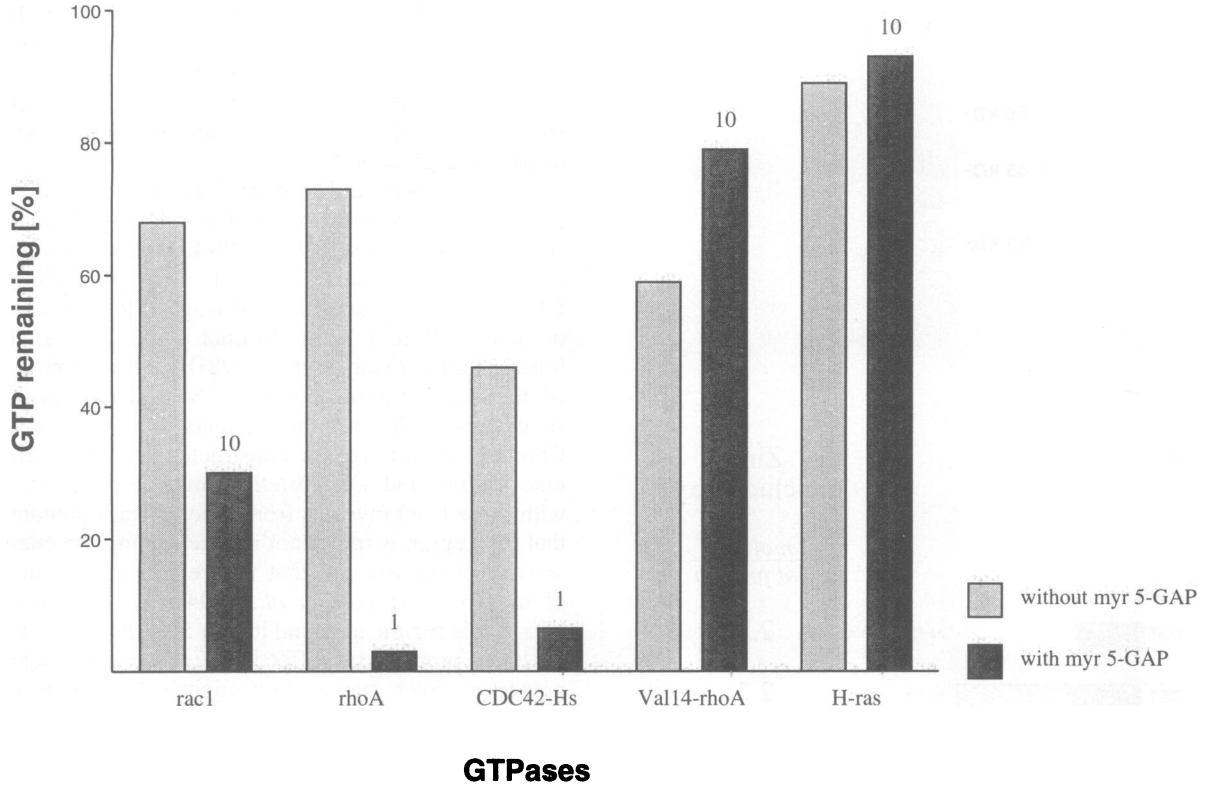

Fig. 6. GAP activity of GST−myr 5 C_6H_2 motif GAP homology fusion protein on various GTPases. Each GTPase was preloaded with [γ-^{32}P]GTP, incubated with or without purified myr 5 fusion protein (10 indicates 5 μg, 1 indicates 0.5 μg of myr 5 fusion protein), and the radioactivity remaining on each GTPase after 5 min was determined in a nitrocellulose filter binding assay (Settleman *et al.*, 1992a). The percentage of radioactivity remaining is relative to the initial radioactivity on each GTPase.

Binding of the putative ligand to the C_6H_2 motif may regulate myr 5 GAP activity, as suggested for n-chimaerin which also contains a C_6H_2 motif followed by a GAP domain (Ahmed *et al.*, 1994).

A fusion protein encoding the region in the myr 5 tail homologous to GAPs of the rho subfamily stimulated specifically the GTPase activity of rhoA and Cdc42Hs *in vitro*. A lower GTP-stimulating activity was observed on rac1. Rho and Rac have been demonstrated to be essential components of signal transduction pathways linking growth factors to the regulation of the actin cytoskeleton and to contraction of the actin-based cytoskeleton (Ridley and Hall, 1992; Ridley *et al.*, 1992; Jalink *et al.*, 1994). It seems plausible, therefore, that myr 5 could be involved in the regulation of the organization of the actin filaments along which it might produce force and movement. Myr 5 could move signalling complexes along actin filaments and thereby act as an effector of rho family GTPases. The identification of functional domains associated with signal transduction in molecular motors of the myosin superfamily, such as a GAP domain for Rho GTPases and a C_6H_2 motif in myr 5, a kinase domain in the *Drosophila* ninaC myosin (Montell and Rubin, 1988) and an SH3 domain in several myosin I molecules (Pollard *et al.*, 1991), underscores the importance of myosins and the actin cytoskeleton in signal transduction pathways.

Important issues for further myr 5 studies are the determination of its physiological role in signalling pathways requiring members of the rho subfamily, the characterization of the mechanochemical properties of the myosin head domain and the analysis of the regulation and interplay of the various separate functional domains.

Materials and methods

Cloning of myr 5

A myr 2 cDNA probe (nucleotides 324–510; EMBL database accession number X74800) obtained by PCR was used to screen 2×10^6 recombinant phages from a rat brainstem/spinal cord λ ZAP II cDNA library (Stratagene). In this screen four overlapping clones coding for myr 5 were isolated. The library was screened repeatedly to obtain additional overlapping myr 5 clones. Finally, two cDNA clones coding for the C-terminus of myr 5 were isolated from an adult rat testis λ ZAP II cDNA library (Stratagene). Positive clones were purified and plasmids containing the cDNA inserts were excised from the λ ZAP phage according to the instructions of the manufacturer. For sequencing, a series of nested deletions were constructed with an Erase-a-base kit (Promega, Madison, WI). Nucleotide sequences were determined by the method of Sanger *et al.* (1977) using double-stranded DNA templates and the USB Sequenase version 2.0 kit (US Biochemicals, Cleveland, OH). All sequences were determined for both strands. Computational analysis was performed using the GCG software package (Devereux *et al.*, 1984). The myr 5 nucleotide sequence is available from the EMBL database under the accession number X77609.

Production of antibodies

For antibody production, fusion proteins were constructed in the pQE8 6×His tag vector (Qiagen). Nucleotides 1992–2490 and 5553–5940 were amplified by PCR using primers with flanking *Bam*HI and *Hin*dIII restriction sites, respectively. The 6×His tagged proteins were expressed in *E.coli* and purified under denaturing conditions over Ni-NTA-resin as described by the supplier. Antibodies were raised in rabbits and affinity purified over the purified fusion protein coupled to CNBr-activated Sepharose 4B.

Actin binding of myr 5

To enrich for myr 5, adult rat testis tissue was homogenized in 0.32 M sucrose, 5 mM HEPES (pH 7.4) with a Dounce homogenizer and

centrifuged at 30 000 *g* for 40 min. The supernatant was loaded onto a MonoQ (HR 5/5) column (Pharmacia) pre-equilibrated in 20 mM Tris−HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EGTA and 1 mM NaN_3. Applying a linear gradient (150 mM−1 M), myr 5 eluted as a broad peak starting at ~370 mM NaCl. Fractions containing myr 5 were pooled and dialysed against 150 mM NaCl, 50 mM Tris−HCl (pH 7.4). After dialysis, the sample was centrifuged (rotor TLA 100.2, 75 000 r.p.m., 20 min, 4°C) and concentrated in a Centricon-30 (Amicon, Beverly, MA). Actin binding assays, SDS−PAGE and immunoblotting were performed as described (Ruppert *et al.*, 1993).

Determination of GAP activity

Myr 5 cDNA fragments obtained by PCR were inserted into *Bam*HI and *Eco*RI restriction sites of a GST vector, pGEX-2T (Pharmacia). *E.coli* cells harbouring these plasmids were grown to an absorbance at 595 nm of 0.5 and expression was induced with isopropyl-β-D-thiogalactoside (0.1 mM) for 2 h. Pelleted cells were lysed by sonication. After the addition of 1% Triton X-100 and PMSF (0.1 mM), extracts containing GST or GST−myr 5 fusion proteins were incubated with glutathione−Sepharose 4B (Pharmacia), washed and the proteins eluted with 5 mM glutathione, 50 mM Tris−HCl (pH 8.0). Recombinant small GTPases were expressed as GST fusion proteins and obtained in purified form after thrombin cleavage, as described (Ridley *et al.*, 1992). To measure GTPase activity, small GTPases (rac1, rhoA, cdc42Hs, V14rhoA and H-ras; 0.5–1.0 μg of each) were preloaded with [γ-^{32}P]GTP (Amersham; 30 Ci/mmol) by incubation for 10 min at 30°C in a solution containing 20 mM Tris−HCl (pH 7.5), 25 mM NaCl, 0.1 mM DTT, 13 mg/ml BSA, 5 mM EDTA, 1 μl [γ-^{32}P]GTP, followed by the addition of 25 mM $MgCl_2$. Preloaded GTPases were then incubated in 30 μl of a solution containing 20 mM Tris−HCl (pH 7.5), 0.1 mM DTT, 1 mM GTP, 13 mg/ml BSA with or without GST−myr 5 fusion protein for 5 min at 20°C. Samples were diluted into 1 ml cold 50 mM Tris−HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$ and filtered through nitrocellulose (BA85; Schleicher & Schüll). Filters were washed with 10 ml of the same solution and radioactivity remaining on the filters was determined.

Determination of zinc stoichiometry

For the determination of zinc stoichiometry, fusion proteins were expressed in *E.coli* XL-1 blue as described by Quest *et al.* (1994b). Zinc was quantitated by flameless atomic absorption on a Perkin-Elmer 400S atomic absorption spectrometer. Protein concentrations were determined by quantitative amino acid analysis or by the methods of Lowry (Peterson, 1983) and Bradford (1976) using bovine serum albumin (BSA) as a standard. Values were corrected for the purity of fusion proteins as determined by densitometry of Coomassie blue-stained SDS gels. Stoichiometries were calculated assuming a molecular mass of 27.5 kDa for GST. Molecular masses of myr 5 fragments were derived from the deduced amino acid sequences. GST and GST−truncated myr 5 GAP fusion protein were used as controls and contained negligible amounts of zinc.

Phorbol ester binding assay

Purified recombinant proteins were incubated in 50 mM Tris−HCl (pH 7.5), 4 mg/ml BSA, 100 μg/ml phosphatidylserine, 2 mM $CaCl_2$ and 20 nM [^{3}H]PDBu (20.7 Ci/mmol from DuPont NEN) for 30 min at 30°C. The [^{3}H]PDBu bound to recombinant proteins was separated from free [^{3}H]PDBu by filtration through prewetted Whatman GF/C glass fibre filters. Filters were washed with 2× 1 ml of ice-cold 25 mM Tris−HCl (pH 7.5) and air dried. Radioactivity remaining on the filters was measured in a liquid scintillation counter (Beckmann, LS6000TA).

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